Gene Therapy for the Special Senses

1. Retinal Gene Therapy in X-Linked Retinitis Pigmentosa Caused by Mutations in *RPGR*: Results at 6 Months in a First in Human Clinical Trial

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X-linked retinitis pigmentosa (RP), caused by mutations in the RP GTPase regulator *(RPGR)* gene, is the most common form of recessive RP. It is characterised by a primary degeneration of rod and cone photoreceptors in childhood, leading to visual field constriction and early, severe sight loss. Currently, there is no treatment for *RPGR* related RP. The inherent instability of the *RPGR* coding sequence, which has a repetitive purine rich region, presents challenges to translate this therapy into human trials. Codon optimisation of the sequence has increased *RPGR* stability and fidelity, providing the basis for this Phase I/II dose escalation retinal gene therapy clinical trial. In total, 18 patients with genetically confirmed variants in *RPGR* were recruited in the trial and received increasing subretinal doses of adeno-associated viral vector serotype 8 encoding codon-optimized human *RPGR* driven by the photoreceptor specific rhodopsin kinase promoter (AAV8.*RK.coRPGR)*. The vector uses codon optimisation to disable the alternate splice donor site and stabilise the open reading frame (ORF) 15 region of *RPGR*. As a result, the ORF15 domain of the retinal isoform of the RPGR protein is preserved and fully functional, as evidenced by glutamylation levels which are indistinguishable from the wildtype protein. The primary outcome of the study was safety and secondary outcomes included visual acuity, microperimetry and central retinal thickness. Apart from steroid-responsive subretinal inflammation in patients at the higher doses, a gene therapy trial with AAV8.*RK.coRPGR* vector did not have any dose limiting toxicities, meeting the pre-specified primary endpoint. Visual field improvements beginning at one month and maintained to the last point of follow-up were observed in six patients. One patient with an exceptional visual improvement had evidence of possible outer segment regeneration seen on retinal imaging. In conclusion, this first in human gene therapy trial with AAV8.*RK.coRPGR* vector had no significant safety concerns.The observed reversal of visual field loss may relate to regeneration of outer retinal structures following successful gene transfer.

2. Therapeutic Efficacy of ARCUS Meganuclease Gene Editing - Arrest of Rod Degeneration and Restoration of Rod Function in a Transgenic Pig Model of Autosomal Dominant Retinitis Pigmentosa

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The P23H mutation in the rhodopsin (RHO) gene represents the most common form of autosomal dominant retinitis pigmentosa (adRP) in North America. Patients progress from night blindness to tunnel vision as rods degenerate. Disease onset occurs in early adulthood and may progress to complete blindness when cones degenerate in the absence of rods. This autosomal dominant genetic disease is caused by a toxic gainoffunction mutation in rhodopsin and does not respond conventional gene replacement strategies. Here we report a replication of our original allelespecific genome editing approach in a transgenic pig model of P23H human adRP. We show that this gene editing approach produces a sustained reduction of mutant RHO following a single administration of an engineered ICreI homing endonuclease (ARCUS). In our well characterized transgenic swine model of human P23H adRP (Tg hP23H), we treated one eye with a single subretinal injection of an self-complementary AAV5 (scAAV) vector containing (ARCUS) between postnatal day 3 and 7 (P3-7). The fellow eye served as an internal control and either received an injection of vehicle or no injection. A range of viral titers was tested to determine both the minimal effective dose as well as any dose response function. Non-invasive structural and functional assessments were made at regular intervals after treatment (weeks post injection, wpi), using full-field electroretinogram (ffERG), spectral domain ocular coherence tomography (OCT) and fundus imaging. Following up to 42 weeks post-injection, animals were sacrificed and the morphology of their retinas examined using immunohistochemistry. Untreated Tg hP23H retinas have no rod function from birth onward. All Tg hP23H eyes treated with scAAV5ARCUS (2×10^9 to 6×10^{10}; n=32) showed a significant rod driven response (scotopic ERG flash = 0.001 cd/m2) that was first measured at ~6wpi (p=0.0016), increased up to ~ 12 wpi and plateaued through 42 wpi. The outer retinal morphology was rescued by scAAV5ARCUS, e.g., rod photoreceptors were maintained along with rhodopsin expression. Cone function and cone morphology also was retained in the treated retinas. The minimum effective dose to induce rod function is to 6×10^9 and there is a correlation of dose with ffERG b-wave amplitude (2×10^9 to 6×10^{10}). Along with treating Tg hP23H mutants, we tracked the safety of this therapeutic approach in WT littermates. To the best of our knowledge, this is the first collection of data to demonstrate positive preclinical efficacy of gene editing in a large animal model of hP23H adRP. These results suggest that this scAAV ARCUSbased gene editing of P23H RHO may be applicable to treat adRP patients with the same P23H mutation.

3. Gene Therapy in a Novel Large Animal Model of Stargardt Disease

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Stargardt disease type 1 (STGD1), due to bi-allelic mutations in *ABCA4*, is the most common form of inherited macular degeneration, for which no therapeutic options are currently available. We have recently developed a gene therapy approach for STGD1 which relies on the use of two adeno-associated viral vectors (AAV) each encoding for one of the two halves of the ABCA4 protein flanked by split-inteins, which catalyze trans-splicing of the two ABCA4 half proteins following AAV administration. We have previously shown that subretinal administration of the AAV intein vectors results in precise reconstitution of the full-length ABCA4 protein and in therapeutic efficacy in *Abca4-/-* mice. However, the mouse retina largely differs from that of humans both in terms of size and retinal architecture. Given the similarity between the swine and human retina, we have recently generated pigs through somatic cell nuclear transfer from primary fibroblasts in which ABCA4 has been knock-out (KO) using CRISPR/Cas9. We have found that ABCA4 KO pigs have increased lipofuscin accumulation in the retinal pigmented epithelium, as it also occurs in STGD1 patients, up to 24 months, the longest time-point evaluated. In addition, subretinal delivery of AAV-*ABCA4* intein vectors in ABCA4 KO pigs results in efficient reconstitution of the ABCA4 protein at 2 months post-injection. Further analysis of the impact of subretinal delivery of AAV intein on lipofuscin accumulation will provide important insights towards the clinical translation of this platform for gene therapy of STGD1.

4. Preclinical Testing of AAV9-PHP.B for Transgene Expression in the Non-Human Primate Cochlea

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Hereditary hearing loss often results from mutation of genes expressed by cochlear hair cells. Gene therapy has recently made tremendous progress in animal models through transgene delivery to the inner ear with adeno-associated virus. However, significant challenges remain before AAV-based technologies can be translated to the clinic. Recently our group reported transmastoid injection of an AAV9 capsid variant, PHP.B, via the round window membrane (RWM) in a cynomolgus monkey. PHP.B mediated efficient transgene expression of a GFP reporter in many cochlear cells including both inner and outer hair cells. However, results were inconsistent, as a second monkey injected with a lower dose showed limited transduction with no obvious transduction of hair cells. The difference might be attributed to steep dose dependency, to a pre-existing immunity to the AAV vector, or to the technical reproducibility of RWM injections in larger mammals. In order to further define the transduction potential of AAV9-PHP.B, we performed a dosing study and assessed GFP expression in the cynomolgus macaque inner ear after RWM injection of AAV9-PHP.B via the transmastoid route. Four animals were studied and four doses tested. Three female juvenile/adult macaques were administrated with doses of $1x10^{11}$ VGs (n=1), $2x10^{11}$ VGs (n=1), $3.5x10^{11}$ VGs (n=2), and $7x10^{11}$ VGs (n=2); the fourth animal served as a vehicle (PBS) control. At the higher doses ($3.5x10^{11}$ and $7x10^{11}$ VGs) AAV9-PHP.B transduced nearly 100% of both IHCs and OHCs, uniformly from base to apex. Anti-GFP staining was also present in the supporting cells of the organ of Corti, the spiral ligament, the spiral limbus, spiral ganglion neurons and Reissner's membrane, from the base to the apex. No specific anti-GFP staining was observed in the uninjected ear. However, at lower doses there was a steep reduction in viral transduction. In a cochlea administrated with $2x10^{11}$ VGs we observed ~50% of IHCs and ~60% of OHCs transduced, and very limited transduction in a cochlea injected with $1x10^{11}$ VGs. The steep dose dependency probably explains the limited transduction previously reported with a lower dose. In conclusion, AAV9-PHP.B efficiently transduces the IHCs and OHCs of nonhuman primates, but shows a striking dose dependency. Together, these data support a feasible path towards clinical development of gene therapy for hereditary hearing loss with AAV9-PHP.B.

5. Dual AAV Delivery of Otoferlin Durably Rescues Hearing in Congenitally Deaf Preclinical Models

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Otoferlin deficiency secondary to biallelic mutations of the *OTOF* gene causes permanent congenital severe-to-profound deafness. Otoferlin is expressed in the primary sensory receptors of the ear (the inner hair cells) and enables synaptic transmission between the inner hair cells and the dendrites of the auditory nerve by functioning as a calcium sensor for exocytosis during synaptic vesicle release, vesicle trafficking, and replenishment. Of the 1 in 500 neonates who are born with hearing loss annually in the US, 50 to 200 are caused by Otoferlin

deficiency. There are no approved therapies for Otoferlin deficiency; infants with biallelic *OTOF* mutations are currently managed with assistive devices. We generated a mutant mouse line that mimics a common human *OTOF* mutation ($OTOF^{Q828X/Q828X}$) to evaluate the use of Adeno-Associated Virus (AAV)-based gene transfer therapy for restoration of hearing in an Otoferlin deficiency model. Because human *OTOF* cDNA exceeds the packaging capacity of a standard AAV, we used a dual AAV system to locally deliver Otoferlin to the inner ears of $OTOF^{Q828X/Q828X}$ mice. $OTOF^{Q828X/Q828X}$ mice replicated the phenotype of patients with orthologous mutations. Physiologic hearing assessments in these mice demonstrated stable profound deafness. Histological analysis revealed maintenance of the sensory epithelia in the ear. Specifically, inner hair cells with no detectable Otoferlin did not degenerate with age, offering a robust cellular substrate for an AAV-based gene transfer approach. Using a dual hybrid AAV system, we packaged human OTOF cDNA under the control of a cell-type specific Myo15 promoter that restricted transgene expression to inner ear hair cells. Dual AAV vectors were locally delivered to the inner ear by direct viral injection through the round window membrane. Four weeks after injection, *OTOF* transgene expression was detected in inner hair cells throughout the cochlea. We assessed hearing in dual AAV injected and sham injected $OTOF^{Q828X/Q828X}$ mice using the auditory brainstem response test, the same physiologic measure used to assess hearing in infants. No physiologic auditory responses were detected in sham injected $OTOF^{Q828X/Q828X}$ mice, consistent with the stable profound deafness seen in untreated mice. By contrast, hearing was rescued to normal sensitivity in injected mice. Furthermore, rescued hearing with dual AAV-OTOF was maintained at least 6 months post-treatment. We assessed the translational potential of AAV-Myo15 in non-human primates by locally injecting AAV through the round window membrane into the ears of cynomolgus monkeys, leveraging a surgical approach that is routinely performed in children to place cochlear implants. Using in situ hybridization and immunohistochemistry we found that AAV-Myo15 produced transgene expression in the inner hair cells of non-human primates. Dual-AAV-*OTOF* delivery provides robust and durable functional recovery in rodent disease models. Clinical delivery of a normal copy of the *OTOF* gene to inner ear hair cells in patients with Otoferlin deficiency should instate protein production and provide hearing to congenitally deaf children.

6. Optimizing the Safety of Dual AAV-Based Treatments for *MYO7A* Usher Syndrome (USH1B) in *Myo7a*$^{-/-}$ Mice

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PURPOSE: Usher syndrome is the most common combined blindness-deafness disorder with Usher Syndrome 1B (USH1B), caused by *MYO7A* mutations, resulting in the most severe phenotype. USH1B patients have deafness/vestibular defects from birth, progressive retinal degeneration, and vision loss within the first decade. Our goal is to develop a safe and effective dual Adeno-associated virus (AAV)-based gene therapy for the treatment of USH1B. We previously showed that both hybrid and overlap dual AAV vector platforms drive full length MYO7A expression in subretinally injected mice. The hybrid system produces higher levels of full length *MYO7A* relative to the overlap platform, but it also produces a truncated protein that causes a slight loss of rod-mediated function in mice. This truncated MYO7A retains a portion of the protein's tail domain that may interact with and thereby disrupt the function of other retinal proteins. We hypothesized that moving all tail domain coding sequence from the front to the back half vector would eliminate the functional decreases observed. We also identified potential in-frame stop codons downstream of the *MYO7A* coding sequence in the front half hybrid vector. Three potential stop codons were located within the alkaline phosphatase (AP) intron and one within the AP head sequence. We hypothesized that substitutions in these sequences would eliminate the production of truncated protein altogether. **APPROACH:** Hybrid dual AAV-*MYO7A* vectors were optimized by altering the split point of the gene between the two vectors (hybrid-v2 dual vectors). The hybrid-v2 vectors were then further altered by eliminating potential in-frame stop codons in the front half sequence. Two codon modified hybrid front half vectors (CM) were created: 1) hybrid-v2_CMv1: modification of 3 potential in-frame stop codons in the AP intron, 2) hybrid-v2_CMv2: modification of 3 potential in-frame stop codons in the AP intron + 1 potential in-frame stop codon in the AP head coding sequence. Hybrid dual AAV vectors were packaged into AAV2 or AAV8(Y733F) via triple transfection. All vectors used for preliminary *in vitro* testing in HEK293 cells were packaged in AAV2. Cells were collected three days post infection and analyzed for the presence of dual vector-mediated MYO7A genomes/transcript and protein using qRT-PCR and western blot (WB), respectively. *Myo7a*$^{-/-}$ mice were subretinally injected with 5E8 vg of optimized hybrid dual vectors packaged in AAV8(Y733F). Control eyes were injected with BSS. Retinal structure and function were analyzed using optical coherence tomography (OCT) and electroretinogram (ERG) at 6 weeks post injection. Retinas from all experiments were analyzed using qRT-PCR and WB. **RESULTS:** Retinal function/structure were preserved in mice injected with the hybrid-v2 dual vectors compared to eyes that received the original hybrid dual vectors. In addition, the hybrid-v2 dual vectors produced comparable levels of full length MYO7A to the original hybrid vectors. Preliminary *in vitro* testing revealed that the hybrid-v2_CMv1 hybrid vectors still produce truncated protein. However, the production of the truncated protein is eliminated in hybrid-v2_CMv2 vectors *in vitro*. *In vivo* testing of the hybrid-v2_CMv2 dual vectors is on-going. **CONCLUSIONS:** By altering the split point of *MYO7A* in the hybrid dual vectors, we eliminated toxicity mediated by truncated MYO7A. Our preliminary results suggest that removal of adventitious stop codons in the AAV-*MYO7A* hybrid dual vectors has the potential to eliminate truncated protein expression altogether, thus increasing the safety and translatability of this dual vector system.

7. Multi-Center Blinded Preclinical Efficacy Study Shows Significant Differences in Promoter Performance for Gene Replacement Therapy of SMARD1/CMT2S

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Mutations in IGHMBP2 lead to a spectrum of rare, autosomal recessive disease phenotypes characterized by neurodegeneration and muscle atrophy. Currently there is no evidence of a close genotype-phenotype correlation, as even siblings carrying the same mutation present a broad spectrum of clinical features ranging from distal muscle weakness with fatal respiratory distress/failure (Spinal muscular atrophy with respiratory distress type 1-SMARD1) to sensory and milder motor neuropathies with lesser respiratory involvement (Charcot Marie Tooth Disease 2S- CMT2S). Current treatment strategies only focus on management of symptoms and do not impact disease progression. Thus, there is an urgent need to develop an effective therapy for SMARD1/CMT2S. Previous studies by us and others show that intravenously delivered adeno-associated virus 9 (AAV9), carrying a functional IGHMBP2 cDNA rescues the disease phenotype in an intermediate mouse model. However, prior to moving this therapy to clinic, the delivery method and promotor selection required further optimization. We designed two transgene cassettes using different promoters driving IGHMBP2 cDNA expression suitable for clinical vector production. Both constructs were packaged in AAV9 and efficacy testing was performed in three independent groups simultaneously in a blinded manner. To gain confidence that the treatment would work in the context of severe SMARD1 disease, the therapeutic constructs were also tested in a new severe mouse model developed by the Cox laboratory (survival untreated ~ 21 days). The focus of this study is to evaluate the efficacy of two different AAV9.IGHMBP2 constructs in multiple *in vivo* disease models in preparation for IND-enabling studies. All labs included are blinded to the nature of the constructs and received either 2 or 3 viral vectors labelled Virus A, B and C (comprising in random order empty capsid, AAV9-Promoter1-IGHMBP2; AAV9-Promoter2-IGHMBP2). Outcome measures of this study have a strong focus on clinical translation and evaluation of potential biomarkers. Animals received a single intracerebroventricular injection on postnatal day 1 to spread the viral vector via CSF. All labs independently found that virus A and C improved survival of either the severe or milder mouse model. Similar to previous studies, animals showed improvement in body weight and survival. While the 2 viral vector constructs performed comparable in the milder disease model, the severe model revealed differences in performance. Virus C treated animals showed superior strength performance at early time points measured by the hanging wire test, while virus A showed a slower gain of strength, ultimately catching up at later time points. Both virus A and C show improvement on histological and pathological analyses of muscle, with a larger % of fully innervate neuromuscular junctions in virus C treated animals compared to virus A. In both animal models, electrophysiological measures previously used in Spinal Muscular Atrophy type 1 pre-clinical and clinical trials showed marked improvement in treated animals. In summary, strong *in vivo* efficacy data shows CSF delivery is a viable route for AAV9.IGHMBP2-mediated therapy and testing in a more severe animal model revealed differences between the two constructs that are less evident in the milder disease form. Clinically applicable outcome measures were evaluated in both animal models and will support development of upcoming clinical studies.

Genome Editing: Preclinical & Rare Disease

8. Clonal Tracking Uncovers Barriers and Validates New Strategies to Enhance Gene Editing in Human Hematopoietic Stem Cells

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The scope of genome engineering in hematopoietic stem/progenitor cells (HSPCs) has broadened from random to precise genome insertions for treating genetic diseases of the blood lineages. Targeted editing of inherited mutant genes allows *in situ* correction and functional reconstitution with preserved expression control. Improvements in HSPC editing efficiency have been made by delivering CRISPR/Cas nucleases as pre-assembled ribonucleoprotein and the donor template for homology-directed repair (HDR) by a serotype-6 adeno-associated viral vector (AAV6). However, we recently showed that both the induced double-strand DNA breaks and the AAV6 genome trigger a p53-dependent DNA damage response in HSPC delaying proliferation and decreasing hematopoietic reconstitution after xenotransplantation. Suppression of this response by transient expression of a dominant negative p53 released cell-cycle block and rescued hematopoietic reconstitution. Yet, the underlying biology remained unknown as well as the impact of gene editing on clonal dynamics of HDR-edited HSPC upon transplantation. Moreover, it has long been contended that the quiescence of primitive HSC constrains HDR-mediated gene editing, thus limiting its perspective clinical applications in several diseases. Here, we first overcame such constraints by transiently expressing the adenovirus 5 protein E4orf6/7, which operates the major cell cycle controller E2F, together with the nuclease. By global and targeted gene expression analysis we showed engagement of targeted cells in S/G2 phases with concomitant upregulation of all major components of the

HDR machinery, thus increasing the efficiency of targeted transgene insertion. Combined E4orf6/7 expression and p53 inhibition enhanced >50% HDR efficiency within human graft surpassing the levels reported until now in the literature. Such outcome was reproducible across several HSPC donors and sources, genomic loci and conceivably portable to most types of editing platforms. In parallel, we devised a novel technology (BAR-seq) which enables clonal tracking of individual HDR-edited HSC by introducing a unique heritable barcode in the AAV6 template. Deep sequencing of integrated BARs in human hematochimeric mice showed that only few (5-10) dominant clones of edited HSC robustly contributed to the hematopoietic graft long-term after transplant. Transient p53 inhibition during editing enabled substantial increase in polyclonal graft composition without altering individual HSC output, thus explaining the improved engraftment and highlighting the p53-mediated response as culprit of an otherwise oligoclonal hematopoiesis. Importantly, BAR-seq provided the first direct evidence that human HDR-edited HSC maintain multilineage potential and undergo multiple rounds of symmetric and asymmetric divisions in primary and secondary xenogeneic hosts. Altogether, we expect that the substantial gains obtained in HDR efficiency and polyclonal repopulation by our improved editing protocol should broaden applicability of HSC gene editing and pave its way to clinical translation.

9. Therapeutic Base Editing of Human Hematopoietic Stem Cells

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Base editing by nucleotide deaminases linked to programmable DNA-binding proteins represents a promising approach to permanently remedy blood disorders, although its application in engrafting hematopoietic stem cells (HSCs) remains unexplored. Here we purified A3A (N57Q)-BE3 protein for ribonucleoprotein (RNP) electroporation of human peripheral blood (PB) mobilized $CD34^+$ hematopoietic stem and progenitor cells (HSPCs). We observed frequent on-target cytosine base edits at the *BCL11A* +58 erythroid enhancer with few indels. Fetal hemoglobin (HbF) induction in erythroid progeny after base editing or nuclease editing was similar. A single therapeutic base edit of the *BCL11A* enhancer prevented sickling and ameliorated globin chain imbalance in erythroid progeny from sickle cell disease (SCD) and β-thalassemia patient derived HSPCs respectively. Amplicon sequencing showed minimal guide RNA-dependent off-target potential with low-level off-targets observed at only 2 of 59 possible off-target sites. Moreover efficient multiplex editing could be achieved with combined disruption of the *BCL11A* erythroid enhancer and correction of the *HBB* -28A>G promoter mutation. Finally base edits could be produced in multilineage-repopulating self-renewing human HSCs with high frequency as assayed in primary and secondary recipient immunodeficient NBSGW mice, resulting in potent HbF induction in vivo. Compared to non-engrafting progenitor cells, quiescent HSCs favored C to T editing. Together these results demonstrate the potential of RNP base editing of human HSPCs as a feasible alternative to nuclease editing for HSC-targeted therapeutic genome modification.

10. Expansion with E478 Significantly Increases the Rate of CRISPR-Mediated Homology Directed Repair (HDR) and Improves Engraftment of Human Hematopoietic Stem Cells

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Background. Site-specific gene correction of hematopoietic stem cells (HSCs) via HDR is a curative approach for many genetic diseases. HDR requires active cycling of cells, but conditions that permit cycling without compromising HSC number and function remain elusive. Therefore, most HDR protocols minimize culture time, limiting HDR rates and cell yield. *Ex vivo* HSC expansion may circumvent this limitation. Aryl hydrocarbon receptor (AHR) antagonism is a clinically validated method to achieve high numbers of HSCs and rapid and robust engraftment following transplant in malignant and non-malignant settings (Wagner 2019; Orchard 2019). Here, we show for the first time that *ex vivo* expansion of human mobilized peripheral blood cells with a novel, potent AHR antagonist, E478, leads to higher rates of HDR and up to a ~175-fold increase in engraftment of gene-modified HSCs in primary and secondary recipients. ***Results***. In limit dilution studies, E478 expanded the number of NSG-engrafting mobilized blood CD34+ cells by 10-fold compared to uncultured CD34+ cells ($p<0.001$, n=8 mice). Cells were gene modified by lentiviral vector (LVV) or CRISPR/Cas9 and expanded for 1-7 days in the presence of E478, resulting in up to 10-fold higher multilineage engraftment of LVV-transduced cells and CRISPR/Cas9 knockout cells compared to cells in culture for 2 days (n=2 donors, $p<0.001$, n=8 mice). Transduction (~40%) and editing rates (~80%) were maintained *in vivo* at 16 weeks post-transplant. Gene correction approaches rely on cytokines to promote HSC cycling, but this leads to differentiation and loss of durable engraftment. A conventional HDR protocol uses a 2 day pre-stimulation period followed by 1 day of culture post-electroporation (herein called a 2+1 culture), but 33±1.8% of CD34+CD90+ cells remain quiescent after 2 days in culture, whereas only 0.92±0.06% of CD34+CD90+ cells were quiescent after 3-4 days (n=2 donors). To determine whether more active cycling is associated with higher HDR rates, we cultured cells for 1, 2, 3, and 4 days prior to electroporation with CRISPR gRNA targeting the beta-globin gene and transduction with a GFP-containing adeno-associated virus (AAV) donor template. With a 4 day pre-stimulation followed by a 4 day expansion with E478 (4+4 culture), we saw a 6-fold increase in HDR rates and a 134-fold increase in the number of HDR+ CD34+CD90+ cells relative to conventional 2+1 cultures ($p<0.01$). Transplant into NSG mice led to 10-fold higher engraftment (Fig A, $p<0.001$, n=8 mice), 18-fold higher HDR rates (Fig B, $p<0.001$) and a 169-fold increase in the number of HDR+ NSG-engrafting cells relative to 2+1 cultures (Fig C, $p<0.001$) at 16 weeks post-transplant. Notably, a 2+1 culture with E478 led to 12-fold higher engraftment versus 2+1 cultures with DMSO (Fig C, $p<0.001$). Multi-lineage engraftment was observed in all groups. Compared to 2+1 cultures, >175-fold increase in engraftment was observed with E478-expanded cells in secondary recipients ($p<0.001$, n=8 mice). ***Conclusions***. Culture with E478 enables higher HDR rates and up to >175-fold higher numbers of NSG-engrafting cells after gene modification by CRISPR/Cas9 and LVV based approaches.

E478 may enable reduced cost-of-goods and vector requirements and represents a promising approach to realize the full potential of HSC-based gene therapy, including targeted gene correction, for a variety of genetic diseases.

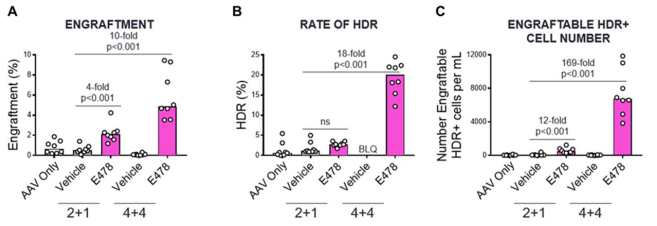

Figure: E478 Optimizes Frequency and Number of HDR+ NSG-Engrafting HSCs. Transplant of 4+4 cultures with E478 led to higher levels of hCD45 engraftment in sublethally-irradiated NSG mice **(A)**, a higher rate of HDR in mice **(B)**, and significantly higher levels of NSG-engrafting hCD45+HDR+ cells **(C)** at 16 weeks post-transplant. Bars represent median and each symbol represents an individual mouse (n=8). BLQ = Below limit of quantification.

11. Development of AAV-Based CRISPR/Cas9 Therapies for Correcting Duchenne Muscular Dystrophy by Targeted Genomic Integration

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Genome engineering technologies are the foundation of exciting potential therapies for correcting genetic diseases. Specifically, Duchenne muscular dystrophy (DMD) is the most prevalent lethal heritable childhood disease occurring in ~1:5000 newborn males. Progressive muscle weakness leading to mortality in patients' mid-20s is a result of mutations in the dystrophin gene. In most cases (~60%), the mutations consist of deletions in one or more of the 79 exons that disrupt the reading frame of the dystrophin transcript. Previous therapeutic strategies typically aim to generate expression of a truncated but partially functional dystrophin protein that recapitulates a genotype corresponding to Becker muscular dystrophy, which is associated with milder symptoms relative to DMD. For example, several groups have adapted the CRISPR/Cas9 technology for gene editing in cultured human DMD cells and the mdx mouse model of DMD to restore the dystrophin reading frame by removing specific exons. However, there remains a need to develop gene editing strategies to restore the complete, fully functional dystrophin protein. This could be accomplished by the targeted insertion of the exonic regions that are lost from the patient's genome. Previously, AAV delivery of CRISPR/Cas9 for homology-independent targeted integration (HITI) was developed for genome editing of neurons *in vivo*. Here, we demonstrate AAV-based HITI-mediated gene editing therapies for correcting the dystrophin gene. Specifically, we adapt the CRISPR/Cas9 gene editing technology to direct the targeted insertion of missing exons into the dystrophin gene. As a therapeutically relevant target, we optimize HITI-mediated genome editing strategies in a humanized mouse model of DMD in which exon 52 has been removed in mice carrying the full-length human dystrophin gene (*hDMDΔ52/mdx* mice). To achieve targeted integration, an AAV vector containing the deleted genome sequence including exon 52 was co-delivered with AAV encoding Cas9/gRNA expression cassettes. Following local injection in the tibialis anterior muscle, we have confirmed targeted exon 52 genomic integration, splicing of exon 52 in dystrophin transcripts, and dystrophin protein restoration in our *hDMDΔ52/mdx* mouse model. Current studies include assessment of HITI-mediated editing following systemic injection. Additionally, to address a larger patient population, we are evaluating insertion of a single "superexon" that encodes the complete dystrophin cDNA sequence downstream of exon 51 in cultured cells and in our *hDMDΔ52/mdx* mouse model. Combined with AAV delivery, the development of HITI-mediated strategies for targeted insertion of missing exons provides a method to restore full-length dystrophin, which could potentially lead to improved functional outcomes.

12. Regulated Allele Specific Gene Editing for Huntington's Disease

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Huntington disease (HD) is a fatal dominantly inherited neurodegenerative disorder caused by CAG repeat expansion within *huntingtin* exon 1. Although mutant huntingtin (mHTT) is ubiquitously expressed, the brain shows robust and early degeneration. To exclusively mitigate expression from the mutant allele, we developed allele-specific genome editing tools that take advantage of single nucleotide polymorphisms that when present in heterozygosity allow us to generate targeted deletions of mHTT exon1. The efficacy and specificity of our tools have been validated *in vitro* in fibroblasts from HD patients, and *in vivo* in a HD mouse model using viral-based delivery methods. Here, we show a novel drug-inducible cassette that allows us to control protein translation both *in vitro*, and *in vivo* in brain and peripheral tissues. To demonstrate the efficacy of our approach, the drug-inducible cassette was cloned to control translation of the SaCas9 protein. Our results show that SaCas9 protein is only transalated when the drug is administrated, and subsequently HTT exon 1 is eliminated. Overall, this unique regulated system will improve the safety of viral-based delivery methods for gene editing.

13. Engraftment and Persistence of HDR-Edited Hematopoietic Stem and Progenitor Cells in Nonhuman Primates

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Introduction: Gene editing of hematopoietic stem and progenitor cells (HSPCs) has shown great promise for diseases including HIV-1 and hemoglobinopathies. To date, most efforts have focused on the generation of semi-random insertions and deletions (indels) via the non-homologous end joining (NHEJ) pathway. For many pathologies however, indels may be more deleterious than the targeted mutation. To address this, numerous groups have targeted the homology directed repair (HDR) pathway to precisely correct a gene of interest. Although a growing body of evidence suggests that HDR cells may engraft less efficiently than NHEJ cells, overcoming this barrier has proven especially challenging. We are interested in addressing limitations

related to the scalability, immunogenicity, and engraftment potential of HDR HSPCs. We have focused our efforts in nonhuman primates (NHPs), an immunocompetent, autologous large animal model that is ideally suited for preclinical studies. **Methods:** Autologous $CD34^+$ HSPCs were isolated from mobilized pigtail macaques (*M. nemestrina*) and edited either within the coding region of the CCR5 gene, or in the promoter of the gamma globin (HBG) locus. Preliminary experiments utilized the Zinc Finger Nuclease (ZFN) mRNA platform coupled with adeno-associated viral vector (AAV2 or AAV5) DNA donors, while later experiments transitioned to CRISPR-Cas9 ribonucleoprotein complexes (RNPs) and single-stranded oligodeoxynucleotides (ssODNs). Small molecules driving stem cell expansion or cell cycle progression/quiescence were also evaluated. **Results:** Our previous studies demonstrated persistent but low-level engraftment of CCR5 ZFN-edited HSPCs in macaques, whereas more recent experiments, namely using CRISPR RNPs directed to HBG have resulted in markedly higher levels of engraftment. We find that CRISPR RNPs support a substantially more stable editing approach than ZFN mRNA, and that ZFN-edited cells' engraftment is significantly lower when HSPCs also received AAV2 or AAV5 donor vectors to drive HDR. Although the size of genetic alterations generated by ssODNs are more limited than AAV, we find that CRISPR/ssODN approaches support higher levels of engraftment of HDR cells in NHPs, relative to ZFN/AAV. HSPC expansion compounds modestly enhance the efficiency of indel formation *ex vivo*, but do not impact engraftment of HDR cells *in vivo*. **Conclusions:** Our macaque model of gene edited HSPC engraftment represents a powerful tool to quantify the long-term persistence of HDR HSPCs. Our work is consistent with and highly complementary to data in immunodeficient mouse models, which similarly suggest that engraftment of HDR HSPCs represents a higher bar than engraftment of NHEJ HSPCs. By focusing on a particularly efficient HDR approach (CRISPR/ssODN), we can rapidly optimize conditions to maximize engraftment and address other potential limitations, for example vector-specific immune responses to AAV donors, and the often-dichotomous requirements for efficient HDR versus long term engraftment of gene edited HSPCs.

14. Highly Efficient Base Correction of Adult Dystrophic Mice Using iABE-NG

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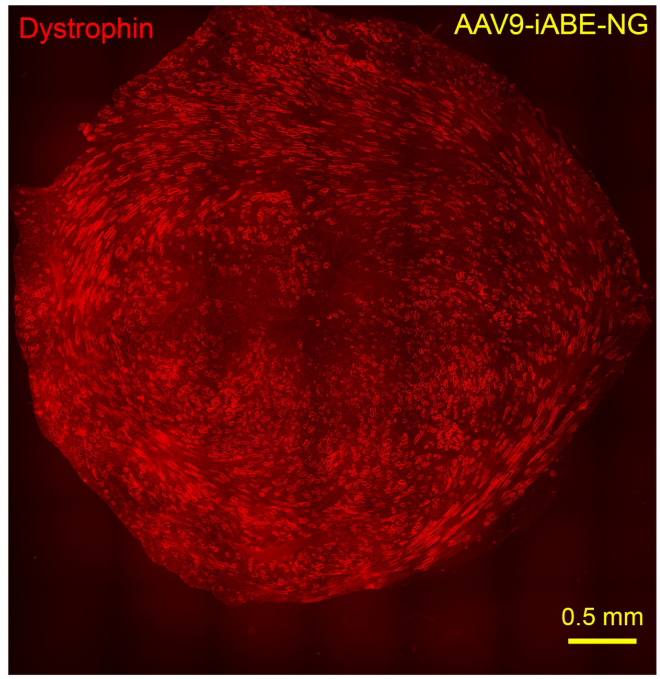
CRISPR genome editing holds great promise for targeting monogenic diseases, including Duchenne muscular dystrophy (DMD). Precise correction of disease-causing mutations in postnatal tissues, however, remains challenging. Recent advances in base editors offer a promising approach to precisely correct the disease-causing single nucleotide variants. More than half of human disease-causing point mutations could be potential targets for base editing correction; however, the majority of these targets could not be suitable for SpCas9 base editing due to the lack of the 5'-NGG PAM sequence within the suitable distance from the mutations. Here we developed a novel adenine base editor with broadened protospacer adjacent motif recognition and eliminated RNA off-targeting editing activity (iABE-NG). Moreover, we engineered super-fast intein-splits of iABE-NG for efficient package into adeno-associated virus 9 (AAV9). Systemic delivery of AAV9-iABE-NG into an adult mouse model of DMD led to widespread rescue of dystrophin expression in the heart muscles (Figure 1). Analysis of the ClinVar database showed that over 70% G>A or T>C point mutations in DMD patients could be potentially targeted for repair by iABE-NG. This study highlights the great promise of iABE-NG for permanent base correction of monogenic diseases.



Cancer - Targeted Gene and Cell Therapy

15. Defining the Role of Spatial and Molecular Adaption of Glioblastoma to Personalized Neural Stem Cell Therapy Using Integrated *In Vivo* and *Ex Vivo* Models

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Intro: Genetically engineered neural stem cells (NSCs) are a promising therapy for the highly aggressive brain cancer glioblastoma (GBM), yet treatment durability remains a major challenge. We sought to define the events that contribute to dynamic adaption of GBM during NSC treatment and develop strategies to convert initial tumor kill into sustained GBM suppression. Using a unique hybrid tumor model treated with human skin-derived induced NSCs (iNSCs) releasing the pro-apoptotic agent TRAIL, we investigated how spatial distribution of tumor and iNSCs affects GBM adaption throughout recurrence. **Methods:** Heterogeneous GBM tumors were grown orthotopically in nude mice using two cell types originating from the same human patient biopsy. Treatment into different tumor regions was initiated 28

days after tumor implant, allowing tumors to develop a large primary mass and significantly invade into the contralateral hemisphere. Serial bioluminescent imaging (BLI) was used to track tumor volumes *in vivo*. In an initial experiment, a subset of mice from each treatment group was sacrificed 6, 13, and 20 days post-treatment to harvest brains and generate living *ex vivo* tissue slices. Tumor burden was spatially mapped in each slice and quantified using fluorescence imaging. Functional drug resistance was assessed in slices containing recurrent tumor by re-treatment with TRAIL *ex vivo*. These results then served a guide for a final *in vivo* survival study, followed by correlative RNAseq analysis to define molecular changes in the primary and invasive tumor tissue regions following iNSC therapy. **Results:** Live animal BLI showed iNSC-TRAIL treatment rapidly decreased tumor volumes when delivered into the primary tumor mass; however, minimal impact on tumor growth was observed when cells were delivered into distal regions of the brain. In contrast, high-resolution imaging of living brain sections showed extensive impacts of iNSC-TRAIL therapy that could not be detected at the macroscopic level with BLI. Slice imaging revealed that iNSC-TRAIL treatment delivered into the primary tumor decreased the solid, but not the invasive, tumor region. Interestingly, delivery of iNSC-TRAIL therapy into the lateral ventricles induced significant tumor kill only in invasive tumor regions, and was more effective at maintaining that inhibition than infusion of iNSC-TRAIL directly into the contralateral parenchyma. A subsequent survival study showed that select multifocal placements of iNSC-TRAIL treatment significantly increased survival. RNAseq analysis of post-mortem samples revealed significant differences in primary and invasive tumor regions, as well as in tumors before and after treatment, but did not show widespread changes in the TRAIL apoptosis pathway. This genetic data supported a TRAIL re-treatment study which showed that brain slices containing recurrent tumor maintained TRAIL sensitivity. **Conclusions:** In this study, we were able to combine the fidelity of *in vivo* studies with the speed and spatial resolution of living brain slice technology. The methods described herein allowed us to observe and quantitatively assay resistance/recurrence mechanisms of aggressive, late-stage tumors in response to iNSC-TRAIL therapy. These findings begin to provide important guidance toward optimizing iNSC/NSC therapeutic infusion and choosing the appropriate therapeutic agent for redosing as we advance toward the ultimate goal of improving GBM patient care in the clinical setting.

16. Transforming Challenges into Opportunities: The T Cell Exhaustion Signature Can Guide the Identification of Patient-Derived, Tumor-Reactive TCRs and Promote TCR Gene Editing for AML

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Allogeneic Hematopoietic Allogeneic Stem Cell Transplantation (AlloHSCT) in the context of haematological malignancies can exert an immune pressure able to eradicate cancer cells surviving conditioning. Nonetheless, disease relapse and transplant-related toxicities are still common events after AlloHSCT. A new therapeutic strategy to improve such outcome, delivering a tumor-specific immunological pressure while mitigating side-effects, could be represented by Adoptive T cell therapy but its use is still hampered by different hurdles, such as the difficulty to isolate tumor-specific T cells for therapeutic purposes. In order to study and isolate circulating tumor-specific T cells, we screened the peripheral blood of N=35 blood cancer patients at serial timepoints after alloHSCT with a 20-colour Flow Cytometry panel including Dextramers to stain peptide-specific T cells using 9 Tumor-Associated Antigen (TAA) peptides and 2 viral peptides. Together, we followed the T-Cell Receptor (TCR) clonal dynamics by high throughput sequencing. TAA-Specific T cells circulate as soon as 30 days after alloHSCT, and unbiased clustering analysis evidenced that they express multiple inhibitory receptors at higher levels when compared with either the total CD8 T cell population or viral-specific T cells, independently from major clinical variables (disease status at transplant, disease type, GvHD incidence). This inhibitory signature was the most pronounced 4 months after alloHSCT and was followed by a substantial contraction of the pool of TAA-specific T cells, particularly of the early-differentiated (Stem Cell Memory and Central Memory) subpopulation. We then established a rapid, highly specific protocol for Dextramer-based isolation and expansion of TAA-specific T cell that yielded 24 colonies from n=13/20 screened patients and from 6 different TAA peptides. TCR sequencing showed that these colonies were oligoclonal, enriched with a dominant T cell clone. When the colonies were challenged with peptide-pulsed targets, however, they displayed minimal lytic activity but when the TCR sequences were cloned into lentiviral vectors and transferred into recipient T cells after Crispr/Cas9 disruption of the endogenous TCR (TCR-edited lymphocytes), cells proved highly efficient and specific in lysing matched pulsed targets. These observations suggested that TAA-specific TCRs can be isolated ex vivo but they are expressed by exhausted T cells. We then tried to broaden the search of Tumor-reactive T cells without relying on Dextramer sorting, exploiting the exhaustion signature to purify a T cell subset enriched with unknown anti-tumour reactivities. We sorted T cells from 3 patients affected by Acute Myeloid Leukemia (AML) on the basis of the inhibitory receptors (IR) expression and stimulated both IR+ and IR- cells with matched Leukemic Dendritic Cells (LDC). Serial LDC stimulations promoted the expansion of dominant clones over time in the IR+ but not in the IR- fraction and IR+ T cells proved superior in killing autologous AML blasts in vitro. Again, dominant TCRs were identified in IR+ cultures of all three patients, and TCR-edited lymphocytes expressing the isolated TCR efficiently and selectively recognized autologous blasts (2/2 of the tested patients). Our findings shed some light on the in vivo dynamics of tumor-specific T cells and introduce two models for the isolation of tumor-specific TCRs, one requiring the knowledge of the target peptide, the other exploiting the IR signature to discover novel tumor reactivities.

17. Tumor-Selective Gene Circuits Enable Highly Specific Localized Cancer Immunotherapy

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The treatment of certain cancers has been revolutionized by immunotherapy, but not all patients benefit due to a limited mechanism of action and tumor-mediated immune suppression. More potent and broadly acting immunotherapies have received considerable interest, but their narrow therapeutic window upon systemic administration creates the need for spatiotemporal regulation, which has yet to be realized in a clinical setting. To address these challenges, we developed a cancer-specific gene circuit platform that enables targeted, multifactorial immunomodulation of tumors in a highly specific and localized manner to drive efficacy through the therapeutic window. First, we constructed a library of 6,107 synthetic promoters built from tandem arrays of all human transcription factor DNA binding sites. We used next generation sequencing and machine learning to screen the library for promoters that expressed specifically and robustly in a particular cancer cell type. When applied to ovarian cancer, breast cancer, and glioblastoma, this approach yielded promoters with activities >100-fold higher in each respective cancer cell type than in corresponding non-cancerous primary cells. Second, we further enhanced expression specificity by using these promoters to build AND logic-gated gene circuits in which expression of therapeutic payloads requires two independent cues - the activity of two distinct transcription factors, or, alternatively, a transcription factor and a microRNA. When we armed an ovarian cancer-specific gene circuit with multiple immunomodulatory payloads (a T cell engager that enables antigen-independent T cell responses, the cytokine IL-12, the chemokine CCL21, and an anti-PD1 checkpoint inhibitor antibody) and delivered it to cells, the gene circuit triggered ovarian-cancer-specific immune responses both in vitro and in vivo. This approach resulted in statistically significant reduction in in vivo tumor burden (>6-fold reduction in tumor burden at day 39 post tumor implantation; $p < 0.005$) and prolonged mouse survival in a humanized ovarian cancer model (all control mice died by day 55 post tumor implantation, whereas 80% of mice survived to the end of the study in the treatment group; $p < 0.005$). These results suggest that these AND logic-gated gene circuits can be readily reconfigured for different cancer types through modular replacement of the promoters and/or miRNA-binding sites. Thus, this technology has the potential to enable highly targeted and effective treatment of cancer via precise immunological programming.

18. MyD88/CD40 (MC) Enhances Chimeric Antigen Receptor Natural Killer (CAR-NK) Cell Proliferation, Cytokine Release and Anti-Tumor Efficacy Against BCMA$^+$ Tumors

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Background: Engineering of chimeric antigen receptors (CAR) into donor-derived, allogeneic Natural Killer (NK) cells holds promise as an off-the-shelf cell therapy for cancer. NK cells offer high anti-tumor potency with low risk for development of GvHD relative to αβT cells but suffer from poor growth and persistence *in vivo.* Our published work (*Mol. Ther 25:2176 (2017), Mol Ther Onc 12:2124 (2018), Leukemia 33:2195 (2019))* demonstrated that MC provides potent costimulatory activity to CAR-T cells. MyD88 acts a signaling node for the NK-activating receptors for IL-18 and IL-1, therefore we evaluated the effects of the engineered signaling molecule MC in CAR-NK cells. Here we describe the engineering of MC-enabled CAR-NK vectors targeting B cell Maturation Antigen (BCMA) to control multiple myeloma. **Methods:**Natural Killer cells from up to 9 donors were isolated by CD56$^+$selection, activated with Il-15 and K562 cell targets and transduced with γ-retrovirus (rv) encoding four genes (iC9-BCMACAR.ζ-2a-MC-IL15, and control combinations) in which MC is partially membrane tethered and constitutively active and inducible caspase-9 (iC9) encodes a rimiducid-inducible proapoptotic safety switch. In the dual switch configuration (iMC-BCMACAR.ζ-IL15; iRC9-ΔCD19) iMC activity was inducible by chemically induced protein dimerization with rimiducid and the iRC9 safety switch was directed orthogonally with rapamycin or its prodrug temsirolimus. Co-culture assays were performed against BCMA-expressing myeloma targets NCIH929, RPMI2101, U266, and THP1 AML cells in Incucyte imaging incubators. Cytokine production at 48 hours was measured by Luminex multiplex assays. For *in vivo* assays of NK cell growth and tumor control, immunodeficient NSG mice were injected i.v. with up to 1.5 x 10^6NCIH929-GFPffluc or THP1-GFPffluc cells and challenged subsequently with to 1-5 x 10^7BCMACAR-NK cells. **Results:** NK cell transduction was efficient at 40-60% in the one or two-vector configurations. Induced or constitutive MC activity in transduced cells stimulated NK cell growth which selectively expanded to ~90% of the NK cultures in one week. MC activity or IL-15 autocrine expression alone failed to support human NK cell engraftment in NSG mice, but in combination produced a synergistic outgrowth and persistence in animals beyond 6 weeks even without an activating tumor target. MC activity greatly enhanced innate and BCMA CAR-specific NK cell cytotoxicity in cocultures with myeloma and AML target cells *in vitro.* MC-driven cytotoxicity did not correlate with alterations in the expression profile of innate NK activating and inhibitory receptors relative to controls but rather to induction of MAPK (p38, JNK, ERK), Akt, NF-κB and Vav1 signaling leading to increased levels of Fas ligand, granzymes and perforin. Furthermore, MC activity drove higher level production of cytokines and chemokines normally produced by activated NK cells (eg. IFN-γ, TNF-α, TNF-β, CCL2). The combination of MC activation with autocrine IL-15 supported the persistence and antitumor activity of 1stgeneration BCMA.CAR-NK cells *in vivo*. Dual-switch BCMA.CAR-NK cells given as a single

10^7cell dose controlled THP1 tumor outgrowth for six weeks, but only with rimiducid treatment were persistent CAR-NK cells evident in spleen and bone marrow upon sacrifice. Constitutive MC activity from the single BCMA-CAR vector configuration at a higher dose supported complete NCIH929 myeloma control beyond six weeks. These results support the development of MC enabled BCMA-targeted CAR-NK cells as potent therapy for bone marrow resident malignancies with the further potential for use as an allogeneic transplant.

19. Abstract Withdrawn

20. Nanoparticles for Targeted Theranostic Gene Delivery to Hepatocellular Carcinoma

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Introduction Hepatocellular carcinoma (HCC) develops predominantly in the inflammatory environment of a cirrhotic liver caused by hepatitis, toxin exposure, or liver disease. A targeted therapeutic approach may enable efficacy without causing toxicity and liver failure. Nucleic acid therapeutics can be designed for selective expression in cancer cells using cancer-specific promoters for transcriptional targeting, but safe and effective delivery remains challenging. Biodegradable poly(beta-amino) ester (PBAE) nanoparticles (NPs) have been developed for biomaterial-based selective transfection of HCC cells over hepatocytes. Here, PBAE NPs were used to deliver transcriptionally-targeted theranostic DNA with high selectivity towards alpha fetoprotein (AFP)-producing HCC cells. **Methods** AFP-producing human HCC cell lines (Huh7 and Hep3b) and hepatocytes (THLE3) were transfected with PBAE NPs harboring plasmid DNA. After transfection, the cells were exposed to a 1.25 μg/mL dose of ganciclovir (GCV), which is cytotoxic when phosphorylated by the SR39 enzyme. To study radiotracer accumulation, SR39-transfected cells were also incubated for one hour with 10-12 μCi/mL of 9-(4-^{18}F-fluoro-3-[hydroxymethyl]butyl)guanine ([^{18}F]FHBG), a radiotracer for positron emission tomography (PET) that accumulates in cells after phosphorylation by SR39. For *in vivo* studies, athymic nude mice were subjected to a laparotomy, and 10^6 Hep3b cells were injected under the liver capsule. Xenograft-bearing mice were injected intravenously with PBAE NPs carrying a 25 μg dose of firefly luciferase (fLuc) plasmid DNA. 24 hours later, animals were injected intraperitoneally with 150 mg/kg D-luciferin, imaged using IVIS, and bioluminescence was measured over regions of interest. **Results** We selected AFP for transcriptional targeting because its expression decreases rapidly after birth, but transcription-level changes drive high expression in HCC. We constructed two therapeutic plasmids harboring a SR39 suicide gene: constitutively expressed CMV-SR39 and transcriptionally targeted AFP-SR39. Therapeutic DNA was delivered using PBAE 536 NPs. Following a single transfection with CMV-SR39 and treatment with prodrug GCV, viability of HCC (Huh7 and Hep3b) and hepatocytes (THLE3) was reduced below 20% by Day 7 (Fig 1A). When transcriptional targeting was employed using AFP-SR39, THLE3 viability was maintained above 90%, while Hep3b and Huh7 viability fell to 15% and 25% by day 9, respectively (Fig 1B). When [^{18}F]FHBG was added to transfected cells, AFP-SR39 facilitated 43 and 50-fold higher accumulation in Huh7 and Hep3b HCC cells over THLE3, respectively (Fig 1C). Further, systemically administered PBAE NPs harboring fLuc enabled efficient gene delivery to HCC tumor xenografts (Fig 1D). Average flux 24 hours after NP administration was significantly increased over background and was localized to the tumor, with a 7- fold higher average radiance in tumor over liver tissue. **Conclusion** Transcriptionally-targeted SR39 delivery to HCC furthers the development of a theranostic platform for simultaneous treatment of the cancer with ganciclovir therapy and tumor monitoring with [^{18}F]FHBG and PET/CT.

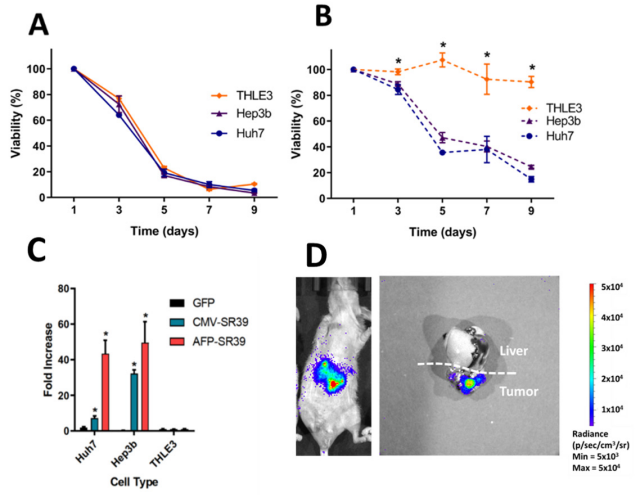

Fig 1. Cytotoxicity of (A) CMV-SR39 and (B) AFP-SR39 NPs + GCV (C) [^{18}F]FHBG uptake in transfected HCC, normalized to hepatocytes (D) fLuc delivery to xenografts by PBAE NPs

21. Tumor-Tropic Liposome-Mediated Therapeutic Delivery of mRNA for T Cell Malignancies

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Introduction: Treatment options for relapsed T cell leukemia are limited, and the prognosis remains dismal. Therefore, the development of new therapies is crucial. A liposomal delivery system has been increasingly recognized as a promising strategy for delivering both reagents and nucleic acids. However, little is known about whether the liposomal delivery of mRNA could be employed for cancer treatment. Herein, we propose a novel strategy for the treatment of T cell malignancies using tumor-tropic liposomes that can selectively deliver mRNAs of interest to leukemia cells. **Methods:** We tested liposomes

with various lipid compositions. Leukemia cells or peripheral blood mononuclear cells (PBMCs) were cultured with rhodamine-labeled liposomes and then analyzed for rhodamine expression to investigate the selective uptake of the liposomes. To examine the selective translation of the encapsulated mRNA, the cells were incubated with liposomes loaded with firefly luciferase (ffLuc) mRNA and examined for luciferase activity. The cells were also treated with liposomes loaded with inducible caspase 9 (iCas9) mRNA, with or without B/B homodimerizer (chemical-induced dimerization, CID), to examine the *in vitro* anti-leukemic effects. To investigate the *in vivo* anti-tumor effects, NSG mice were inoculated with Jurkat-ffLuc cells, and then they were intravenously treated with either liposomes loaded with iCas9 or control liposomes on days 4 and 15 after tumor inoculation. Every 24, 48, and 72 h after liposome infusion, the mice were treated intraperitoneally with CID. Bioluminescence imaging was performed twice weekly to track the leukemia cell burden. **Results:** The screening analysis identified two types of liposomes (No. 43 and No. 79) that could selectively deliver mRNA to the cancer cells. Flow cytometry analysis showed that these liposomes labeled with rhodamine were efficiently taken up into Jurkat cells, whereas they were minimally taken up into PBMCs. Furthermore, when these two liposomes were loaded with ffLuc mRNA (43-ffLuc and 79-ffLuc), they could efficiently deliver mRNA to the cancer cells, with enhanced luciferase activity, but they minimally delivered ffLuc mRNA to PBMCs. Consistently, when these two liposomes were loaded with a suicide gene iC9 (43-iC9 and 79-iC9) in combination with CID, they effectively killed Jurkat cells and CCRF-CEM cells but minimally killed PBMCs *in vitro*. Furthermore, in a xenograft model of Jurkat-ffLuc, the mice treated with 79-iC9 showed significantly suppressed tumor growth compared with the mice treated with control liposome in combination with CID (Figure 1). These results suggested that the tumor-tropic liposomal delivery of iC9 mRNA could be employed for the treatment of T cell leukemia. **Conclusions:** Tumor-tropic liposomes can selectively deliver mRNAs of interest to leukemia cells. Moreover, tumor-tropic liposomes loaded with iCas9 in combination with CID showed anti-leukemic activity both *in vitro* and *in vivo*. Thus, liposomal delivery could be a promising alternative for the treatment of T cell malignancies.

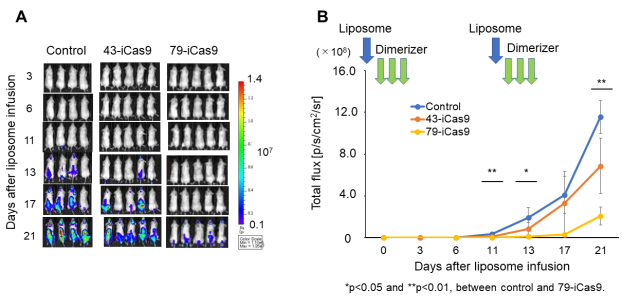

Figure 1. Tumor-tropic liposome suppressed leukemia progression in a xenograft mouse model.
(A) Bioluminescence imaging and (B) summary of total luminescence from Jurkat-ffLuc cells in control and treated mice. iCas9, inducible caspase 9.

AAV Vectors - Virology and Vectorology III

22. Evolution and Investigation of Engineered AAV Capsids Exhibiting Enhanced Transduction of the Central Nervous System with or without Murine Strain Specificity

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Recombinant adeno-associated viral (rAAV) capsids are increasingly used as gene delivery vectors in science and in therapeutics. However, there is room for further improvement on the efficiency and specificity to transduce the central nervous system (CNS) via non-invasive systemic delivery. We have shown in the past that marked improvement is possible using a Cre recombination-based AAV targeted evolution (CREATE) platform to identify AAV-PHP.B and AAV-PHP.eB capsids, which broadly transduce the CNS (Deverman et al, 2016; Chan et al, 2017). While CREATE was successful in identifying efficient CNS vectors in two rounds of evolution, the method was limited by its ability to identify only a few top candidates from the selection. To truly utilize the potential of a large selection design (such as a library of ~1.28 billion theoretical size using 7-mer-NNK mutagenesis strategy) across multiple different selection targets (such as endothelial cells, neurons and astrocytes), we developed Multiplexed-CREATE (M-CREATE). This method involves: (1) application of a positive selection pressure for on-target delivery and post-hoc negative selection against off-target delivery using next generation sequencing, (2) an unbiased selection design, and (3) a novel data analysis platform. M-CREATE identified distinct families of variants based on shared amino acid motifs. These include a family of PHP.B-like variants that appear to be dominant across *in vivo* selections for CNS transduction in Cre-transgenic adult mice. While some variants from this family showed similar tropism to PHP.B as expected, two variants (PHP.Vs) were distinct by exhibiting biased transduction to the vascular cells forming the blood-brain barrier (BBB). In addition, the new analysis platform allowed us to further mine the selection that yielded PHP.eB from its PHP.B parent (Chan et al, 2017) and therefore uncover PHP.N, that differs by 3 amino acids from PHP.B and shows greater specificity in transducing neurons over other CNS cell-types. This further demonstrates that small sequence variations within a family can yield different tropisms. Recent reports on the non-permissibility of PHP.B or PHP.eB in BALB/cJ (Hordeaux et al, 2018; Matsuzaki et al, 2019), and the subsequent identification of LY6A as the receptor underlying the improved BBB entry (Hordeaux et al, 2019; Huang et al, 2019; Batista et al, 2019) has motivated us to investigate the non-dominant families with distinct amino acid signatures in our 7-mer NNK library. We identified several non-dominant families (PHP.Cs), exhibiting better transduction of the CNS compared to parent AAV9 while also being efficient in crossing the BBB across mouse strains, including BALB/cJ. This suggests that

variants with different amino acid motifs from the PHP.C-like families may have different mechanisms of BBB transmission, thereby making them promising candidates for further study toward translation of AAV vectors across strains and species.

23. Engineering and Evaluation of Novel AAV Serotypes for Gene Delivery to the Central Nervous System

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Efficient gene delivery to the central nervous system (CNS) remains a barrier to the clinical translation of gene therapies. AAV capsid engineering is a promising approach to improve CNS delivery, and research on serotypes such as PHP.B has highlighted the importance of applying cell type-specific functional selections in models that exemplify the genetics and physiology of the human CNS. Here we have developed and evaluated two functional selection strategies across multiple models, including cynomolgus macaques, in order to identify AAV variants that transduce neurons following administration into the cerebrospinal fluid. In these selections we evaluated capsid libraries containing peptide insertions of 7, 10, and 15 amino acids in AAV1, AAV2, AAV3B, AAV6, AAV8, and AAV9. The first selection strategy employed an EGFP-Histone H2B fusion expressed from a neuron-specific human synapsin1 (hSyn1) promoter to incorporate EGFP into the chromatin of neurons. AAV-transduced neuronal nuclei were isolated by fluorescence-activated sorting (FACS) from brain tissue and analyzed by next-generation sequencing (NGS) to quantify enrichment of AAV variants. Nuclei contain transcriptionally active AAV vector genomes and are readily sorted due to their uniform morphology, minimal autofluorescence, and absence of cellular projections. The second selection strategy utilized high-throughput DNA synthesis in selection round 2 to link each AAV variant to a unique barcode located in the three prime untranslated region of the EGFP-H2B mRNA. In both strategies the hSyn1 promoter was placed in an opposite orientation to the p40-driven capsid library. This design avoids *in vivo* transcription of the *cap* mRNA and enables head-to-head comparison of libraries derived from multiple parental serotypes using universal NGS primers. During round 2 library synthesis we included degenerate sequences for each AAV variant, added control serotypes, and specified barcodes that were computationally designed to minimize transcriptional bias and NGS collisions. To track the progression of AAV selections we conducted NGS on library plasmid DNA, manufactured virus, and variants recovered following screening. A custom bioinformatics suite was created to rank variant performance based on metrics of enrichment and noise and to advance our understanding of the impact of library size on selections. Capsid libraries were evaluated in cynomolgus macaques, C57BL/6J mice, human iPSC-derived neurons, primary mouse cortical neurons, and Neuro2A cells. After two rounds of library selection and NGS analysis we identified multiple engineered AAVs that were substantially and robustly enriched as compared to parental serotypes in cynomolgus macaques across 13 CNS regions and both genders. In ranking enrichment of engineered AAVs across species we found capsids that were potent in multiple models as well as those that were disparate in performance. A subset of 34 high-performing novel variants and several control serotypes were selected for further evaluation as a barcoded pool. For this evaluation we designed an expression cassette including both the hSyn1 and hU6 promoters in order to rank AAV variant transcription in neurons and all cells respectively. The results of serotype evaluation in cynomolgus macaques, Sprague-Dawley rats, C57BL/6J mice, human iPSC-derived neurons, and primary mouse cortical neurons will be shared.

24. Cross-Species Evolution of Synthetic AAV Strains for Clinical Translation

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Recombinant adeno-associated viral (AAV) vectors continue to show promise for gene therapy of rare genetic disorders such as spinal muscular atrophy, muscular dystrophies and myopathies. As we expand the scope of AAV gene transfer applications, an important challenge that remains unaddressed hinges on differences in AAV tropism across different species. The latter often results in non-linear vector dose-biodistribution relationships when scaling from small to large animal models and subsequently impacts clinical translation. For instance, studies evaluating AAV spread in the brain and spinal cord or AAV transduction in systemic organs such as the heart, liver or lung have demonstrated that vector biodistribution and transduction can vary significantly for a given dose between mice, xenograft models, canine, pig, non-human primates and ultimately, in patients. One approach to address this unmet need is to engineer AAV biology to demonstrate greater translatability across species. Here, we describe a cross-species evolution approach that involves sequential cycling of AAV capsid libraries in multiple animal models. Briefly, AAV surface loops were subjected to saturation mutagenesis to generate AAV libraries, which were injected systemically into mice and pigs in alternating cycles. New AAV variants enriched in different regions of the brain, spinal cord, heart, skeletal muscle and satellite muscle cells derived from one species were amplified, pooled under different subgroups, injected into a different animal model and subjected to further cycling. We characterized the expression profiles of several novel, lead AAV variants in mouse and pig models following intravenous, intracisternal or intrathecal injections. Robust expression in heart, skeletal muscle, liver, kidney, vasculature and other organs were observed at doses 4-5 fold lower than those currently being utilized in DMD and SMA clinical trials. Intrathecal infusion in pigs afforded gene expression in motor neurons, white and grey matter of the spinal cord as well as the cerebellum. While final rounds of cycling in non-human primates are

currently underway and the CNS transduction profile of these cross-species compatible vectors (ccAAVs) is forthcoming, our intermediate results support the hypothesis that cross-species cycling of AAV libraries can afford new capsids demonstrating greater compatibility across different animal models. We envision that these new ccAAV strains can provide an efficient gene transfer platform with improved correlation in dose-response as we scale from preclinical animal models into patients.

25. A Highly Efficient Dual AAV Technology for Therapeutic (epi)Genome Editing Applications

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Recombinant adeno-associated viral vectors (rAAVs) are the gold standard in gene therapy. Many genes, however, exceed the packaging capacity (4.7 kb) of rAAVs such as various CRISPR/Cas modules harboring high therapeutic potential. To circumvent the limited rAAV payload, dual rAAV vector approaches are used focusing on the reconstitution of split large genes at the genome level (concatemerization- or recombination-dependent) or at the split intein-mediated protein level. Nevertheless, gene reconstitution at the genome level leads to low reconstitution efficiencies, whereas split inteins strongly depend on the split position and generate potentially pathogenic proteins or protein fragments. Here, we present an optimized dual vector strategy for the reconstitution of genes via *trans*-splicing at the mRNA level. Using a fluorophore-based splice reporter assay, we show that this technology is highly efficient, accurate, position-independent and broadly applicable *in vitro* reaching reconstitution efficiencies of up to 78 %. To demonstrate the principle capability of this technology *in vivo*, we aimed at reconstituting the catalytically active split *Sp*Cas9 fused to the transcriptional activator domains VPR (*Sp*Cas9-VPR). As the spacer length of single guide RNAs (sgRNAs) determines the enzymatic activity of Cas9, *Sp*Cas9-VPR can be used to knockdown and activate different genes simultaneously by multiplexing different sgRNAs. Such an approach can e.g. be utilized to treat gain-of-function mutations, which require an efficient gene knockdown followed by gene supplementation. One well-characterized gain-of-function model for retinitis pigmentosa are mice carrying the common P23H mutation in rhodopsin. Accordingly, *Sp*Cas9-VPR could be applied in this mouse model by knocking down rhodopsin (*Rho*) while simultaneously activating its functional equivalent M-opsin (*Opn1mw*). To test this strategy, dual rAAVs expressing split *Sp*Cas9-VPR, *Opn1mw*- and *Rho*-targeting sgRNAs were delivered subretinally into C57Bl/6J wild type mice. The *Sp*Cas9-VPR reconstitution efficiency of our optimized mRNA *trans*-splicing technology was compared to the split intein approach via qRT-PCR. Four weeks post-injection the mRNA *trans*-splicing approach yielded a similar *Opn1mw* activation efficiency and an increased *Rho* knockdown efficiency when compared to the split intein approach. Finally, reconstitution via mRNA *trans*-splicing also led to efficient *Rho* knockdown and *Opn1mw* activation in the P23H mouse model. In conclusion, we present an optimized, versatile and highly efficient dual rAAV approach suitable for reconstitution of large genes at the mRNA level. In the experimental setup used herein, this approach is at least as effective as the split-intein technology, but offers a more convenient design and most likely a more favorable safety profile. Using this technology, we also provide initial experiments designed to treat gain-of-function mutations with a split *Sp*Cas9-VPR-based multiplexing strategy.

26. A Novel AAV9 Variant Exhibiting Strong Preference for Neuronal Transduction in Mice and Non-Human Primates Following Systemic Administration

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The arrival of adeno-associated virus (AAV)-mediated gene therapy to the clinic promises to revolutionize the treatment of central nervous system (CNS) diseases. Of particular clinical interest is the potential for global transduction of the CNS via non-invasive, systemic administration of AAV vectors. However, there remain multiple challenges to the future of AAV-mediated CNS gene therapy including (1) relatively poor transduction and a strong bias for transduction of astrocytes over neurons in the primate brain, and (2) prominent dissemination of AAV vectors to the liver leading to decreased bioavailability to the brain and increased immunogenicity. Previously, we reported application of the transcription-dependent directed evolution (TRADE) system to an AAV9-based variant library harboring a liver-detargeting mutation. High-throughput characterization of neuronal transduction using a human synapsin I-based DNA/RNA barcoding system identified the AAV9-N272A-HN1 variant that exhibits enhanced brain neuronal transduction in both mice and non-human primates (NHP). We validated this finding in C57BL/6J and BALB/cJ mice using a conventional approach of administering AAV-GFP reporters and performing immunohistology. Here, we report validation of AAV9-N272A-HN1 enhancement in NHP using an epitope-tagged GFP reporter approach. In order to overcome the challenges of using a small number of highly heterogeneous non-human primates, we developed a method that allowed for within-subjects comparisons of AAV9 and AAV9-N272A-HN1 transduction at the level of transgene protein expression. In brief, AAV9 and AAV9-N272A-HN1 capsids were packaged with CAG promoter-driven transgene cassettes encoding GFP tagged on the N-terminus with either HA or FLAG epitopes. These vector genomes also incorporated DNA/RNA barcode libraries (i.e. AAV-CAG-HAnlsGFP-BCLib and AAV-CAG-FLAGnlsGFP-BCLib). Two libraries were mixed with inverted capsid-epitope relationships. Each library was then administered into a rhesus macaque at a dose of 3×10^{13} vg/kg intravenously. Animals were sacrificed three weeks post-injection and brain tissues were processed

for immunofluorescent staining. Total transduction drastically differed in the two animals, supporting our rationale for using a within-subjects methodology. Our preliminary analysis confirmed that AAV9-N272A-HN1 neuronal (NeuN+) transduction efficiency is several fold greater than that of AAV9 across a variety of brain regions. Remarkably, AAV9-N272A-HN1 exhibited >10-fold greater transduction of neurons compared to AAV9 in the motor cortex of the animal exhibiting higher transduction efficiency. Additionally, we found that the AAV9-N272A-HN1 variant transduces neurons in the rhesus brain with a specificity of up to 94% in certain brain regions. This strong capsid-mediated neuronal tropism was confirmed in mice, where we observed a neuronal specificity of 96%. Finally, we utilized AAV RNA BC-seq to assess transduction efficiency in peripheral tissues. We observed that the AAV9-N272A-HN1 variant is moderately detargeted from many major organs, including the liver, relative to AAV9. In conclusion, we have characterized the TRADE-derived AAV9-N272A-HN1 variant in NHPs and found that it demonstrates enhanced brain transduction with strong capsid-mediated neuronal specificity, while simultaneously exhibiting reduced transduction of many major organs.

27. Sequencing of Barcoded AAV Libraries Enables Reprogramming of AAV Capsids with Respect to Liver Tropism

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Study of naturally occurring adeno-associated virus (AAV) capsids has revealed a broad range of phenotypes such as assembly, biodistribution , tissue tropism, and host response. Additionally, researchers have generated large mutational libraries to further interrogate and even engineer the extent to which mutations in the capsid govern these relevant biological functions. The ability of AAV capsids to successfully traffic to and transduce cells within the liver has been extensively reported on. In some gene therapy approaches, liver tropism is a highly desired property of the capsid, while in other approaches liver tropism may be undesirable. Here, our group reports the discovery of a single amino acid mutation with the potential to dramatically affect the capsid's ability to transduce the liver. We generated a barcoded library of 2048 viral capsids through ancestral sequence reconstruction around the most recent common ancestor of AAVs 1-3, 6-9 (BC-Anc80Lib). The library was spiked with known amounts of barcoded AAVs known to transduce murine liver to varying degrees. This pooled library was injected into mice, and tissues were collected at different timepoints. To ascertain the transduction efficiency of the injected AAVs, barcodes were isolated from both gDNA and mRNA and subjected to deep sequencing. Following analysis, we recapitulate many of the findings that the field has previously reported regarding the ability of AAVs such as AAV2, 8, and 9 to differentially transduce the liver. Moreover, we uncovered a novel single amino acid change within our library which appeared to modulate the ability of these capsids to transduce liver by up to 64-fold. To confirm this finding, we clonally characterized Anc80 viral capsids predicted to have altered liver tropism. We produced and injected them individually in mice and assessed DNA/RNA amounts of a reporter gene by digital-droplet qPCR. We report greater than a 100-fold reduction in expression in Anc80 vectors predicted to be deficient for liver tropism. When our group examined transcript abundances in the hearts of these mice, the liver-off vectors only demonstrated a modest (~3-fold) reduction in expression, suggesting that this single amino acid change might have differential effects across tissues in the same species. Finally, our group sought to determine whether this single amino acid change was sufficient to retarget other AAVs beyond our Anc80 vectors, including AAV3B, a vector that in recent years has garnered interest due to its reported ability to efficiently transduce primate hepatocytes, but which also has been reported to transduce murine liver poorly. Upon grafting the 'liver-off' state into AAV3B viruses, we report a greater than 10-fold decrease in liver expression relative to wildtype AAV3B. Grafting the 'liver-on' state into AAV3B resulted in an average increase of expression within the liver of greater than 10-fold when compared to wildtype - levels that we show to be comparable to those of known murine liver-tropic vectors such as AAV9. Taken as a whole, we present evidence that a single point mutation discovered through deep sequencing of barcoded AAV-libraries may be able to broadly modulate liver tropism of AAV capsids.

28. Deamidation of Adeno-Associated Virus Capsid Impacts Transduction Activity in AAV9

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Very little is currently known about how post-translational modifications (PTMs) of the adeno-associated virus (AAV) capsid impact efficacy. One such PTM is deamidation, which occurs when the amide group of an asparagine is lost after a nucleophilic attack from an adjacent main-chain amide. Our group previously found that asparagine deamidation in AAVs is common. In fact, the extent of modification reaches nearly 100% at some sites. In sites with sequence asparagine glycine (NG), deamidation occurred rapidly in AAV8 and AAV9 and was correlated with up to a 60% loss of *in vitro* transduction efficiency. Here, we explore the impact of global vector deamidation on factors that are important for AAV function *in vivo*. Using barcode methodology in mice, we found that AAV9 deamidation impacts transduction efficiency in all tissues tested (i.e. brain, heart, liver, kidney, skeletal muscle, spleen, and gonads) following IV administration. Transcripts originating from low-deamidation vectors were, on average, four-fold more abundant than transcripts originating from a co-delivered, high-deamidation vector. We also present data on the impact of deamidation on AAV8 and AAV9 susceptibility to neutralizing antibodies. A decrease in susceptibility to these antibodies could be a critical factor for intravenous administration and preventing suppression of gene therapy vectors. Finally, we discuss strategies for engineering capsids that can identify amide-stabilized variants of AAV9 with improved function.

CAR T-Cell Therapies I

29. Results of Four Clinical Trials using Autologous T-Cells, Genetically Modified at the CCR5 Gene by Zinc Finger Nucleases, in HIV-Infected Subjects

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Although antiretroviral therapy has revolutionized the treatment of HIV-1 infection, there is a need for a life-long therapy aiming at "HIV cure". The first ever *ex vivo* gene editing clinical trial in humans was conducted by Tebas et al [2014] using autologous T-cells genetically modified at the *CCR5* Gene by Zinc Finger Nucleases (ZFN). This study was conducted in subjects with HIV. In parallel, four additional clinical trials were also conducted using autologous T cells which were genetically edited by knock-out of the *CCR5* gene using Zinc Finger Nucleases (ZFN). CCR5 is one of the co-receptors to CD4 for HIV, and subjects who are homozygous for CCR5 deletion are protected against HIV infection [Liu R et al, 1996]. The objective of these studies was to augment HIV-specific T-cells and to reverse or decrease the progressive destruction of CD4 T-cells that leads to clinical AIDS, by protecting these CCR5-deleted T cells from HIV infection. In these 4 studies described in this abstract, the ZFN modification of the T cells was either done via an adenoviral vector (vector SB-728-T) or by electroporation (vector SB-728mR). Different HIV subject populations were studied, and the cells were administered alone or with cyclophosphamide as a conditioning regimen to allow nesting, and space for these modified T cells. In total, 74 subjects with HIV were treated with ZFN-CCR5-deleted T cells, and 47 (63.5 %) underwent analytical treatment interruption or ATI. Six subjects were identified as showing delayed viral rebound or prolonged undetectable viral load. The details of the 4 studies and the results will be presented at the conference. Following the clinical experience gained in these clinical trials, studies are ongoing with academic partners. **Reference**: Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G et al. Gene editing of CCR5 in autologous CD4 T cells of persons with HIV. NEJM 2014; 370:901-910; Liu, R et al, Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell 1996: *86*, 367-377.

30. CT-0508 is an Anti-HER2 Chimeric Antigen Receptor (CAR) Macrophage that Promotes a Pro-Inflammatory Solid Tumor Microenvironment and Eliminates Cancer Cells via Phagocytosis

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Despite recent advances in T cell immunotherapy for the treatment of human cancer, metastatic solid tumors remain an intractable challenge. Macrophages are often the most abundant immune cell in the tumor microenvironment (TME) where, as immunosuppressive tumor associated macrophages (TAMs), they participate in disease progression. Currently, most macrophage based immunotherapeutic approaches are focused on the depletion, repolarization, or phagocytic disinhibition of TAMs. We have developed a new paradigm based on the adoptive transfer of genetically engineered CAR macrophages (CAR-M) for the treatment of human cancer. CAR-M can be efficiently produced using the chimeric adenoviral vector Ad5f35. We have previously shown that the primary mechanism of action of CAR-M is antigen dependent phagocytosis, and that a single dose of primary human anti-HER2 CAR-M leads to significantly improved overall survival in multiple solid tumor xenograft models. Given that Ad5f35-transduced anti-HER2 CAR-M (CT-0508) adopt a unique pro-inflammatory M1-like phenotype, we hypothesized that CT-0508 may have the capacity to reprogram the TME toward an activated state. Functional evaluation and transcriptome-wide characterization revealed that CT-0508 maintain a pro-inflammatory phenotype despite challenge with immunosuppressive environments *in vitro*. By engrafting immunodeficient mice with human hematopoietic cells and human cancer cells we established a novel xenografted human TME model. We demonstrate with single cell resolution that CT-0508 maintain their M1 phenotype within the human TME. Additionally, CT-0508 augmented the human TME by inducing a pro-inflammatory signature in surrounding immune cells, characterized by induction of MHC-II and TNF. To further investigate the potential of CT-0508 for TME activation, we modeled the interaction of CT-0508 with primary human M2 macrophages, dendritic cells, and T cells in vitro. CT-0508 repolarized bystander M2 macrophages toward a pro-inflammatory phenotype, induced activation and maturation markers on immature dendritic cells, and recruited resting as well as activated T cells in chemotaxis assays. CT-0508 demonstrated enhanced antigen presentation when compared to control human macrophages and cross-presented tumor derived intracellular antigens to CD8 T cells after tumor phagocytosis. Our results show that in addition to direct anti-tumor activity, the anti-HER2 CAR macrophage cell product CT-0508 is capable of promoting a pro-inflammatory tumor microenvironment and has the potential to induce epitope spreading via T cell recruitment and antigen presentation.

31. Mechanisms of Adoptive T Cell Micropharmacies

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Adoptive T cell therapy reprograms endogenous T cells, ex-vivo, to kill antigen positive cancer cells. Adoptive cell therapy has shown promise for treating B cell malignancies and melanoma, but many barriers limit their efficacy in other solid tumors, such as antigen heterogeneity and an immunosuppressive tumor microenvironment. We hypothesize that adoptive T-cells can be used to deliver small cytotoxic molecules to solid tumors to overcome these barriers. Thus, we will couple enzyme prodrug therapies with adoptive T cell therapy, generating SEAKER (Synthetic Enzyme Armed KillER) cells. SEAKER cells secrete an enzyme, beta-lactamase, in addition to their cytotoxic function. Beta-lactamase cleaves cephalothin motifs. We generated cephalothin-based prodrugs that mask the cytotoxic activity of the nucleoside analog AMS. Beta-lactamase can unmask nontoxic ceph-AMS prodrug, generating toxic, active AMS that kills cancer cells. As SEAKER cells localize and accumulate in the tumor microenvironment, systemically administered prodrug will only be unmasked at the tumor site. Here, we characterize the SEAKER platform using syngeneic, solid tumor mouse models. We demonstrate that the SEAKER platform is feasible and broadly applicable to a wide range of adoptive cell therapies. Murine SEAKER cells have sufficient transduction efficiency, beta-lactamase secretion, and CAR-mediated cytotoxicity. We also demonstrate that our SEAKER platform is able to eliminate both antigen positive and negative cancer cells better than conventional adoptive T therapies, in vitro.

32. CAR T-Cells Targeting the EDB Splice Variant of Fibronectin Have Potent Anti-Tumor and Anti-Vasculature Activity in Preclinical Solid Tumor Models

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Background: The outcome for recurrent/refractory solid tumors remains poor. While chimeric antigen receptor (CAR) T cell therapy holds the promise to improve outcomes, activity has been limited in early phase clinical studies. Lack of efficacy is most likely multifactorial, including a limited array of targetable antigens. Targeting the cancer-specific splice variant of fibronectin (FN-EDB, EDB) might overcome this limitation since it is abundantly secreted by cancer cells and adheres to their cell surface. The goal of this project was now to generate EDB-CAR T cells and characterize their function. **Methods/Results:** We constructed an EDB-CAR with a CD28.zeta endodomain and generated CAR T cells by retroviral transduction. *In vitro*, EDB-CAR T-cells were activated by recombinant EDB and recognized & killed EBD+ human tumor cells (A549 [lung], LM7 [osteosarcoma], U87 [glioma]). *In vivo*, 1x10^6 EDB-CAR T-cells had potent antitumor activity in both subcutaneous and systemic tumor xenograft models resulting in a significant survival advantage in comparison to mice that had received non-transduced (NT) T-cells. Tumors treated with EDB-CAR T-cells revealed a significant decrease in CD31+ endothelial cells in comparison to control CAR T-cells, indicating that EDB-CAR T-cells also target the tumor vasculature. The anti-vasculature of EDB-CAR T-cells was confirmed by live-animal vasculature imaging. Mechanistic studies with U87 FN1-/- (EDB knocked out) cells revealed that EDB secretion by tumor cells is critical for the observed anti-tumor and anti-vasculature activity. Lastly, since human and murine EDB are 100% homologous, we performed toxicity studies with 1x10^7 EDB-CAR T-cells and observed no dose limiting toxicities. **Conclusion**: We demonstrate here that EDB-CAR T-cells have potent anti-tumor and anti-vascular activity. While our findings warrant further active preclinical development of EDB-CAR T cells, they also provide the rationale of exploring other cancer-specific splice variants as targets for CAR T cells.

33. Pooled Knock-In Targeting for Genome Engineering of Cellular Immunotherapies

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Genetically-engineered immune cell therapies have proven effective to treat some types of cancer, but most tumors still cannot be cured. Forward genetics with CRISPR loss-of-function screens in T cells have identified some mutations to enhance immunotherapies, however these methods do not yet take full advantage of the power of knock-in targeting to engineer cell functions. Here, we developed a robust new platform to assess the functional effects of pools of knock-in constructs targeting the same locus in parallel and determine which would be most effective at promoting anti-tumor activity. We used high-efficiency nonviral gene editing to introduce large panels of candidate immunotherapeutic knock-in constructs into a defined genome position in human T cells allowing us to compete the cells against each other and test which constructs enhance T cell function. We designed a 36-member library of barcoded knock-in templates that included published and novel dominant negative receptors, synthetic "switch" receptors with engineered intracellular domains, and heterologous transcription factors, metabolic regulators or receptors. High-throughput pooled screening of targeted cells identified distinct members of the library to promote T cell fitness under various resting, stimulated and immunosuppressive *in vitro* conditions. Direct competition among adoptively transferred human T cells targeted with the pool of constructs into immunodeficient mice bearing human melanoma cells revealed a subset of constructs that promoted *in vivo* accumulation of tumor infiltrating lymphocytes (TILs). Pooled knock-ins combined with single-cell sequencing of template barcodes and transcriptomes independently confirmed the constructs that promoted *in vivo* tumor accumulation, and also revealed high-dimensional cellular phenotypes induced by each construct *ex vivo* and in an *in vivo* tumor microenvironment. A TGFβR2-41BB chimeric receptor enhanced cell fitness in TGFβ-treated cells *in vitro* and tumor accumulation *in vivo* and also promoted gene expression of key effector

cytokines. The knock-in cassette encoding a defined TCR and TGFβR2-41BB improved clearance of a solid tumor model *in vivo*, nominating a potential lead construct for further clinical development. Overall, these studies demonstrate the power of pooled knock-in technology to discover and functionally characterize complex synthetic gene programs that can be written into targeted genome sites to generate more effective cellular therapies.

34. G-CSF Mobilized Apheresis as an Alternative Source of CAR T Cells

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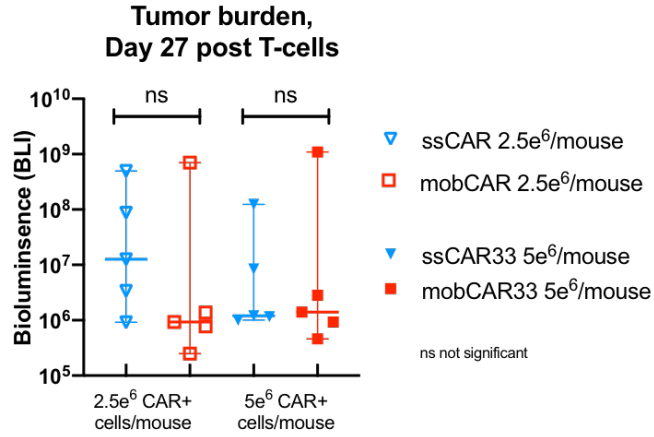
BACKGROUND Cell therapies are traditionally manufactured from a steady state apheresis i.e. without any medication given to the donor prior to apheresis. During development of a new therapy involving ex vivo gene editing of human CD34(+) stem/progenitor cells, which are collected after granulocyte colony stimulating factor (G-CSF) mobilization, we sought to evaluate the feasibility of utilising the CD34(-) fraction manufacturing CAR T-cells. This could be utilized as a means by which allogeneic donors are spared multiple apheresis collections, and a possibility to utilize previously cryopreserved mobilized units. The effect of G-CSF on CAR T-cell function is unknown, prompting us to evaluate if CAR T-cells manufactured after G-CSF exposure (*mob*CAR) may be functionally equivalent to those manufactured from a steady state apheresis (ssCAR). METHODS Untransduced T-cells (UTD) and CAR T-cells (targeting CD33) were manufactured using different starting products from the same donor, collected either at steady state (*ss*) or after G-CSF treatment (*mob*). Healthy volunteers were enrolled on an IRB approved protocol and had *ss* cells collected from 30mL peripheral blood by venepuncture, then received 4-5 days of G-CSF (10ug/kg/daily) followed by apheresis to collect *mob* cells. Effector T-cells were manufactured from *ss* and *mob* T-cells, and their in vitro cytotoxicity was tested. In vivo function was tested at 2 dose levels ($5e^6$ vs. $2.5e^6$ CAR+ cells/mouse) following engraftment into NSG mice of a CD33+ cell line, with assessment of tumor eradication by bioluminescence and mouse survival. In vivo expansion of T-cells was assessed by flow cytometry of mouse blood. RESULTS Paired effector T-cells (*ss*UTD/*ss*CAR & *mob*UTD/*mob*CAR) were manufactured with lower expansion of *mob*CAR [4 vs. 7 population doublings, $p<0.01$]. CAR expression was 78.6% in *ss*CAR vs. 64.7% in *mob*CAR, and in vitro cytolytic function was comparable against a CD33+ leukemia cell line. Preliminary in vivo data shows no difference in tumor eradication between the two CAR products (*Figure 1, median tumor burden; 95% CI; $p>0.05$*), and in vivo expansion of *mob*CAR33 was higher at Day 27 post infusion in the higher dose group [median CAR+ cells/uL blood; *ss*CAR 5.8 vs. *mob*CAR 38.6, $p=0.008$]. No differences in body weight, graft versus host disease, or survival have been observed to date. Additional analysis of T-cell memory subsets, exhaustion markers and cytokine profile are ongoing.



CONCLUSIONS We were able to successfully manufacture a CAR T-cell product from G-CSF mobilized cryopreserved cells, and despite slightly lower *ex vivo* expansion and transduction efficiency we found comparable in vivo anti-tumor efficacy, with evidence of enhanced T-cell expansion of the mobilized product in a murine xenograft model. Assessment of T-cell in vitro and in vivo function are ongoing, and additional donors and CAR-T phenotyping are planned. Successful completion of this work may support the use of cryopreserved or fresh G-CSF stimulated apheresis units for a range of cell therapy applications.

35. Enhanced tgTCR T Cell Product Attributes Through Process Improvement of CRISPR/Cas9 Engineering

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Adoptive cell therapy using T cells expressing transgenic (tg) tumor antigen-targeting T cell receptors (TCRs) has become an attractive modality to treat hematological and solid cancers due to a broader array of accessible targets relative to CAR-T cell therapies. However, the clinical development of TCR T cell therapies has been challenging in part due to their more complex T cell engineering requirements. Specifically, the ability to engineer multiple attributes in TCR T cell therapy is dependent on the ability to efficiently perform edits in multiple targeted genes, including knockouts and in locus insertions, while retaining viability and the desired cell phenotype.

While CRISPR/Cas9 genome editing has been demonstrated to be highly efficient, simultaneous edits in different loci could result in increased translocations, potentially impairing the quality and safety of the cell product. Moreover, existing cell engineering technology cannot support the necessary T cell quality and yield using a sequential editing process due to the cumulative toxicity of current protocols, necessitating the development of improved processes. Here we show an example of multiplexed T cell editing to replace the endogenous TCR with a therapeutic TCR. When tgTCR α and β chains are transferred into donor T cells, they can mispair with the endogenous TCR α and β chains, which limits tgTCR surface expression levels and poses a safety risk due to the potential of novel TCR chain combinations with unknown specificities. To overcome this, we have developed a highly efficient CRISPR/Cas9-based gene editing process to knockout the endogenous TCR chains utilizing highly specific sgRNAs targeting the TRAC and TRBC loci, while inserting the tgTCR into the TRAC locus at the CRISPR cut site using AAV donor templates. By developing an improved process, we have been able to achieve multiple sequential gene edits in primary human T cells, leading to knockout of the endogenous TCR with up to 99% efficiency and insertion of tgTCRs targeting Wilms' Tumor 1 (WT1) into 55-80% of the cells. Process improvement of CRISPR/Cas9 engineering of WT1-targeting TCR T cells resulted in substantial advantages over traditional methods. Specifically, we demonstrated marked improvement in viability post-editing and a greater than three-fold increase in expansion, thereby shortening the required time for T cell manufacture and increasing the yield. Notably, the expanded T cells have a favorable early-stem cell memory phenotype. Functionally, *in vitro* assays demonstrated that these engineered WT1-targeting TCR T cells have increased cytokine production (IFN-γ and IL-2) and continued proliferation following repeated stimulations when co-cultured with WT1 presenting *HLA-A*02:01*$^{+}$ tumor cells. Additionally, the low toxicity profile readily allowed for sequential CRISPR/Cas9 gene knockout in T cells. We demonstrate that this improved process led to the near-complete editing of three genes with no measurable target-to-target translocations, which were detected in cells engineered using traditional processes. These results show that process improvements in CRISPR/Cas9 cell engineering can address challenges associated with the complex engineering of T cell therapies and provide a robust modular platform for multiplexed editing of TCR or CAR-T cells.

Oligonucleotide Therapies for Genetic Diseases

36. A Novel Oligonucleotide-Based RNA Base Editing Therapeutic Approach for the Treatment of Hurler Syndrome

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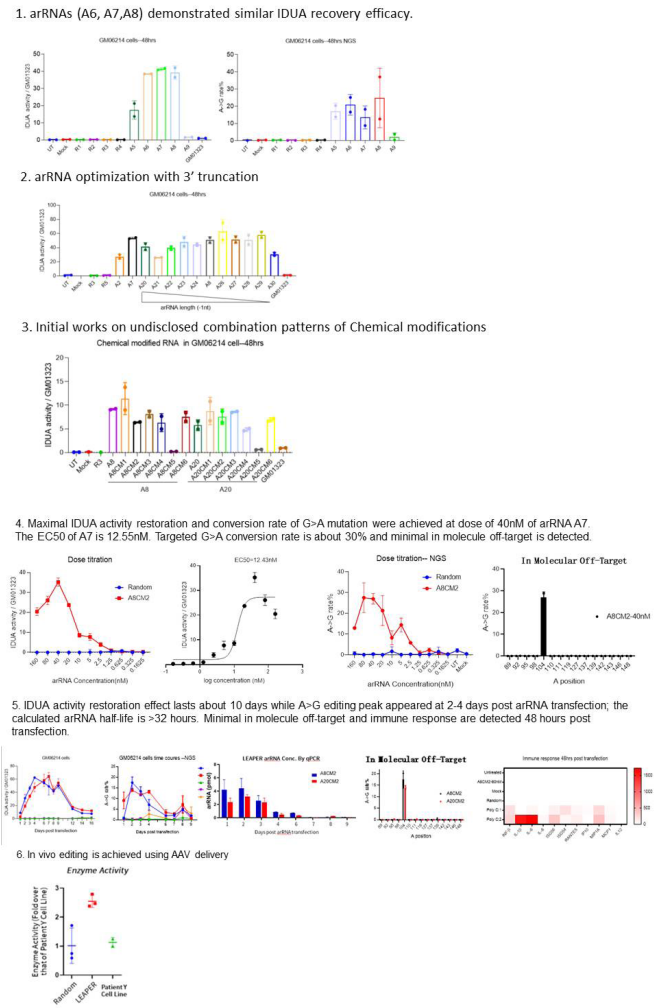
Mucopolysaccharidosis I-Hurler (MPS I-H) is the most severe form of metabolic genetic disease caused by a deficiency of the lysosomal enzyme α-L-iduronidase (IDUA), which is responsible for the hydrolysis of glycosidic bonds in terminal α-L-iduronic acid residues of the complex glycosaminoglycans dermatan sulfate and heparan sulfate. MPS 1-H patients develop multiple organ morbidities from glycosaminoglycans accumulation within lysosomes, and if untreated, often die before reaching age 10. Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) and Enzyme Replacement Therapy (ERT) are considered standards of care for children with severe MPS I-H. Allogeneic HSCT requires HLA matching and long term immunosuppression, is not curative and does not ameliorate cardiac valvular or skeletal manifestations. ERT involves intravenous weekly infusion of exogenous proteins which do not cross the blood-brain barrier and thus does not improve the CNS symptoms. Due to such unmet needs, it is highly desirable to develop new therapeutics with better safety and efficacy profile as well as ease of administration. For a significant number of the MPS 1 patients, the IDUA deficient is caused by a single G>A mutation, creating a stop codon at position 402 (W402X). We are developing a novel therapeutic approach by utilizing a novel RNA base editing technology-Leveraging Endogenous ADAR for Programmable Editing of RNA (LEAPER), to convert this specific adenine into an inosine, which acts like a guanine during translation, and enable endogenous production of wild type IDUA protein. This technology uses an oligonucleotide only, which can potentially be developed in the path of approved RNA-based therapeutics like antisense oligonucleotide or siRNA. Previously we in collaboration with a research lab reported that a 111nt oligonucleotide (ADAR-recruiting RNA, i.e.,arRNA) produced precise and sequence specific A>G conversion at Codon 402 and can partially restore IDUA enzymatic activity in MPS1 patient-derived primary fibroblasts. In this abstract, we report optimization of the lead arRNAs to achieve high RNA editing rates and sufficient IDUA enzymatic activity restoration in MPS1 patient primary fibroblasts GM06214. Through optimization of arRNA length, editing position and chemical modifications, lead arRNAs of 70-90nt in length can produce specific A>G RNA editing rate at up to 30-40%, and IDUA enzymatic activity at up to 60 fold of that in GM01323, derived from a MPS 1 patient with mild symptoms. Current arRNA has a half-life of 30-40 hours, and the restored IDUA enzymatic activity can last for 10 days or longer. Genome wide off-targeting for LEAPER is minimal, and limited in-molecule off-target RNA editing was observed, which can be minimized through chemical modification. No immune response is detected 48 hours post transfection. In addition, we showed that an arRNA delivered via AAV in an MPS 1 transgenic mouse model resulted in significant A>G editing and higher IDUA enzymatic activity. Taken together, these results demonstrated that arRNA, based on LEAPER technology, could be further developed as a potential treatment of these patients with Hurler Syndrome.



37. Targeting DNA Damage Response Genes with Oligonucleotides for Therapeutic Modulation of Somatic Instability at Disease Gene Repeats

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DNA damage response ("DDR") genes are potent modifiers of the onset and severity of diseases caused by expansion of short DNA repeats including Huntington's disease (HD), myotonic dystrophy, and spinocerebellar ataxias, and act via modulation of repeat somatic expansion at causal disease loci (e.g. *HTT, DMPK, ATXN1/2/3/7*). We developed antisense oligonucleotides ("ASOs") and small interfering RNAs ("siRNAs") to knock down specific DDR genes selected for somatic instability phenotypes and loss of function tolerance. Our approach operates upstream of disease gene targeting approaches and targets a fundamental unifying pathway driving multiple DNA repeat disorders. DDR gene knockdown slowed repeat expansion over time in both HD patient-derived cell lines and HD model mice. Single doses of DDR targeting ASOs administered via multiple routes to non-human primates were well tolerated and drove significant knockdown in relevant brain regions. Repeat intrathecal dosing was safe and well tolerated; we further characterized target knockdown under this dosing paradigm. In parallel with preclinical development, we have initiated SHIELD HD, a multinational natural history study in HD gene expansion carriers, including prodromal and early manifest individuals, to assess somatic expansion, DDR gene expression, and various outcomes of disease progression over time. These activities will be discussed in the context of a coordinated development program seeking to stop manifestation and progression of multiple DNA repeat disorders via halting somatic DNA repeat expansion.

38. SINEUPs: A New Antisense, Long Non-Coding RNA-Based Platform to Increase Endogenous Protein Levels for Therapy

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SINEUPs represent a new platform to increase endogenous protein levels of target mRNAs for therapeutic purposes. They are antisense long non-coding RNA (lncRNAs) that stimulate translation of sense mRNAs. Their activity depends on the combination of two domains: the overlapping region, or binding domain (BD), dictates SINEUP specificity, while an embedded inverted SINEB2 element acts as effector domain (ED) controlling the enhancement of mRNA translation. Their modular structure can be employed to artificially engineer their BD and design synthetic SINEUPs to specifically enhance translation of virtually any target gene of interest. They usually increase target protein expression of 2-3 fold, thus avoiding massive over-expression. Moreover, they are active only on cells that express target mRNAs, thus limiting the side effects. As representative examples, SINEUP-GDNF RNA increases endogenous GDNF protein levels both *in vitro* and *in vivo*, when injected in the mouse striatum. Furthermore, 6 months after injection, SINEUP-GDNF protects mice from neurodegeneration in a neurochemical model of PD. SINEUP-frataxin RNA increases endogenous frataxin protein levels restoring mitochondrial activity in Freidreich's Ataxia patient's cells proving SINEUPs as a therapeutic strategy for haploinsufficiencies.

39. Restoration of Full-Length Dystrophin Expression in the Dup2 Mouse Induced by Systemic Delivery of a Peptide-Conjugated Morpholino Oligomer

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Duchenne muscular dystrophy (DMD) is an x-linked recessive genetic disorder caused by mutations that disrupt the reading frame of the *DMD* gene and lead to lack of dystrophin protein. There are two approved gene corrective therapies that use antisense phosphorodiamidate morpholino oligomers (PMOs). These therapies alter splicing to exclude exon 51 or exon 53 from the mRNA, restoring translation of a truncated, yet functionally active protein. However, in the presence of single exon duplications, the antisense PMO therapies allow restoration of a wild-type (WT) *DMD* transcript and dystrophin protein. Here we report the application of cell-penetrating peptide-conjugated PMO (PPMO) to target the *DMD* exon 2 splice acceptor site in Dup2 mice. These mice carry a duplication of exon 2, which is the most common single duplication mutation in DMD patients, accounting for approximately 10% of all duplication mutations. In the presence of an exon 2 duplication, skipping can result in either of two therapeutic transcripts. Skipping a single copy results in a WT transcript, whereas skipping both copies results in a transcript lacking exon 2 entirely (Del2), but from which functionally active dystrophin protein can be expressed via utilization of an exon 5 internal ribosome entry site (IRES). The dose-escalation and time point studies were conducted in 12-week-old Dup2 mice which were intravenously injected with different doses of PPMO. Scrambled PPMO and saline-injected mice were used as controls. Dup2 mice (n=5-6) were sacrificed at 7 days post-injection in dose-escalation study and at 15-, 30- and 60 days post PPMO administration in time point study. Five muscles—tibialis anterior (TA), gastrocnemius (Gas), quadriceps (Quad), diaphragm (Dia) and heart—were harvested for analysis of *DMD* mRNA transcripts by RT-PCR, and for dystrophin protein expression by western blot and immunofluorescence staining. The single injection in dose escalation study demonstrated a positive dose response; robust skipping of the duplicated exon 2 (both WT and Del2 transcripts) was seen at the higher does in all skeletal muscles: TA (40.8%), Gas (39.7%), Quad (53.5%), and Dia (53.1%), with minimal skipping observed in heart muscle (8%). The time-point study was performed using the highest dose from the escalation study and showed robust skipping of the duplicated exon 2 in most skeletal muscles at 15 days post-injection: TA (68.2%), Gas (45.1%), Quad (55.1%), Dia (62.4%) and heart (14.8%). The dystrophin expression was determined by western blot. The highest level of dystrophin was observed at 30 days post-injection with an average of 11% in heart, 40-52% in TA, Dia and Gas, and 71% in Quad. Dystrophin expression decreased in all skeletal muscles at 60 days post-injection with peak expression in Gas still showing an average of 19%. Future studies will evaluate longer term expression, with a goal to develop a meaningful therapy for patients with exon 2 duplications and potentially other 5' *DMD* mutations.

40. A Novel Lentiviral Vector for Gene Therapy of β-Hemoglobinopathies: Co-Expression of a Potent Anti-Sickling Transgene and a microRNA Downregulating *BCL11A*

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β-globin (*HBB*) gene mutations lead to adult hemoglobin (Hb) defects causing β-thalassemia and sickle cell disease (SCD). SCD is due to a single missense mutation causing the formation of β^S-globin chain. The sickle Hb (HbS) has the propensity to polymerize, resulting in the production of sickle-shaped red blood cells (RBCs) that cause occlusions of small vessels, leading to multiple organ damage. Transplantation of autologous hematopoietic stem cells (HSCs) transduced with lentiviral vectors (LVs) expressing an anti-sickling β-globin transgene (βAS) is a promising curative treatment that is partially effective in patients affected by severe forms of β-hemoglobinopathies. Here, we aim to improve LV to further boost the levels of therapeutic β-like globins without increasing the mutagenic vector load in HSCs. In particular, we developed a novel LV expressing βAS together with an artificial microRNA (amiR) targeting the fetal Hb (HbF) repressor BCL11A (βAS/amiR-LV). By downregulating BCL11A, this amiR re-activates the expression of the endogenous anti-sickling γ-globin fetal chains, which, together with βAS, should improve the clinical course of β-hemoglobinopathies. To limit BCL11A downregulation to the erythroid lineage and reduce potential cellular toxicity and off-target effects, the amiR was inserted in the intron 2 of βAS under the control of β-globin promoter/enhancers. First, we tested two different position of βAS intron 2 for the insertion of amiR and showed that in a human erythroid cell line (HUDEP2) none of them affected LV titer and βAS expression as compared to a LV containing only βAS (βAS-LV).We then evaluated the potency of this βAS/amiR-LV in downregulating BCL11A. Transduced HUDEP2 cells showed a 3-5 fold decrease in BCL11A expression, which led to γ-globin gene de-repression and a high proportion of HbF^+ cells, as evaluated by qRT-PCR, flow cytometry and HPLC. Importantly, the total amount of therapeutic β-like globins was substantially higher in βAS/amiR-LV- than in βAS-LV-transduced cells, without any impairment in cell viability or erythroid differentiation. Then, we tested this bifunctional LV in primary adult HSCs derived from healthy donors. We achieved significant transduction level and γ-globin de-repression coupled with βAS expression resulted in a 2-fold increase in the total amount of therapeutic β-like globins per vector copy number (VCN) in RBCs derived from βAS/amiR-LV- compared to βAS-LV-transduced HSCs. It is worth noting that neither the BCL11A down-regulation nor the expression of the amiR affected erythroid differentiation and generation of enucleated RBCs. Finally, we validated these findings in clinically

relevant plerixafor-mobilized HSCs from SCD patients. Efficient transduction of HSCs by βAS/amiR-LV led to a substantial γ-globin re-activation in HSC-derived erythroid cells and a 2-fold increase of the amount of total therapeutic Hb (HbF + HbAS) per VCN in βAS/amiR-LV- compared to the βAS-LV-transduced erythroid cells. This is a crucial point as a 2-fold improvement of therapeutic Hb per cell could represent a major clinical benefit, since current gene therapy trials do not reach the amount of β-like globin per cell necessary to correct severe β-thalassemia and SCD. Overall, these results show that the combination of gene replacement and gene silencing strategies can improve the efficacy of current therapeutic approaches and represents a novel treatment for β-hemoglobinopathies.

41. Engineering Backsplicing Introns for Robust Expression of Synthetic Circular RNAs from AAV Vectors

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Circular RNAs (circRNAs) are highly stable RNA molecules, making them attractive templates for expression of therapeutic proteins and non-coding RNAs. Unlike self-splicing introns, the potential of the spliceosome for generating synthetic circRNAs *in vivo* has not been fully explored. Here we describe SCRIBE, a robust platform for generating Synthetic Circular RNAs from Intronic Backsplicing Elements, which consists of a modular assembly of promoters, internal ribosome entry sites (IRESs) and engineered backsplicing elements flanking exons and/or non-coding RNAs on a single DNA template. By engineering natural introns into smaller, synthetic elements, we achieve markedly improved expression of circular exons. Further, we demonstrate tandem expression of backspliced circRNAs and tRNA intronic circRNAs from the same RNA molecule. Delivery of these constructs in mice with recombinant AAV vectors enables robust circRNA expression and translation in skeletal muscle tissue. In summary, SCRIBE is a robust new platform for the biogenesis of natural and synthetic circRNAs and expands the flourishing class of gene-based medicines.

42. Delivery of mRNA and CRISPR in CNS with a Novel Polymer Nanoparticle

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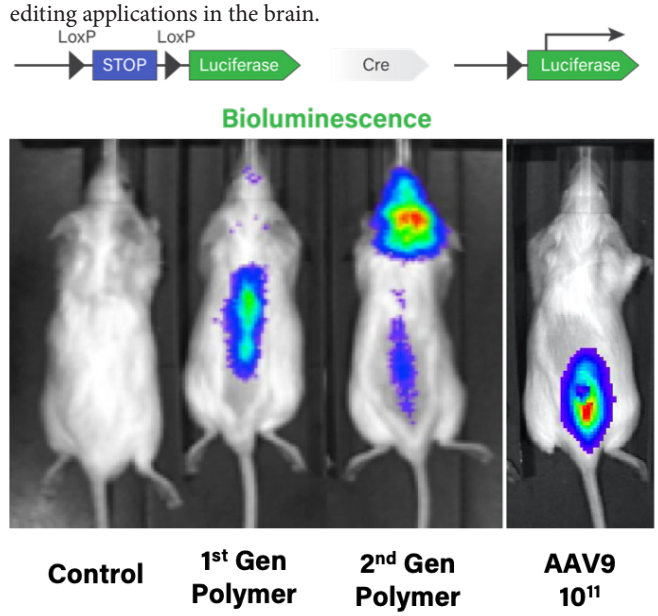
Despite the potential of gene therapy and gene editing, the lack of efficient targeted delivery systems limits the areas of therapeutic application. Even the most advanced delivery system, adeno associated viruses (AAVs), faces issues like inefficient targeted delivery, pre-existing immunity, and genome integration. Non-viral delivery systems are an alternative with several advantages: low manufacturing cost, low chance of pre-existing immunity, and no genome integration. However, efficient targeted delivery beyond the liver has been a recurring challenge. Previously, our group demonstrated that CRISPR/Cas9 ribonucleoprotein (RNP) can be delivered to the brain in mice by polymer nanoparticles via direct intracranial (IC) injection. Compared to invasive IC injections, intrathecal lumbar (IT-L) injection is a minimally invasive alternative. Our study focused on the IT-L delivery of polymer nanoparticle encapsulated mRNA and RNPs. We synthesized a library of novel polymers and screened it in vitro to identify polymers that efficiently encapsulated mRNA and transfected primary human neural progenitor cells. We then evaluated candidate polymers to deliver CRE mRNA in vivo in a Loxp luciferase reporter gene mouse model. Iterative medicinal chemistry was conducted on the candidates to improve the efficiency of delivery in vivo. Our process identified a polymer that can efficiently encapsulate and deliver macromolecule cargos to the brain via IT-L administration. Figure 1 shows the result of Cre mRNA delivery with candidate polymer nanoparticles. A second generation of nanoparticle showed higher efficient delivery of Cre mRNA and expression of luciferase in the brain. H&E staining showed no visible inflammation in the target tissue. In summary, we have identified a polymer that can deliver nucleic acids and CRISPR RNP via IT-L administration for gene therapy and gene editing applications in the brain.



Immune Responses Against AAV Vectors

43. IL-1R/MyD88-Dependent CD8+ T Cell Responses to Hepatic AAV Gene Transfer

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Immune responses to the adeno-associated virus (AAV) vector and their transgene products present one of the greatest hurdles to the success of gene therapy. Several studies have implicated innate immune sensors such as Toll-like receptors (TLR) 2 and 9 and their downstream adaptor molecule myeloid differentiation primary response protein 88 (MyD88) in sensing viral capsid and genome. A

clear link between TLR9-MyD88 signaling and CD8+ T cell responses to capsid and transgene products has been established, prompting the development of CpG-depleted expression cassettes. However, little is known about other signaling pathways that may lead to immune activation. Previously, our lab has shown that although liver gene transfer is capable of inducing immunological tolerance to AAV encoded transgene products, levels of gene expression as determined by vector dose and design play a critical role. For instance, low levels of hepatic gene expression may elicit an adaptive CD8+ T cell response to an AAV encoded transgene, resulting in loss of the model antigen ovalbumin (OVA) in C57BL/6 mice or of FIX expression in hemophilia B mice. In order to understand the underlying mechanisms of transgene specific CD8+ T cell activation in the hepatic microenvironment, we interrogated a broad spectrum of innate immune sensing pathways. Wild-type C57BL/6 mice and various knockout mice of specific innate sensing molecules (such as TLRs 2, 9 and MyD88) on C57BL/6 background (n=8 per experimental group) were intravenously injected with $1x10^9$vg particles of hepatotropic AAV8 virus expressing OVA, which we previously found to activate CD8+ T cells (Mol Ther 25:880, 2017). Four weeks after vector administration, PBMCs were analyzed by flow cytometry for OVA-specific CD8+ T cells using a class I MHC tetramer. In 50-75% of wild type mice, OVA-specific CD8+ T cells were observed at frequencies of 2-32%. Unlike our previous experience with AAV-capsid specific CD8+ T cell responses, innate sensor TLR9 was dispensable for transgene specific CD8+ T cell response, as 60% of TLR9$^{-/-}$ mice developed a response. No significant differences were seen in TLR2$^{-/-}$, TRIF$^{-/-}$, IFNaR$^{-/-}$, or MDA5$^{-/-}$ mice. Interestingly, adaptor protein MyD88$^{-/-}$ mice did not elicit CD8+ T cell response to OVA, implying an important role for MyD88 in mediating transgene specific cellular immune response. Since MyD88 is an essential adaptor protein not only for TLRs but also for interleukin-1 (IL-1) signaling pathways, we next analyzed IL-1R$^{-/-}$ mice. Similar to MyD88$^{-/-}$ mice, IL-1R$^{-/-}$ mice did not show OVA specific CD8+ T cells (*p=0.0063, 0.0075* respectively), indicating that transgene specific adaptive responses are mediated via IL-1R/MyD88 signaling. CD4-deficient mice also failed to elicit an immune response to OVA, likely reflecting a requirement for CD4+ T help. While MyD88 and CD4 were also required for antibody responses against the viral capsid and the transgene product, IL1-R was not. Surprisingly, in comparison to wild-type mice, TLR9$^{-/-}$ mice had a significantly (*p=<0.0001*) diminished antibody response to AAV capsid (but not the transgene product). TLR2- and IL1R-deficient mice had a modest reduction in antibody formation against ovalbumin, and these strains as well as MyD88$^{-/-}$ mice showed a significant increase in circulating ovalbumin levels (reaching 100-200 ng/ml). In summary, we uncovered a novel signaling pathway that can activate CD8+ T cell responses in AAV gene transfer that is independent of endosomal DNA sensing and can unlikely be avoided by CpG depletion. The IL-1R/MyD88 pathway drives CD8+ T cell activation against the transgene product at lower levels of hepatic transgene expression, while at higher levels tolerance is induced.

44. A Vector Independent Method of Neutralizing Antibody Evasion Potently Protects AAV for Efficient Gene Delivery

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Approximately 1 in 10 people in the US suffers from a rare genetic disease, which can seriously impact life-span, quality of life, independence, and economic potential. Gene therapy is one of the most promising forms of treatment for the correction of heritable diseases. Among gene therapy delivery vehicles, adeno-associated virus (AAV) vectors have shown robust therapeutic effects in numerous clinical trials and are among the first FDA approved gene therapy products. The number of clinical trials for AAV gene therapy has grown substantially due to its safety and success in targeting many different types of organs for long-term therapeutic gene expression. Despite clinical success, the major barrier to AAV mediated gene delivery is the high prevalence of neutralizing antibodies (NAbs), which block vector transduction of target tissues. Greater than 90% of the general population has been exposed to AAV through natural infection, and over 50% of people are serum positive for NAbs against AAV. Identification of pre-existing NAbs during clinical trial screening can disqualify patients from enrollment, since NAbs severely attenuate therapeutic effectiveness and cause outcome variability. To overcome AAV NAbs several approaches have been employed however most of them suffer from low efficiency, unwanted side effects, or alteration of AAV tropism. We have developed a vector independent protein-based method to universally block NAbs and establish that this method is effective against a broad range of pre-existing NAb concentrations. We identified a synthetic large molecule, termed Protein-M, and pioneered its use to enable successful gene delivery by preventing AAV recognition and neutralization. We demonstrate Protein-M mediated NAb escape using polyclonal serum from AAV immunized mice or human (IVIG) sources. In vitro studies show protection of AAV over a 100-fold concentration of neutralizing serum. Using passive transfer of neutralizing serum, we achieve protection of AAV over a 1,000-fold dilution of serum concentration in vivo. Additionally, we demonstrate Protein-M can protect AAV to enable redosing, and provide proof-of-concept in vivo after primary immunization with AAV vectors. To our knowledge this is the most efficient method demonstrated to enable AAV neutralizing antibody escape. We show that Protein-M can be administered alone prior to AAV administration, or formulated with AAV for NAb evasion in vivo. The effectiveness of this approach depends on the interaction of Protein-M with immunoglobulins before AAV can be neutralized. Finally, our unique approach can be used independently of any AAV serotype to overcome NAbs while maintaining the unique or beneficial properties of each vector capsid for specific gene therapy applications.

45. Effect of CpG Depletion on Anti-Capsid CD8+T Cell Priming to AAV Gene Therapy

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Adeno-associated virus (AAV)-based gene therapy has received regulatory approval for treatment of two genetic diseases and is currently in clinical development for many more. One of the challenges for long-term success of gene therapy is the development of immune response to the viral vector or the transgene product. $CD8^+$ T cell responses have been linked to TLR9-MyD88 signaling. Therefore, depletion of immune stimulatory CpG motifs, which are expected to be unmethylated in viral DNA and to therefore enhance innate signaling though this pathway, has been explored as strategy to reduce the risk for $CD8^+$ T cell activation. Here, we first evaluated the impact of CpG contents of the vector genome on the response to AAV capsid. A vector was constructed based on an entirely CpG-free expression cassette. It contained a CpG-free edited sequence of the coding region for human coagulation factor IX (FIX) (hyperactive FIX-Padua variant) and a synthetic intron under transcriptional control of a CMV enhancer/EF1alpha promoter combination and SV40 polyA signal. This cassette was inserted in between AAV2 ITRs and packaged into a modified AAV2 capsid that contains a surrogate, ovalbumin-derived $CD8^+$ T cell epitope (AAV2-SIINFEKL). C57BL/6 mice received IM injections (1x10^11 vg/mouse) of AAV2-SIINFEKL depleted of CpG motifs (AAV2-SIINFEKL-dCpG) or CpG rich control vector (AAV2-SIINFEKL-rCpG; containing native, not CpG depleted sequences). Notably, CpG depletion markedly reduced anti-capsid $CD8^+$ T cell response as observed by the decrease of % $tetramer^+$ $CD8^+$ T cells in mice that received AAV-SIINFEKL-dCpG. In contrast, we did not observe differences in capsid-specific antibody formation in mice injected with AAV2-SIINFEKL-dCpG compared to those injected with AAV2-SIINFEKL-rCpG. Next, we evaluated the adaptive immune response to FIX in male hemophilia B (C3H/HeJ $F9^{-/Y}$) mice injected with AAV1-dCpG or AAV1-rCpG (1x10^11 vg/mouse, n=5/group). The immunogenic intramuscular route was chosen for these experiments, as hepatic gene transfer typically results in tolerance induction to FIX. Within the first month after gene transfer, IgG2a titers against AAV1 capsid were similar. IgG1 formation against hFIX was >10-fold lower at 2 weeks and ~2.5-fold lower at 4 weeks for AAV1-dCpG compared to AAV1-rCpG. All animals formed inhibitory antibodies against FIX as determined by Bethesda assay, were only modestly reduced for AAV1-dCpG (average of 53.6 BU/ml vs 34.5 BU/ml). While no FIX antigen was detectable in circulation, low FIX activity of 0.3% of normal was detected in mice treated with AAV1-dCpG. We are currently evaluating $CD8^+$ T cell infiltration in transduced skeletal muscle. Thus far, our results are consistent with literature data that sensing of the AAV genome via TLR9 more substantially affects $CD8^+$ T cell than humoral immune responses, although it is encouraging that antibody titers against FIX were reduced.

46. Transgene-Specific T Cell Immunomodulation is Achieved in Mice by a Subretinal Co-Injection of the AAV Gene Transfer Vector with Peptides from the Transgene

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While subretinal injection of adeno-associated virus (AAV) gene therapy vectors can successfully treat several inherited retinal diseases, some patients display transient inflammatory events requiring the use of corticoids as an adjunct treatment. The eye is known as an immune-privileged site but anti-capsid humoral and cellular immune responses have been reported in some patients. Possibly, cellular immune responses against the transgene could also occur and may contribute to the loss of gene-modified cells. To better characterize and to control immune reactions to intraocular AAV gene therapy, we developed a murine model using a well-defined HY male antigenic system. We previously reported that the injection of AAV8 vector-mediated subretinal gene transfer triggers a systemic anti-transgene T-cell response in a dose-dependent manner. We have also demonstrated that an antigen introduced into the subretinal space can provide a systemic antigen-specific immunosuppression referred to as subretinal-associated immune inhibition (SRAII). We now hypothesize that immunodominant peptides, part of a corresponding transgene product, could be exploited as SRAII inducers, when they are injected simultaneously to an AAV gene transfer vector. **We thus investigated here whether a single subretinal co-injection of the AAV gene transfer vector with peptides from the transgene can lead to a systemic anti-transgene specific T-cell inhibition.** A transgene encoding the HY male antigen, containing MHC class I- and MHC class II-restricted T cell epitopes (UTY and DBY peptides immunodominant in H-2b female mice), was packaged into AAV8 under PGK promoter and injected subretinally with or without HY peptides at day 0 in C57BL/6J female mice. We evaluated the primary response in mice sacrificed 2 to 14 days post injection. To explore the memory T-cell response, a group of mice was challenged at day 14 subcutaneously with the HY peptides adjuvanted in CFA and their immune responses were analyzed at day 21. We found that: **(i)** subretinal injection of 2.10e9 or 5.10e10 vg of AAV8-PGK-GFP-HY triggered a dose-dependent systemic primary and memory anti-transgene Th1/Tc1 responses, **(ii)** the simultaneous co-injection of AAV8 and of HY peptides inhibited both $CD8^+$ and $CD4^+$ T-cell specific primary and memory responses against HY even at high dose (5.10e10 vg) of AAV. SRAII phenomenon seems to be a powerful systemic immunosuppressive mechanism specific to a transgene expressed in an eye. Even whether we have to confirm these results in a pathophysiological context, co-injection of the transgene product and the therapeutic vector may be considered as a new immunomodulatory strategy to control inflammatory reactions in the context of ocular gene therapy.

47. Systemic Immunity Baseline Alterations in X Linked Retinoschisis: Do They Confound to AAV8-RS1 Gene Therapy Outcomes?

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Introduction: We have encountered intraocular inflammation in our human Phase I/IIa trial for XLRS (Clinical Trial.Gov NCT02317887). We wanted to discern whether systemic cell mediated immunity correlates with ocular inflammation. This study evaluates changes in immune cell subpopulations and/or pathways altered in XLRS patients at baseline and after AAV8-RS1 injection. Methods: Eleven participants were dosed, 3 at 1e9 vg/eye, 3 at 1e10 vg/eye, 4 at 1e11 and 1 at 3e11 vg/eye. One eye per participant received intravitreal AAV8-RS1 vector and the un-treated eye was neither masked nor randomized. The cell mediated immunity was analyzed at baseline and day 14 using flow cytometry and luminex assay. Twelve healthy controls were included in the study. Mann Whitney U test was used for comparisons and $p<0.05$ was considered significant. Spearman's correlation coefficient was calculated for linear association between altered immune factors and ocular inflammation. Results: We found that CD4/CD8 ratio was upregulated at baseline, which mostly persisted after vector injection in 9 of 11 human XLRS subjects. The mean CD4/CD8 ratio in healthy subjects was 2.23±0.53 and for XLRS was 3.52±0.93 ($p=0.001$). Typically, the CD4/CD8 ratio in a clinically healthy individual is ~ 2. The CD4/CD8 ratio after vector injection (3.42±0.81) weakly correlated to inflammation scores in XLRS ($R^2=0.47$, $p=0.02$). CD16+ Natural Killer T ($p=0.02$), CD56+ Natural Killer ($p=0.04$) and $CD14^+CD16^+$ cells ($p=0.01$) were also upregulated in XLRS at baseline. Upregulation in these pro-inflammatory cells may result in inflammation upon exposure to self /non-self-antigens. CD11b+CD11c+ ($p=0.02$) and CD11c+ dendritic cells (DC, $p=0.003$) were downregulated, but CD123+ DC ($p=0.02$) showed upregulation indicating an imbalance in the DC type frequency. We also found upregulation in IFN-g ($P=0.014$), TNF-a ($P=0.04$) and IL-6 ($P=0.02$) while EGF ($P=0.0004$) and IL8 ($P=0.01$) were downregulated in baseline XLRS. Baseline IFN-g ($R^2=0.66$, $p=0.002$) also correlate linearly to inflammation scores after vector injection. These changes indicate towards a pro-inflammatory state of cell mediated systemic immunity in XLRS. Several XLRS subjects with altered baseline immune activity exhibited further changes after vector administration ($1e^9$vg-$3e^{11}$vg/eye). CD11b+CD11c+ cell frequency was further downregulated ($p=0.045$), while CD8+HLADR+ cytotoxic cells ($p=0.043$) and CD68+CD80+ macrophages ($p=0.046$) were upregulated. Vector dosing also led to upregulation of Granzyme-B in 8/11 subjects ($p=0.039$), secreted by cytotoxic T cells. Nine of 11 subjects had elevated IFN-g ($p=0.044$), and 7 of 11 had elevated TNF-a levels ($p=0.04$) at day 14 after vector injection, which may indicate a Th1/Th2 imbalance and possible activation of TNF/MAPK/TLR signaling pathways upon inflammation in both ocular and systemic microenvironment. Conclusions: The high CD4/CD8 ratio and the imbalance between regulatory and effector immune cell frequencies indicate a systemic chronic inflammatory state in XLRS at baseline. The upregulation of pro-inflammatory cytokines (IFN-g, TNF-a and IL-6) implicates a systemic Th1/Th2 imbalance in XLRS. Subjects with a higher vector dose (1e11vg, 3e11vg) showed some degree of inflammation and had higher neutralizing antibody levels in circulation, which suggests that vector load may play a role that exacerbates the pre-existing baseline immune imbalance and propels inflammation. It is unclear if these alterations in systemic immunity are a cause or consequence of XLRS linked ocular inflammation.

48. Elastin-Like Polypeptide Mediated AAV Delivery Improved Transduction Efficiency and Reduced Immune Response

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Adeno-associated virus (AAV) has been developed as a major gene therapy vector and approved by the FDA for direct patient administration. However, a significant barrier to therapeutic gene delivery is when the vector encounters a pre-existing humoral immune response upon administration. Here we utilized a thermoresponsive elastin-like polypeptide (ELP) to crosslink with AAV serotypes 2, 8, and 9, and formed polyplex AAV vectors that create a shield of non-viral polymer around them in nanoscale (100-200 nm). We show that the encapsulation of the capsid of AAV reduced potential immune response without significantly affecting transduction efficiency. The ELP-AAV polyplex exhibited similar (~70%) transduction efficiency to free AAV and effectively expressed targeted genes both in vitro and in vivo, and no adverse events were observed after subretinal or tail vein injection. We performed in vitro AAV neutralizing antibody (Nab) assays that reveal the ELP-AAV polyplex significantly improves gene expression at high Nab titer. With the presence of Nab in vivo, the polyplexes also significantly increased the transduction at different time points. The ELP cross-linked to the AAV capsid also decreased the major histocompatibility complex class 1 and cytotoxic related cytokines production in vitro. This method has strong potential to overcome the AAV associated humoral immune response in gene therapy.

49. A Systematic Analysis of the Immunologic Effects of Intrathecal AAV9 Mediated Gene Transfer Targeting the Nervous System in Giant Axonal Neuropathy

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OBJECTIVE: We are conducting a first-in-human intrathecal (IT) AAV9 mediated gene transfer trial for giant axonal neuropathy or GAN to attempt to restore gigaxonin function in the nervous system. GAN is a recessively inherited and fatal pediatric neurodegenerative disease with progressive sensorimotor neuropathy, cerebellar dysfunction, and CNS involvement due to a lack of protein gigaxonin, a regulator of intermediate filament (IF) turnover, thus resulting in widespread IF accumulation in cells and axons. Within the framework of a clinical trial of IT delivery of scAAV9-JeT-GAN for patients with GAN (NCT02362438), we report here on the systematic analysis of safety and immunologic profiles following IT gene transfer. We evaluate the impact of host factors (eg: baseline seropositivity or CRIM negative status) and treatment factors (dose level and co-administration of immune modulation) on safety and immune phenotype post gene transfer. METHODS: Twelve subjects with GAN have been injected in a dose escalating study (doses ranging as follows: 1x= $3.5x10^{13}$ vg; 3.3x=$1.2x10^{14}$ vg; 5x=$1.8x10^{14}$ vg; 10x=$3.5x10^{14}$ vg), with follow up ranging from 3 months to 3 years. Transient broad immunosuppression is used in all subjects with corticosteroids, but subjects with predicted 'CRIM negative' (Cross reactive immunologic material based upon biallelic null mutations) status (N=4) received additional T-cell targeted immunosuppression with tacrolimus (for 6 months) and rapamycin (ongoing). Immune analysis included pre-and post- gene transfer immunologic data, including: neutralizing antibody (NAb) titers in serum and cerebrospinal fluid (CSF), IFN-γ ELISpot data, CSF cell counts, oligoclonal band formation, IgG index, vector shedding, cytokines, and in a subset of patients analysis by flow cytometry. IFN-γ ELISpot analysis to GAN and AAV9 epitopes pre-and post-gene transfer is performed, and the impact of immune modulation on these markers is evaluated. Further exploratory immune studies include flow cytometry performed on blood and CSF and a pilot analysis of intracellular cytokine staining. RESULTS: Following IT gene transfer, 11 of 12 subjects have shown a rise in CSF white blood cell count (WBC), with peak values ranging from 8/mm3 to 113/mm3 and with onset ranging from 3 weeks to 6 months post gene transfer. CSF WBC elevation is lymphocyte predominant, and appears to be steroid responsive. Baseline seropositive subjects (N=3) showed a more rapid rise in serum anti-AAV9 NAb's as well as earlier clearance of detectable circulating vector in blood. Subjects who received added T-cell targeting immune modulation appeared to have an attenuation of the anti-capsid IFN-γ ELISpot response. No subjects developed an anti-transgene ELISpot response. SUMMARY: Overall, IT gene transfer up to a dose of $3.5x10^{14}$ vg appears to be safe and well tolerated clinically, and the CSF WBC elevation does not thus far correspond to clinical or neuroimaging findings of neuroinflammation. AAV9 baseline seropositivity does not appear to impact the safety of IT gene transfer, but may lead to earlier vector clearance due to a statistically significant earlier rise in anti-AAV9 serum NAb responses. GAN subjects who are predicted CRIM-negative can safely receive IT gene transfer with targeted immune modulation, and T-cell mediated immune modulation may be beneficial in all subjects.

Assay Development

50. Development of a Flexible *In Situ* Sequencing Method with Single-Cell Resolution

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To preserve spatial information associated with cells' transcriptome to a higher or lesser degree significantly improves our understanding of the relationships among events taking place in living organisms. However, despite the efforts, throughput and financial demands remain a common obstacle. For this reason, we ventured to develop a method enabling *in situ* transcript detection with single-cell resolution. The development of this method followed the use of spatial transcriptomics, which enables the preservation of spatial information associated with transcriptome data to ≈200 μm level. Spatial transcriptomics was used for analysis of the integration of dopaminergic transplants into dopamine deficient rats (*Rattus norvegicus*). However, as it only allows for the assignment of identity to 20-40 cell conglomerates, the resolution is not sufficient for distinguishing rare cell populations even with extensive data processing. In order to maximally maintain spatial information present in a tissue with close to single micron resolution, the new method, presented here, adopts modified slides capable of mRNA immobilization. Furthermore, it incorporates the use of PCR-maintained padlock probe constructs, grown on a gene chip array, targeting mostly non-overlapping marker genes. Padlock probes are linear single-stranded DNA (ssDNA) constructs with sequence (e.g. transcript) specific ends, which upon binding to their target allow for ends' ligation. Following molecule circularization, rolling circle amplification (RCA) takes place, creating an easily detectable rolling circle product (RCP) due to the thousand-fold amplification of the original detection sequence. Padlock probes in these gene-specific libraries are associated with unique barcodes embedded in the backbone, connecting sequence-specific ends. The barcoding enables gene detection without the need for sequencing long sequences *in situ*. The development of this method relies on the creation of libraries with thousands of sequence-specific ssDNA padlock probes cut out from PCR-maintained (dsDNA) gene array constructs. The padlock probes can be targeted to genes in their *in situ* position, potentially reaching subcellular resolution. The method also provides the additional benefit in the form of the potential to trace barcodes associated with viral vectors delivered into tissues and to monitor their spread throughout cell types. This can be accomplished with sequence-capturing, i.e. gapped, padlock probes hybridizing on both ends of the sequence of interest, enabling high throughput connectomics studies with single-cell resolution. In this poster, we present the steps taken and the current state of the development from the design and generation of the padlock constructs, aimed at the analysis of cell types within rat brain, to their detection via fluorescent probes or barcode readout. The information obtained through

this method is presented alongside the readout of spatial transcriptomics. The throughput and reproducibility of the method can be increased by the implementation of a microfluidic chip, unifying sample processing, and diminishing errors caused by manual manipulation. With high throughput being made possible by the relatively low cost of the method, it is feasible to eventually create inferred 3D maps of specific tissues with single-cell resolution as well as connection graphs within the nervous system.

51. Using Nanopore Sequencing for Quality Control Analysis of Viral Vectors for use as Gene Therapies

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Packaging gene therapy elements into viral vectors provides an efficient method to introduce the constructs in vivo. Adeno-associated virus (AAV) and lentivirus (LV) are the two viral vectors most commonly used for this purpose and are both produced following transfection of HEK 293 cells. The resultant viral particles predominantly contain the ssDNA or ssRNA encoded between the two ITR and LTR sequences respectively for AAV and LV, however mispackaging of the plasmid or HEK293 DNA has been shown to occur at low proportions. The single strandedness of the DNA and RNA makes sequencing the cargo inside viral vectors challenging, but recent developments in sequencing technology have provided a number of solutions. Recently, Radukic et al. have demonstrated long read sequencing of AAV vectors could be achieved using the Nanopore sequencing library kit and compatible MinION flow cell. To test whether the smaller and cheaper Flongle flow cell could be used for quality control of viral vectors produced for in vivo experiments we extracted the ssDNA from an AAV1 stock virus produced within a GMP facility. This virus contained an RNAi expression cassette for *ATXN1* silencing. From this experiment we achieved 35k reads within 13 hours of loading the Flongle flow cell. Alignment of the reads to the three plasmids used for triple transfection of HEK293 and the human genome demonstrated that packaging included the DNA sequence within the ITRs in greater than 99% of the reads. The backbone of the expression plasmid was observed to be packaged at below 1%, consistent with what has been previously reported. Rare packaging events of the RepCap plasmid and pHelper plasmid were observed only sporadically. Additionally, we identified four loci within the ITRs where a single nucleotide mutation accumulated to a frequency of 16-41% compared to the reference sequence. Using this methodology, the RNAi viral vector was processed and loaded onto the Flongle flow cell in under a day, with over 10k reads generated within the first 3 hours. The library preparation and flow cell was under $200, and the MinION sequencing platform can be purchased for $1000. This provides a quick and cost effective approach to analyze AAV particles prior to using in vivo, and can be readily incorporated in a virus production pipeline.

52. Establishment of a Validated Assay for Clinical Vector Integration Site Analysis: S-EPTS/LM-PCR

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The analysis of vector integration sites (IS) within the host genome constitutes an essential step to evaluate gene therapy safety. Early studies with γ-retroviral vectors showed that vector integration in oncogenes e.g. LMO2 and MECOM led to insertional mutagenesis and severe adverse events triggering the need for IS monitoring in gene therapy trials. We developed and optimized our 'Shearing Extension Primer Tag Selection Linear Mediated PCR' (S-EPTS/LM-PCR). The method comprises random DNA shearing, thus avoiding the use of restriction enzymes, and a reduced number of PCR cycles. These features overcome the restriction and reduce the amplification biases. In addition, specificity and sensitivity are increased by the capture of the target DNA on a solid phase. Here, we present the preliminary data obtained during the development phase. LV-transduced LS174T cells expressing low transgene levels were sorted and cultured in order to obtain single cell-derived clones bearing a single integration event, as well as a polyclonal bulk control. After confirming the presence of a single integration site (IS) in a non-repetitive region, two candidates were selected for the generation of the validation samples. The integration events were located in the vicinity of RERE and PTPN13, respectively. After determination of vector copy numbers (VCN) by qPCR (RERE: 0.9; PTPN13: 0.9; Bulk: 1.2), validation samples of known clonalities were generated by mixing the clone DNA into a polyclonal background (Bulk) resulting in eight control samples (Table 1).

Table 1. Sample clonal composition and summary of the results obtained by S-EPTS/LM-PCR

		Expected			S-EPTS/LM-PCR						
		RERE	PTPN13	Bulk	**RERE**	CV (%)	Accuracy (%)	**PTPN13**	CV (%)	Accuracy (%)	**Bulk**
Sample ID	A	69.04	18.40	12.56	**69.72**	0.19	-0.98	**18.98**	4.73	-3.18	**11.30**
	B	46.79	17.45	35.76	**44.78**	0.37	4.30	**18.71**	4.65	-7.21	**36.51**
	C	26.70	16.60	56.69	**26.78**	5.72	-0.29	**17.18**	2.83	-3.48	**56.04**
	D	17.38	16.21	66.41	**16.09**	3.73	7.41	**18.13**	2.82	-11.88	**65.77**
	E	8.49	15.83	75.68	**7.43**	6.22	12.46	**16.90**	8.34	-6.72	**75.67**
	F	4.19	15.65	80.15	**3.774**	13.60	10.03	**16.92**	5.06	-8.08	**79.31**
	G	0.42	15.49	84.09	**0.292**	42.63	29.75	**16.09**	5.64	-3.85	**83.62**
	H	0.04	15.47	84.48	**0.036**	100.36	14.01	**15.79**	3.97	-2.01	**84.18**

Triplicate sample analysis by S-EPTS/LM-PCR enabled the detection of both clones in all the samples revealing a limit of detection (LOD) of 0.04%, which was the lowest clone frequency assayed. Precision, measured as coefficient of variation (CV), and accuracy were considered in order to define the assay's dynamic range setting a cutoff of +/-15% for both parameters, except for the lower limit of quantification (LLOQ) where the threshold was +/-20%. According to these criteria, the LLOQ was established at a clone frequency of 4.19%. For PTPN13, the stable clone spiked in at an average frequency of 16.4%, the average CV and accuracy were 4.76 and -5.80%, respectively. For RERE, the clone spiked in to simulate increasing clonal contributions, the CV and accuracy throughout the assay's dynamic range were on average 4.97% and 5.49%, respectively. For samples G and H, where RERE clonal contributions were 0.42% and 0.04%, RERE was still detected although CV and accuracy were above

the established thresholds with average values of 71.50% and 21.88%, respectively. Thus, the dynamic range determined was 69.04% - 4.19%. Therefore, the S-EPTS/LM-PCR constitutes a reliable and sensitive method for lentiviral vector integration site detection and quantification. Its full validation will be presented and will enable to satisfy the still unfulfilled regulatory requirements for clinical IS analysis monitoring and contribute towards clinical gene therapy risk assessment standardization.

53. Development of a Peptide Immunoaffinity LC-MS/MS Assay for Frataxin Transgene Protein Measurements after AAV9-FXN Treatment of the MCK Mouse Model of Friedreich Ataxia

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Mutations in the frataxin gene cause Friedreich ataxia (FRDA), an autosomal recessive genetic disease with spinocerebellar degeneration that affects functions of the brain, spinal cord, heart, and other tissues. We aimed to assess the efficacy of AAV9 gene therapy to deliver human frataxin in a murine muscle creatine kinase (MCK) conditional frataxin knockout model of FRDA cardiac disease. Frataxin is highly-conserved between species and has multiple isoforms. We developed a high-sensitivity and high-specificity frataxin protein assay based on tissue pellet protein digestion and online peptide immunoaffinity liquid chromatography tandem mass spectrometry, IA-LC-MS/MS, to quantify endogenous mouse, transgenic human, and mouse and human precursor frataxin forms. This method used an online immunoaffinity peptide enrichment step with anti-peptide antibodies directed against a specific peptide coupled to nanoflow LC and selected reaction monitoring MS. This IA-LC-MS/MS method has high sensitivity with a lower limit of quantification of 21 fmol/mL frataxin in lysate and a wide detection range (21-10,589 fmol/mL) that permits detection of both endogenous and exogenous proteins in multiple tissue matrices. Using this novel method, we were able to determine the frataxin protein expression in the MCK mouse model after AAV-mediated gene transfer and related the protein levels to the phenotype.

54. Development and Optimization of a New, Harmonized Droplet Digital PCR (ddPCR) Method for Pharmacokinetic (PK) Monitoring of Axicabtagene Ciloleucel (Axi-Cel) and Association with Outcomes in ZUMA-1

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Introduction: Axi-cel is a US and EU-approved autologous anti-CD19 chimeric antigen receptor (CAR) T cell therapy for patients with relapsed/refractory large B cell lymphoma (R/R LBCL) after ≥ 2 prior therapies. In ZUMA-1, the PK profile of axi-cel was generated using quantitative PCR (qPCR; Method 1) to derive peak levels of CAR gene marked cells/μL of blood (#CAR T cells/μL) and cumulative levels through 1 month posttreatment (Kochenderfer et al. *Blood.* 2012). The PK profile correlated with clinical response and neurologic events (NEs; Neelapu et al. *N Engl J Med.* 2017; Locke et al. *Lancet Oncol.* 2019). Conversely, PCR-based methods used to generate PK profiles of other CAR T cell products for R/R LBCL derived gene copy number/μg DNA (#CAR copies/μg DNA, as with Method 2) and failed to associate with response (Schuster et al. *N Engl J Med.* 2019). To increase assay sensitivity, reproducibility, and accessibility used to generate PK profiles, we describe development of a new, harmonized ddPCR method (Method 3) and a comparative, retrospective analysis of PK methods using ZUMA-1 patient samples. **Methods:** Peripheral blood mononuclear cells (PBMCs) from patient samples collected post-infusion over time were analyzed using PK derivation parameters (Table 1). The PK data conversion algorithm in Method 1 (qPCR) required pre-infusion product for standard curves and time point matched absolute lymphocyte counts (ALCs). Methods 2 (ddPCR) and 3 (harmonized ddPCR) enumerated integrated vector copy number (VCN). The PK profiles generated by qPCR (Method 1 [#CAR T cells/μL]) and ddPCR (Methods 2 [#CAR copies/μg DNA] and 3 [#CAR T cells/μL]) were compared and correlated with ongoing response and NEs. Kruskal-Wallis (KW) test followed by Dunn's test for pairwise comparisons assessed correlation of PK-based ddPCR/qPCR with response, and Wilcoxon test assessed correlation with NEs. **Results:** PBMCs from 97 patients were analyzed (ongoing response, n = 37 for qPCR and 36 for ddPCR; nonresponders, n = 17 and 15, respectively; relapsed, n = 43 and 40, respectively). Method 1 yielded significant association with ongoing response ($P = 0.0018$), but Method 2 did not ($P = 0.2006$; Table 2; Figure A-B). Methods 1 and 2 showed significant association with Grade ≥ 3 NEs ($P = 0.007$ and 0.0002, respectively; Table 2; Figure A-B). The PK profile generated by Method 2 poorly correlated with Methods 1 and 3 (Figure C); a significant linear correlation ($R^2 = 0.9817$; $P < 0.0001$) was observed between Methods 1 and 3 (both utilized #CAR T cells/μL). Method 3 additional sample analysis and correlations are ongoing and will be presented. **Conclusions:** We report successful development and harmonization of a new ddPCR method (Method 3) that offers potential applications for investigational and commercial implementation. Further validation may be required. Method 1 (#CAR T cells/μL) was significantly associated with ongoing response and NEs while Method 2 (#CAR copies/μg DNA) only correlated with NEs. Methods 1 and 3 were highly correlated (both #CAR T cells/μL). These results suggest increased correlative value of PK methods that derive #CAR T cells/μL (Methods 1, 3) versus #CAR copies/μg DNA (Method 2).

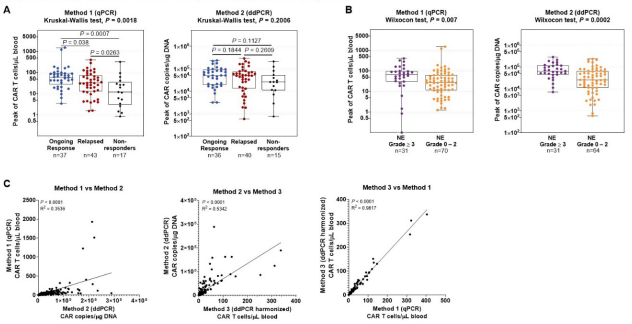
Table 1. Methods and parameters used for PK derivation.

Experimental Method	Units	Formula Parameters
Method 1 (qPCR)	#CAR T cells/µL blood	% CAR/housekeeping gene post infusion, product for standard curve, % transduction, ALC
Method 2 (ddPCR)	#CAR copies/µg DNA	VCN post infusion & no. of cells producing 1 µg DNA
Method 3 (ddPCR harmonized)	#CAR T cells/µL blood	VCN post infusion, product VCN (single test), % transduction, ALC

Table 2. PK profiles generated by Methods 1 and 2 were compared and correlated with **A.** response (ongoing response, nonresponders, relapsed) and **B.** NEs by category (Grade 0-2 and Grade ≥ 3). Data, excluding *P* values, are presented as n | median peak measurement.

	A. Response							B. NEs		
	Response Category			P Value From Dunn's Test			P Value From KW Test	NE Category		P Value From Wilcoxon Test
Method	Ongoing Response	Relapsed	Nonresponders	Ongoing Response vs Relapsed	Ongoing Response vs Nonresponders	Relapsed vs Nonresponders		Grade 0 - 2	Grade ≥ 3	
Method 1 (qPCR) CAR T cells/µL blood	37 | 61.6	43 | 32.0	17 | 12.1	0.038	0.0007	0.0263	0.0018	70 | 27.3	31 | 63.5	0.007
Method 2 (ddPCR) CAR copies/µg DNA	36 | 50868	40 | 49410	15 | 25920	0.1844	0.1127	0.2609	0.2006	64 | 26365.5	31 | 65286	0.0002

Figure. Box plots showing differences in PK methods between **A)** ongoing responders, nonresponders, and relapsed patients (n = 97 patients total) or **B)** NEs by Grade 0 - 2 versus ≥ 3. **C)** Correlations between PK profiles.



55. Implementation of an Automated VCN Platform to Assess Integration of Lentiviral Vectors in a High-Throughput Capacity

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Lentiviral vectors (LVVs) have proven to be efficient gene delivery vehicles in a growing number of gene therapy applications. The accurate quantitation of gene transfer is essential for vector development at both research and clinical stages. Vector copy number (VCN) is the measure of integrated vector genomes per diploid cell within a population of cells, and its measurement is a critical analytic tool used in assessing the transduction efficiency of therapeutic LVVs. However, VCN analysis is currently a manual, multi-step process that necessitates the use of several separate, time-consuming, and laborious steps. First, genomic DNA (gDNA) is extracted from transduced target cells. Next, a multiplexed qPCR assay is used to probe the extracted gDNA for a segment of the lentiviral backbone and an endogenous reference gene. Finally, the relative quantity of the vector segment to the reference gene is used to generate VCN values. Given the fact that thousands of samples are generated annually for screening via the VCN assay, we sought to develop an automated high-throughput approach to shorten wet lab hands-on time, improve data reproducibility and consistency, and increase overall throughput of data delivery. By moving the VCN wet-lab workflow onto a suite of liquid handling instruments, we've increased the hourly throughput of samples moving through gDNA extraction and qPCR analysis by 32-fold. With the introduction of this automated workflow, the number of LVVs screened on the VCN platform increased by 533% monthly, all while reducing wet lab hands-on time spent by as much as ten-fold. This platform has consistenly reduced inter-sample variability measured in the qPCR assay. In addition, an interactive, R-shiny-based VCN analysis application was developed to efficiently process the increase in raw data generated. Users can input raw qPCR data into the application and receive comprehensive graphs outlining VCN values linked to given experimental conditions. With the incorporation of this user-friendly interface to assist in data processing, we have fully automated the VCN analysis pipeline from start to finish. The automation of our VCN workflow drove an increase in the number of samples analyzed that would not have been possible with a manual process and resulted in a decrease in operator associated inter-assay variability and improved data consistency. Automation of critical assays such as VCN is vital to scaling the development of LVV-enabled cellular therapeutics.

56. Development of a Single-Cell Western Assay for Anti-Sickling Beta Globin Drug Products for Sickle Cell Disease

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Sickle cell disease (SCD) results from a point mutation (E6V) in the beta (β)-globin gene, leading to production of abnormal sickle hemoglobin (HbS) in erythroid cells. Under low oxygen conditions, HbS can polymerize causing the characteristic sickled shape of red blood cells (RBCs). These rigid and inflexible sickled RBCs are prone to hemolysis and can obstruct small blood vessels, which leads to significant morbidity and early mortality due to complications such as chronic anemia, painful vaso-occlusive events, stroke, and organ damage. Drug product for LentiGlobin for SCD gene therapy is made by the *ex-vivo* lentiviral vector (LVV)-mediated addition of a modified β-globin gene with the anti-sickling substitution ($\beta^{A\text{-}T87Q}$) into autologous CD34+ hematopoietic stem cells (HSCs). Once transplanted into patients, these transduced cells can differentiate into RBCs that express both anti-sickling $\beta^{A\text{-}T87Q}$ as well as β^{S} (given their progenitors still carry the EV6 point mutation in the β-globin gene). RBCs containing both β^{S} and $\beta^{A\text{-}T87Q}$ have the potential to demonstrate a reduced propensity to sickle as is observed in RBCs from individuals with sickle cell trait. Therefore, it is important to understand the proportion of RBCs expressing the gene-therapy derived $\beta^{A\text{-}T87Q}$. While high-performance liquid chromatography (HPLC) can be used to quantify expression of bulk levels of $\beta^{A\text{-}T87Q}$, this method does not provide information on how $\beta^{A\text{-}T87Q}$ expression is distributed within the RBC population. To measure the $\beta^{A\text{-}T87Q}$ expression across a population of RBCs differentiated from gene-modified HSCs, we developed a single-cell Western (scW) assay. Briefly, scW involves loading a cell suspension onto a commercially available specialized chip with microwells, confirming single-cell dispersion, lysing cells within wells, electrophoresis, cross-linking, and probing with two antibodies: one specific to β^{S} and a second that recognizes normal adult β-globin chain (β^{A}) or $\beta^{A\text{-}T87Q}$. Chips are then read on a microarray scanner to detect bound antibodies. Optimization of each of these parameters was performed using mixtures of healthy donor (HD) and SCD RBCs. Due to the single-cell based nature of this assay we can determine the percentage of cells positive for β^{S} (SCD RBCs), β^{A} (HD RBCs) or both (i.e. in wells that were found to erroneously contain more than one cell or in RBCs derived from transduced HSCs that express both β^{S} and $\beta^{A\text{-}T87Q}$). Assay precision was evaluated for both β^{A} and β^{S} at five relative analyte concentrations, by three operators, over three consecutive days. When the relative

proportions of SCD and HD RBCs were above 25%, inter-assay precision of β^S was 15% and that of β^A was 20%. Robustness of the assay was performed to evaluate instrument-to-instrument antibody variability. scW platform instrument-to-instrument variability resulted in a coefficient of variation (CV) of 3.23% for β^A and 4.44% for β^S. Microarray scanner-to-scanner CV was 0.77% for β^A and 0.95% for β^S. The assay showed good linearity when mixtures of SCD and HD RBCs were evaluated (R^2>0.95). The stability of samples from subjects treated with LentiGlobin for SCD gene therapy was also evaluated. Over 7 days post-collection, 96.8% of samples (n=31) from these subjects were stable with a percent change in $\beta^{A\text{-}T87Q}$-positive RBCs of less than 15.7%. These data demonstrate that the scW assay was able to precisely detect the percent of β^A only-positive, β^S only-positive, and double-positive cells in mixtures of RBCs from HD and patients with SCD. This assay is robust, precise, and suitable for detecting the proportion of RBCs that contain β^A or $\beta^{A\text{-}T87Q}$ in subjects treated with allo-hematopoietic stem cell transplant or gene therapies delivering $\beta^{A\text{-}T87Q}$ for SCD, respectively.

Vector and Cell Engineering, Production or Manufacturing I

57. Akt Inhibition Enhances Memory, Proliferation and Cytotoxicity in CD19 CAR T Cells

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Background: CD19 chimeric antigen receptor (CAR) T cells deliver excellent clinical responses in relapsed/refractory acute lymphoblastic leukaemia (ALL) and high grade B cell lymphoma. However, a significant number of patients do not respond to CAR T cells or relapse post treatment as a result of poor CAR T cell expansion and persistence. Adoptive cell transfer studies have shown that naïve (Tn) and central memory (Tcm) T cell subsets compared to effector/terminal (Te/Temra) subsets, deliver superior T cell expansion, persistence and anti-tumour efficacy in vitro and in vivo. However, in patients the proportion of Tn/Tcm subsets can vary considerably and current manufacturing protocols prioritising mass cell expansion often give rise to Te/Temra skewed products. Strategies to optimise CAR T cell manufacture to enrich Tn/Tcm subsets is of critical importance to the field. Studies have demonstrated that the ex vivo inhibition of Akt signalling, an important mediator of T cell activation and differentiation can enrich such Tn/Tcm populations. **Aim:** Here, we explore the impact of Akt inhibition on CAR T cell manufacture, from pre-clinical assessments to the development of an optimised current good manufacturing practice (cGMP) compliant manufacture method for the generation of enhanced CAR T cell products for clinical use. **Methods:** Pre-clinical assessments were carried out using healthy donor CD4/8 selected T cells. T cells were activated using the TransAct™ reagent and transduced with a lentiviral vector encoding a second generation CD19 targeting 41BBz CAR construct, as used in NCT02443831/02935257 trials. Cells were manufactured for a total of eight days and maintained in TexsMACS™ medium supplemented with human serum, IL7, IL15 in the presence/absence of Akt inhibitor VIII (Akti). The final product was evaluated by immunophenotyping for Tn/Tcm subsets, functional testing for proliferation, cytotoxicity, cytokine release and by transcriptome analysis. Based on this data, cGMP scalability onto the semi-automated, CliniMACS Prodigy® platform was validated through parallel runs using excess ALL patient material from the NCT02935257 trial in the presence/absence of Akti. **Results**: CD8 CAR T cells manufactured in the presence of Akti were significantly enriched for Tcm populations (CD62L+/CD27+/CD28+/CD45RA-/GZMB-) compared to untreated cells. Co-culture of CAR T cells with a CD19+ target cell line (Raji) led to a 3.9 fold increase in CD8 CAR T cell proliferation in Akti treated versus untreated cells. Additionally, increased secretion of IL2, INFg and TNFa following a 72 hour exposure to Raji cells was seen in the treated arm, demonstrating an enhanced effector profile. Following a prolonged 7 day co-culture with Raji's, re-challenge of Akti treated CAR T cells resulted in significantly higher cytotoxicity towards CD19+ targets in comparison to untreated cells at low effector to target ratios. Transcriptome analysis of Akti treated and untreated CD8 populations reveal enriched co-stimulation and T cell activation signatures, complemented by decreased effector function signatures. The analysis also revealed an increase in autophagy related genes, all of which have been implicated in T cell survival and memory formation. We have successfully scaled this novel manufacture method to cGMP on the CliniMACS Prodigy® using ALL patient derived starting material and have shown favourable phenotypic and functional profiles consistent with pre-clinical assessments. **Conclusion**: We report an optimised CAR T cell manufacture method which permits Tcm enrichment and generates a CAR product with enhanced proliferative and cytotoxic capabilities. We plan to adopt this method imminently onto the UCL CAR T cell programme for cGMP manufacture on the CliniMACS Prodigy®.

58. A New Primary Human Muscle Stem Cell Product, PHSats

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Skeletal muscle possesses an own stem cell population, the satellite cells (SCs). The high regenerative capacity makes SCs a perfect source of cells for cell-based therapies of muscle diseases. We invented a new technology that allows for the first time million-fold expansion of human satellite cells and at the same time the delay of their differentiation. We name our product PHSat (**p**rimary **h**uman **sat**ellite cell product). Hypothermia pretreatment eliminates otherwise co-isolated contaminating fibroblasts. Without the need of a cell-sorting procedure our cell colonies are >98% myogenic (desmin-positive). The muscle tissue is gently mechanically prepared, no enzymatic digestion is performed. By this, we generate native (not activated) and highly regenerative satellite cell populations. The regenerative potential of PHSats has been demonstrated in preclinical efficacy studies: 1. Injected PHSats build muscle fibers, 2. They re-populate the satellite cell niche and 3. They regenerate muscle also after re-injury. In order to further develop PHSats as an Advanced Therapeutic Medical Product (ATMP) we transferred the manufacturing process into a Good Manufacturing

Practice (GMP) environment. Especially in the field of cell therapy, it is important to have fixed process parameters in order to have the same output when the starting material from patient to patient varies. Despite the fact that our starting material contains mixed cell populations we can ensure that our final drug product contains primary human satellite cells only. The purity of the resulting PHSats is monitored by immunostaining and FACS. We defined potency-, impurity- and purity markers. Sterility is a major concern, because the final product cannot terminally sterilized and antibiotics are avoided during the entire production process. We validated that PHSats can be stored for 2 years with unchanged regenerative capacity Several time points are tested for all relevant approval criteria. During the process of repeated freeze-thaw cycles, we quantified the viability and potency of the cells. Cell concentrations also have an impact on the viability of cells. Our product is a ready-to-inject vial. We are in the process of preparing the first clinical trial using PHSats.

59. Novel Strategies to Enhance CAR-T Cell Production and Improve Quality During Manufacturing

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Chimeric antigen receptor (CAR)-T cells have shown remarkable efficacy against some blood cancers and can potentially treat many other human diseases. During CAR T-cell manufacturing, T-cells are activated via engagement of the T-cell receptor (TCR), which activates proximal TCR signaling pathways resulting in cellular expansion. However, persistent TCR signaling can induce unchecked activation, differentiation and exhaustion, which can affect CAR-T cell production, quality and efficacy. In addition, patient-derived T-cells, which are commonly used to manufacture CARs, are prone to donor-to-donor variability and can be affected by their disease status and prior therapeutic treatments. These inherent product and process-related challenges have led to manufacturing failures in part due to failure to meet required quality attributes. Thus, strategies to improve CAR-T cell product quality during manufacturing may help improve production and reduce manufacturing failures. Previously, we found that activation of distal TCR signaling in the presence of a Src-kinase inhibitor (SKI) enhanced NFAT1 mediated signaling in human T-cells. Since NFAT1 plays a critical role in T-cell activation, expansion and effector function, we assessed the effect of SKI on CAR T-cell quality attributes. SKI-treatment improved CAR T-cell production by increasing cellular expansion and reducing unwanted cellular activation (CD25), exhaustion (PD1) and differentiation ($CD45RA_{neg}$). SKI-treatment did not negatively affect anti-CD19 CAR T-cell stability, *in vitro* potency or CAR expression compared to CAR T-cells treated with DMSO control. When comparing with CAR T-cells conventionally manufactured by activating proximal TCR signaling (anti-CD3/CD28/CD2), CAR T-cells manufactured by activating distal TCR signing in presence of SKI had an increased CD8/CD4 ratio, decreased cellular activation, exhaustion, differentiation and comparable *in vitro* potency (cytotoxicity), stability and CAR expression. These CAR T-cells also released significantly lower levels of inflammatory cytokines (GM-CSF, IFN-γ) following co-culture with CD19+ve tumor cells. Together, these data suggest that manufacturing CAR T-cells by activating distal TCR signaling in presence SKI improves numerous product quality attributes without affecting potency and stability. **Acknowledgements**: This work was supported by the Intramural Research Program of Center for Biologics, Evaluation and Research (CBER) and the Challenge Grant from the Office of Chief Scientist, U.S. Food and Drug Administration. This project was supported in part by Dr. Gauri Lamture's appointment to the Research Participation Program at CBER administered by the Oak Ridge Institute for Science and Education through U.S. Department of Energy and FDA.

60. A Universal High Density Cell Respirator (HDCR) Bioreactor for Intensified Production of Gene Therapy Vectors

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AAV-based gene therapy vectors are under intense investigation and rapidly becoming established as clinical therapy for gene-based diseases including inherited diseases. Despite their great promise, the single biggest limitation to their widespread use is the inability to manufacture sufficient quantities of high quality AAV vectors rapidly particularly at low cost. This represents the single biggest limitation to the development of clinical AAV gene therapy. The intensification of cell-based production processes holds great promise for increasing the capacity and speed of AAV vector manufacturing while also reducing cost, by saving on space, time, labor, and resources. While much effort to date has focused on improving cell specific productivity (vector genomes (vg)/cell), little progress has been made on increasing cell density, which remain at 10^{6-7} cells/mL due to limitations of either surface area or gas exchange and shear forces (e.g. stirred tank reactors, perfusion reactors). With total productivity being the product of specific cell productivity and cell density, there exists substantial untapped potential in high-density culture. Recognizing the enormous discrepancy in the necessary gaseous exchange rate compared to soluble nutrient/waste exchange rate for most cells (e.g. 90× for HEK293), we hypothesized that 1) decoupling mass transport through novel bioreactor architecture would enable higher cell densities and that 2) the optimized cell niche afforded by this architecture would maintain cell specific productivity even at high cell densities, with an overall impact of significantly elevated production capacity. With the goal of improving AAV vector production capacities and to address issues of cell growth and densities, we designed a high-density cell respirator (HDCR). The HDCR is a scalable bioreactor comprised of stackable, gas and media perfusable membranes, which achieves an oxygen mass transfer coefficient k_La of > 40 / hr via membrane permeation to support high density culture $>10^8$ cells/mL. The micro-architecture of the membranes has been engineered using integrated finite element modeling to guarantee that all cells receive sufficient gas, nutrient, and waste exchange and are protected from shear forces. We present cell growth curves demonstrating the system is compatible with suspension (e.g. CHO-S), adherent (e.g. HEK293, A549), and microcarrier (e.g. CV-1 on Cytodex-3) cultures to $>10^8$ cells/mL making it a universal platform.

Importantly, we demonstrate that the HDCR bioreactor is compatible with AAV vector production, including the steps of cell seeding, expansion, transfection, feeding, and harvesting. We show for the first time that the optimized cell growth niche in the HDCR maintains productivity (within 80% vg/cell of low-density culture) despite increasing cell density by 400% for a total increase in AAV production of 320%. The results of ongoing optimization studies for AAV production in the HDCR will be presented. From these initial empirical results and modelling, we predict that a scaled HDCR bioreactor fitting inside a standard 160L tissue culture incubator would support the expansion of $>2\times10^{12}$ cells and production of up to 10^{17} vector genomes of AAV. By matching mass transport with cellular demands AAV production can be intensified, unlocking the potential for researchers to screen AAV candidates faster and for clinical vectors to be affordably manufactured.

61. Acoustic Affinity Cell Selection: A Non-Paramagnetic Scalable Technology for T Cell Selection From Unprocessed Apheresis Products

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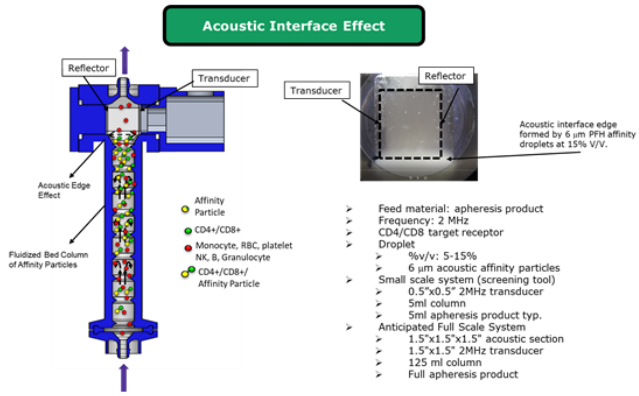
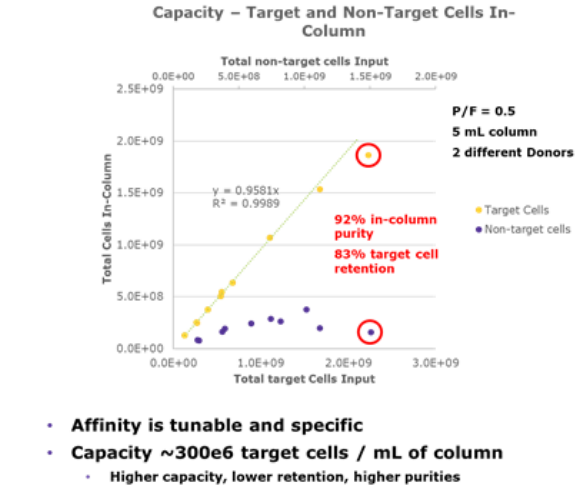
Acoustic Cell Processing is a unique acousto-fluidics platform technology for minimal shear manipulation of cells using ultrasonic standing waves. The platform has broad applications in the field of cell and gene therapy, e.g., cell concentration and washing, cell culturing, microcarrier/cell separation, acoustic affinity cell selection and label-free cell selection. The acoustic radiation force exerted by the ultrasonic standing wave on the suspended cells in combination with fluid drag forces and gravitational forces is used to manipulate the cells and achieve a certain cell processing unit operation, e.g., separate, concentrate, or wash. The technology is single-use, continuous, and can be scaled up, down or out. It therefore allows for a flexible and modular approach that can be customized to process a desired cell count, cell culture volume or cell concentration within a given required process time. Utilizing its proprietary multi-dimensional standing wave platform, MilliporeSigma has been developing the Acoustic Affinity Cell Selection (AACS) system for closed and automated Cell and Gene Therapy manufacturing, e.g., CAR-T immunocellular therapies. The AACS technology is a scalable acoustic affinity cell selection method using acoustic (non-paramagnetic) affinity beads for positive or negative cell selection. A multi-dimensional acoustic standing wave is then used to separate the affinity bead-cell complexes from the unbound cells, thereby completing the process of a negative or positive cell selection. In this work the AACS system has been used to capture CD4+ and CD8+ cells from unprocessed apheresis products. The AACS column and acoustic section (Fig. 1A) allow for a continuous flow of the initial cell population (pre-labeled with biotinylated antibodies) while acoustically retaining the acoustic affinity particles in the column. The affinity particles are functionalized with Neutravidin and thus capture the target cells that are kept inside the column, while the non-target cells are washed out of the column. The small scale 5mL column has a total target cell capture capacity of 1.8B cells (60mL Leukopak volume), of which 83% are retained in the column at a 92% purity (Fig. 1B). The AACS scale up system can process a full apheresis product (300-450mL) in less than 2h.





62. The Lisocabtagene Maraleucel (liso-cel) Manufacturing Process is Designed for Consistency, Purity, and Robustness Across B-Cell Malignancies

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Background. Chimeric antigen receptor (CAR) T cell therapies have demonstrated significant clinical benefit across various hematologic malignancies. However, the autologous nature of this therapeutic modality introduces patient-to-patient variability (including significant variance in the absolute lymphocyte count [ALC] and CD8/CD4 ratio of incoming material) into the manufacturing process and can create challenges for achieving CAR T cell manufacturing consistency

across patient populations and disease indications. Liso-cel is an investigational, CD19-directed, defined composition, 4-1BB CAR T cell product administered at equal target doses of CD8+ and CD4+ CAR+ T cells. Liso-cel is being developed for the treatment of multiple B-cell malignancies, including relapsed/refractory large B-cell non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), and pediatric acute lymphoblastic leukemia (pALL). The liso-cel manufacturing process is designed to be robust and generate consistent product quality across multiple disease indications and heterogeneous patient populations. **Methods.** The liso-cel manufacturing process involves sequential selection of CD8+ and CD4+ T cells from leukapheresis material, followed by independent CD8+ and CD4+ T cell activation, transduction, expansion, formulation, and cryopreservation. There are no ALC threshold requirements to initiate the liso-cel manufacturing process. Liso-cel was manufactured to enable the TRANSCEND NHL 001 (NHL, including large B-cell lymphoma and mantle cell lymphoma), TRANSCEND CLL 004 (CLL/SLL), and JCAR017-BCM-004 (pALL) trials. Flow cytometry was used for phenotypic analysis of T and B-cell composition of leukapheresis, selected T cell starting material, and drug product. **Results.** Enrichment of T cell subsets before activation enables robust manufacturing of drug product across a wide range of ALCs ($0.08\text{-}3.6 \times 10^9$/μL; n=183) and disease histologies. Characterization of leukapheresis material composition with variable ALCs demonstrates significant CD3+ T cell frequency differences, whereas selected intermediates exhibit similar T cell purities and quantities for activation. CLL/SLL leukapheresis material contains increased CD19+ B-cell frequencies (viable leukocytes: median, 13.5%; max, 94.6%; n=28) compared with NHL leukapheresis material (viable leukocytes: median, 0.0%; max, 76.5%; n=178; Wilcoxon rank sum $P=5.3 \times 10^{-10}$). Nevertheless, equivalent clearance of non-T cell impurities, including B cells, was achieved via selection, as indicated by high CD3+ T cell frequencies in selected material (median: CLL/SLL, 99.2%; NHL, 98.3%) and at final drug product (median: CLL/SLL, 99.9%; NHL, 99.9%). Control of the number of T cells entering activation unit operations affords downstream process control and consistency of product quality across heterogeneous patient populations. **Conclusions.** The liso-cel drug product manufacturing process is a robust platform capable of generating highly purified CD19-directed CAR T cell immunotherapy from heterogeneous patient populations comprising multiple B-cell malignancies and disease histologies, with no minimum ALC required. Enrichment of CD3+ T cells through sequential selection of CD8+ and CD4+ T cell lineages upstream in the manufacturing process is a key aspect to reducing between-lot variation across diverse patient populations that also aids in process robustness and consistency of drug product quality.

63. Using Numerical Modelling (CFD) to Design and Improve a 3D Printed Atomizer for the Transfection of Cells with Virus

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Numerous vector-free transfecting methods for cell engineering are currently being developed across the Pharmaceutical industry as more cost-effective alternatives to viral-vector based transduction methods. Spray atomization is a transfecting method which potentially enables high transfection rates of target cells while maintaining high cell viability. Limited knowledge exists about the fluid dynamics of the spray atomization process for cell transfection, particularly when a custom designed 3D printed device is used. Numerical modelling can provide an in-depth understanding of the current process, key features affecting performance and how to optimize the spray delivery method. This can reduce the number of experiments that need to be run by defining the optimal operating conditions and assessing potential 3D designs without the associated cost and time. Numerical models were combined with laboratory-scale experiments to understand the fluid dynamics of a 3D printed spray atomizer, optimize the design and scale the process from laboratory to pilot scale. Liquid- and air-lines were joined in an atomizer via a parallel path connection. The sample payload (in the liquid line) mixed with the air stream, delivering spray droplets to a target area, was located below the atomizer. The transfection rate was dependent on how evenly the droplets were dispersed on the cell well and the momentum of the droplets on impact. Computational Fluid Dynamics (CFD) used direct numerical simulations to model the spray dynamics in the current system and to assess different process scales and/or design features. CFD models were run to characterise the spray for two of the atomizers previously tested at lab scale. Uneven sample dispersion and high air velocities were observed in both lab scale designs. The spray dispersion patterns generated in both designs were replicated in the numerical models. The connection between the air and sample line was shown to cause uneven droplet dispersion on the target area An alternative atomizer was designed for lab scale with the objectives of improving sample dispersion, maintaining the appropriate droplet momentum across the cell well and reducing the dead sample volume. The optimised design contained an elbow in the sample line to direct the payload towards the air jet stream. This allowed the sample line to be narrowed without the associated increase in sample velocity disrupting spray formation. An additional 3D printed dispersion feature (ADF) was numerically designed, tested and added directly after the spray formation to promote even sample dispersion. This achieved the design goals of even sample dispersion and reduced dead volume at laboratory scale. At pilot scale filtered vents were employed to prevent a pressure build-up in the spray chamber that could damage the cells to be transfected. Simulations at pilot scale showed the suitability of the recommended 3D printed design at a larger scale and optimised the location of the vents so the developed spray patterns were not negatively affected and the pressure maximum in the chamber was below critical limits. This integrated approach between numerical models and experiments allowed for the rapid assessment of potential practical solutions on a laboratory scale device. The same approach was then implemented at pilot scale.

CAR T-Cells Targeting Solid Tumors

64. Combining De-Glycosylating Agents with CAR T Cells for Targeting Solid Tumors and Improving Therapeutic Index

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The adoptive transfer of CAR T cells demonstrated impressive results against B-cell malignancies but still limited efficacy against solid cancers. Since solid tumors display a wide range of glycosylation alterations, including increased N-glycan branching, we hypothesized that peptidic epitopes may be masked by glycans from CAR T cell targeting. In the present study, to investigate if sugar chains may be sterically hulking for CAR T cells, we generated N-glycosylation defective pancreatic tumors by knocking-out the expression of the glycosyltransferase Mgat5 using the CRISPR-Cas9 technology.Hampering N-glycosylation resulted in a dramatic increase of tumor elimination by CAR T cells targeting two model antigens (CD44v6: $p<0.001$; CEA $p<0.001$). Interestingly, this effect also associated with stronger intracellular NFAT and NF-kB signals upon CAR engagement and numerous advantageous features in the formation of the immunological synapse, including significantly higher F-actin accumulation (p=0,0027), reduced distance of MTOC to F-actin (p=0,0225) and stronger granule convergence (p=0,0042), suggesting a more proficient antigen engagement in the absence of tumor glycosylation. To exploit this mechanism in order to increase the efficacy of CAR T cells against solid tumors, we sought to block tumor N-glycosylation with the glucose/mannose analogue 2-Deoxy-D-glucose (2DG). Similarly to glycosylation knocked-out cells, treatment with 2DG also sensitized tumors to recognition by CAR T cells, significantly increasing their killing (CD44v6: $p<0.01$; CEA: $p<0.001$), release of inflammatory cytokines and activation of nuclear transcription factors. Accordingly, when challenged against high tumor burdens in vivo, CAR T cells highly benefited from 2DG administration, resulting in more profound antitumor responses (p=0.0036). Interestingly, improved antitumor activity associated with a significantly less exhausted phenotype, as assessed by a lower frequency of cells co-expressing an array of exhaustion and senescence markers (SPICE software analysis, p=0.0105), suggesting that cumbersome N-glycans likely contribute to cancer progression by fostering immune inhibitory signals and inducing a profound immunological dysfunction. Moreover, different types of carcinomas, including those arising from lung and bladder, revealed to be extremely N-glycosylated. As expected, 2DG significantly increased their elimination by CAR T cells, asserting the broad applicability of this approach toward several solid tumor types. Finally, thanks to metabolic deregulation 2DG is expected to selectively accumulate in cancer cells compared to healthy tissues, supporting the safety of the combined approach. Accordingly, we observed that the same doses of 2DG able to enhance tumor recognition by CAR T cells failed to de-glycosylate or to increase killing of healthy cells. Overall, our results indicate that i) the glycosylation status of tumor cells regulates the efficacy of CAR T cells and that ii) combining CAR T cells with the de-glycosylating agent 2DG, which preferentially accumulates in tumor masses, may pave the way for a successful immunotherapy against solid tumors.

65. Overcoming the Breast Tumor Microenvironment by Targeting MDSCs through CAR-T Cell Therapy

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Broadening the application of CAR T cells (CARTs) to target solid tumors has proven challenging, largely due to the hostile immune suppressive tumor microenvironment (TME). In breast cancer (BC), myeloid derived suppressor cells (MDSCs) are the main contributors which inhibit CARTs function and persistence within the TME. To effectively target BC, we developed and validated a CAR, targeting tumor-expressed mucin 1 (MUC1) (Bajgain P et al, 2018). In order to potentiate the expansion and persistence of MUC1 CARTs and regulate the TME, we generated a novel chimeric receptor, TR2.4-1BB, encoding a scFv targeting TNF-related apoptosis-inducing ligand receptor 2 (TR2) linked to the endodomain of the co-stimulatory molecule 4-1BB. We hypothesize that upon engagement with TME-resident MDSCs, which express high levels of TR2, CARTs will receive an activating co-stimulatory signal which will promote T cell persistence and expansion. To evaluate the benefit of TR2.4-1BB, we compared the phenotypic profile, proliferative capacity, cytotoxic activity and anti-tumor effects of T cells expressing either CAR.MUC1 alone, TR2.4-1BB alone, or a combination of both CAR.MUC1 and TR2.4-1BB. T cells were activated with CD3/CD28 antibodies and subsequently transduced with retroviral constructs, resulting in high level of expression of each transgenic receptor (87.3±4%, 80.8±2.7% and 74±5.5% transduction efficiency for MUC1, TR2.4-1BB and MUC1.TR2.4-1BB CARTs, respectively, mean±SEM, n=4). Next, to evaluate the function of TR2.4-1BB, we exposed transgenic cells to recombinant TR2 and evaluated NFKB translocation (due to 4-1BB signaling) by performing ELISA. We found that NFKB translocation was induced only in cells expressing TR2.4-1BB (percent absorbance: 0±0% and 3±0% NFkB translocation, NT (non-transduced) and TR2.4-1BB respectively, mean±SEM, n=3). Co-culture of CD3/CD28 stimulated NT T cells with MDSCs significantly decreased T cell proliferation by 75±5% and IFN-γ production by half compared with T cells cultured alone. Moreover, in presence of MDSCs, cytotoxic activity (4hr cytotoxicity assay) of MUC1 CARTs against MUC1+ BC cell lines was diminished by 25%. However, transgenic TR2.4-1BB expression on CAR.MUC1 T cells induced MDSC apoptosis in a cytotoxicity assay (70±7% vs 15±2% CAR.MUC1 alone) thereby restoring the cytotoxic activity

of CAR.MUC1 against MUC1+ BC lines in presence of TR2.4-1BB (67±8.5%). Finally, to assess the anti-tumor effects associated with co-expression of CAR.MUC1 and TR2.4-1BB on T cells, we established a xenograft model by co-injecting BC tumors with or without MDSCs in the mammary fat pad of NSG mice. Co-injection of MDSCs with tumor cells led to approximately two-fold increase in tumor growth and enhanced distant metastatic spread compared to tumor cells alone. Treatment of these MDSC-containing established tumors with firefly luciferase-labeled MUC1.TR2.4-1BB CARTs resulted in a significant delay in tumor growth (576.5±87.8 mm^3) compared to CAR.MUC1 (1131.9±89.2mm^3) or TR2.4-1BB (1032.6±46.4 mm^3) T cells alone (on Day 49 after T cell injection). The treatment also improved T cell proliferation and persistence at the tumor site as measured by in vivo bioluminescence imaging ($2.14x10^8$±$7.91x10^7$p/s vs $8.02x10^7$±$7.88x10^7$p/s and $1.06x10^7$±$9.10x10^7$p/s for CAR.MUC1 and TR2.4-1BB respectively, on Day 49). In conclusion, our data indicate that CARTs co-expressing our novel TR2.4-1BB receptor have higher anti-tumor potential against BC tumors and infiltrating MDSCs, resulting in TME remodeling and improved T cell proliferation at the tumor site.

66. Induction of Potent Systemic and Tissue Resident Immune Responses by ExoVACC™: A Novel Exosome-Based Vaccine Platform

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Background: Exosomes are an important intercellular communication system facilitating the transfer of complex macromolecules between cells that is mediated by a complex network of proteins and glycoproteins on the exosome surface which play a role in cellular tropism and uptake, as well as, immune cell signaling. We identified PTGFRN and BASP1 as exosome-enriched proteins found on the exosome surface and in the lumen, respectively. Exosomes expressing PTGFRN are preferentially taken up by antigen presenting cells (APCs) which render them an ideal carrier of antigen and adjuvant cargo to selectively deliver and activate APCs. We developed exoVACC™, a novel vaccine platform built on engineering exosomes for the coincident delivery of biologically active, exogenous antigens and adjuvants to induce robust antigen-specific immune responses. Using engineered exosomes containing the model antigen ovalbumin (OVA), we demonstrate the versatility and superior efficacy of the exoVACC platform compared to traditional soluble antigen / adjuvant combinations. **Methods and Results:** Expression of exogeneous biomolecules fused to PTGFRN and BASP1 results in uniform, high-density display on the exosome surface (using PTGFRN) or packaging into the lumen (using BASP1). Exosomes overexpressing a BASP1-OVA fusion protein contained over a hundred copies of antigen per exosome. Mice vaccinated with BASP1-OVA exosomes without adjuvant did not induce OVA-specific immune responses. We stably loaded a STING agonist adjuvant to the BASP1-OVA exosomes (referred hereafter as exoVACC) and assessed immune responses through different routes of administration. A single dose of exoVACC induced approximately 9 to 20-fold greater numbers of splenic antigen specific $CD8^+$ T effector memory cells (T^{EM}) when administered subcutaneously (SC), intranasally (IN) or intravenously (IV) compared to soluble OVA and AddaVax adjuvant administered SC. exoVACC also induced 5-fold greater numbers of splenic antigen specific $CD4^+$ T cells compared to soluble antigen and alum. We investigated whether exoVACC could induce mucosal immune responses. A single IN administration of exoVACC induced robust lung mucosal $CD8^+$ resident memory T cells (47% $CD8^+T^{RM}$) that increased following a second IN dose (67% $CD8^+T^{RM}$). We tested the efficacy of exoVACC in mice transplanted SC with E.G7-OVA tumors. A single prophylactic dose (IN) of exoVACC attenuated E.G7-OVA tumor growth rates in all mice (0.037 mm^3/day) and completely inhibited tumor growth in 50% of mice whereas mice vaccinated IN with soluble OVA and poly(I:C) had tumor growth rates (0.121 mm^3/day) comparable to vehicle (PBS) treated mice (0.113 mm^3/day). Lastly, we enhanced exosome targeting and activation of rare cDC1 cross-presenting DCs by engineering the surface expression of an anti-Clec9A scFab via fusion to PTGFRN. Anti-Clec9A-PTGFRN exosomes demonstrated enhanced tropism for and uptake by cDC1s in vivo when administered IV and, when loaded with a STING agonist, enhanced antigen-specific $CD8^+$ T cell responses in the CT-26 tumor model. **Conclusions:** exoVACC is a versatile vaccine platform that incorporates complex antigen engineering, diverse adjuvant and immunomodulator combinations and cell specific targeting into a single system. This unique combination results in superior systemic and tissue resident immune responses via multiple routes of administration compared to conventional vaccine formulations.

67. Glypican-3-Specific CAR NKT Cells Armored with IL-15 Mediate Potent Anti-Tumor Response Against Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related deaths worldwide with a five year survival rate of only 18%. There are currently no curative therapies for unresectable HCC, highlighting the need for novel approaches to treat this disease. Glypican-3 (GPC3) is a membrane-bound proteoglycan highly expressed in HCC but not on healthy non-neoplastic cells, making it an attractive immunotherapeutic target. Natural killer T cells (NKTs) are particularly promising for the immunotherapy of HCC because: i) HCC produces CCL20, a major chemotactic factor for NKTs, ii) adoptively transferred NKTs suppress HCC in preclinical models, and iii) the presence of NKTs in primary HCC is associated with improved patient survival. Redirecting NKTs with chimeric antigen receptors targeting GPC3 (GPC3-CARs) can combine the natural antitumor properties of these innate lymphocytes and the specificity of CAR-based therapies. Expansion and persistence of therapeutic cells in patients is critical for durable antitumor responses. Interleukin-15 (IL-15) is known to stimulate proliferation and enhance survival of human NKTs in the tumor microenvironment; thus, we hypothesized that NKTs expressing GPC3-specific CAR with IL-15

will induce robust antitumor effect in preclinical models of HCC. **Methods:** We first engineered NKTs to express a set of GPC3-CARs incorporating the CD3ζ signaling domain with CD28, 41BB, or both costimulatory endodomains. We selected the CAR that mediated the most potent anti-tumor activity in preclinical models of HCC. Next, we co-expressed IL-15 with this receptor and systematically examined the ability of CAR NKTs to kill and produce cytokines *in vitro* and to expand, persist, and induce tumor regression in HCC xenograft models *in vivo*. **Results:** We determined that the combination of CD28 and 41BB (G28BBz) in the GPC3-CAR induces the most potent anti-tumor responses with efficient and specific killing of GPC3+ HCC cells *in vitro* and *in vivo* ($p<0.001$; Mantel-Cox analysis). Multiplex cytokine analysis by Luminex assay revealed that GPC3-CAR NKTs secrete a range of effector cytokines including GM-CSF, IFNγ, and TNFα in response to stimulation with GPC3+ HCC cells *in vitro* with no significant difference between G28BBz and 15G28BBz groups (Two-way ANOVA, Tukey's multiple comparison method). Incorporation of IL-15 enhanced the expansion and persistence of GPC3-CAR NKTs *in vivo* (**Fig 1**, $p<0.01$ NT/GFP controls vs 15.G28BBz groups, $p<0.05$ G28BBz vs 15.G28BBz groups; One-way ANOVA, Tukey's multiple comparison method). Most importantly, 15.G28BBz NKTs induced long-term tumor control and significantly improved survival of HCC xenograft bearing mice (**Fig 2**, $p<0.0001$ control groups vs 15.G28BBz group, $p<0.001$ G28BBz vs 15.G28BBz groups; Mantel-Cox analysis). **Conclusion:** Our findings provide the basis for clinical testing of GPC3-CAR NKTs in HCC patients and highlight the potential for further clinical development.

Fig 1.

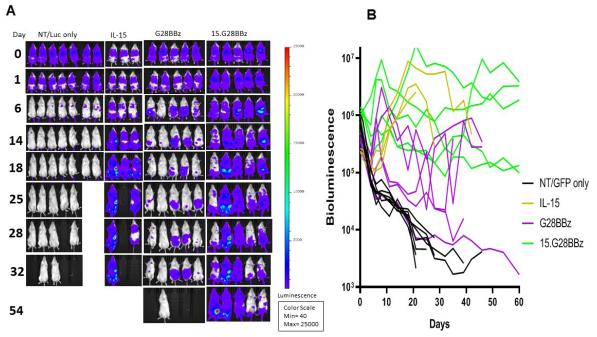

Figure 1. GPC3-CAR NKTs expand in HCC tumor bearing mice. Human NKTs were transduced with Fluc alone (NT/GFP) and in combination with IL-15, G28BBz or 15.G28BBz. NSG mice (n= 3-7 per group) were injected with $2x10^6$ GPC3-positive Huh-7 tumor cells IP. After 7 days, $6 x10^6$ Fluc positve CAR NKT cells were injected into tumor bearing mice. NKT expansion and persistence was monitored with bioluminescence imaging at the indicated time points. **A**) Serial bioluminescence images of mice at indicated time points. **B**) NKT cell bioluminescence images graphed over time. NT/GFP vs 15.G28BBz groups $p<0.01$ and G28BBz vs 15.G28BBz groups $p<0.05$ by Tukey's multiple comparisons test.

Fig 2.

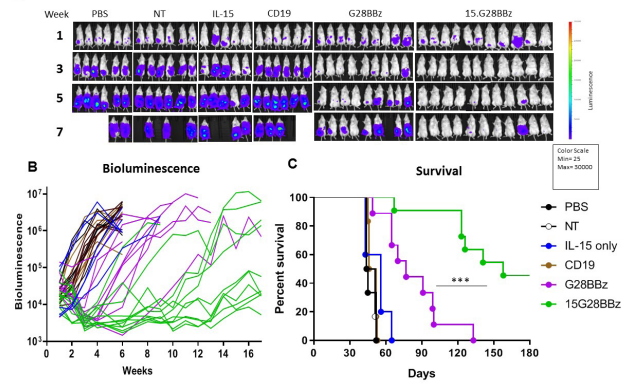

Figure 2. GPC3-CAR NKT cells induce robust antitumor effect and IL-15 further enhances their antitumor activity *in vivo*. NSG mice (n= 5-11 per group) were injected with $2x10^6$ GPC3-positive Huh-7.Ffluc tumor cells IP. After 7 days, $8x10^6$ GPC3-CAR NKT cells or controls (parental NKT cells, IL-15 or CD19-CAR cells) were injected IV on Day 7. **A**) Weekly tumor bioluminescence images of mice for each therapeutic and control group at indicated time points. (**B**) Tumor bioluminescence graphed over time. (**C**) Kaplan-Meier survival curve of tumor bearing mice after treatment with GPC3-CAR NKT cells. Control groups vs 15.G28BBz group $p<0.0001$ and G28BBz vs 15.G28BBz groups $p<0.001$ by Mantel-Cox analysis.

68. Preclinical Study of Oral Cancer Vaccine Using Recombinant *Bifidobacterium* Expressing WT1 Protein in Murine Bladder Cancer Model and Non-Human Primate

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Introduction: Recently, we constructed an oral cancer vaccine using recombinant *Bifidobacterium longum* displaying WT1 protein (*B. longum* 420), and showed WT1-specific T cell immune responses and significant anti-tumor effect in a mouse prostate cancer model in combination with anti-programmed death-1 (PD-1) antibody. Immune-checkpoint inhibitors such as anti-PD-1 antibodies have been considered as one of the most breakthrough in immuno-oncology (I-O) drugs, but induction of tumor-specific immune response is still a fundamental of I-O therapy. Therefore, the combination therapy of immune-checkpoint inhibitors and cancer vaccines are promising modality to increase response rate in cancer patients. In this study, we investigated the anti-tumor effect of *B. longum* 420 in murine bladder cancer model. In addition, we investigated the efficacy and safety of recombinant *B. longum* expressing human WT1 protein (*B. longum* 440) in cynomolgus monkey as preclinical study. **Methods:** One million cells of MBT-2 expressing WT1 were subcutaneously inoculated into mice at day 0. 1×10^9 colony forming units of pasteurized *B. longum* 420, *B. longum* 2012 (non-WT1 expressing control), or PBS were orally administered on days 4-8, 11-15, 18-22, 25-29, 32-36. Anti-PD-1 antibody were intraperitoneally injected on days 9, 11, 15, 18, 22. Tumors, spleens were collected to analyze the immune responses. In another experiment, the anti-PD-1 treatment was initiated ahead of

oral vaccination of *B. longum* 420 to assess the clinical uses. In addition, we administered the 39 mg/kg/day or 77 mg/kg/day of lyophilized *B. longum* 440, which are equivalent to 1g/60kg/day or 2g/60kg/day for human doses of that, into cynomolgus monkeys 5 times a week for 4 weeks to examine immune responses and safety. **Results:** The combination treatment of *B. longum* 420 and anti-PD-1 antibody significantly inhibited the tumor growth and improve survival rate comparing to the other therapies in mice ($p<0.05$). The significant antitumor effect was seen even when anti-PD-1 was injected first 3 times and following oral administration of *B. longum* 420 alone ($p<0.05$). The combination therapy induced significantly higher population of tumor infiltrating CD4T cells than *B. longum* 420 alone ($p<0.05$) and induced substantially higher CD8T cells in MBT-2 tumor tissues. *B. longum* 420 also induced remarkably higher population of WT1-epitope-specific T cells in mouse spleens. Cynomolgus monkey study showed that oral administration of lyophilized *B. longum* 440 did not showed the obvious adverse effects and signs of toxicity. *B. longum* 440 were detected only in feces, but not in peripheral blood and urine after oral administration. Intracellular cytokine staining showed that remarkable increase of IFN-gamma-producing CD4 and CD8T cells that were responding to re-stimulation with WT1 protein and WT1-specific epitopes in splenocytes. **Conclusions:** we demonstrated that the combination of *B. longum* 420 oral vaccination and anti-PD-1 antibody could induce the significant antitumor effect and WT1-specific immune responses to urothelial cancer in mice. In addition, the preclinical study in cynomolgus monkey showed oral administration of *B. longum* 440 showed the feasibility of the oral cancer vaccine in clinical setting. These findings suggested that our oral cancer vaccine could augment the efficacy of pembrolizumab, anti-PD-1 antibody, in the patients with advanced urothelial cancer.

69. T Cells Redirected to the Pan-Cancer Target GRP78 Have Potent Antitumor Activity in Preclinical Models

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Ideal immunotherapy targets should be cancer specific, essential for the malignant phenotype, and expressed in a broad range of cancers. We posit that members of the unfolded protein response (UPR) fulfill these characteristics since the UPR regulates hallmarks of cancer including the ability of cancer cells to resist cell death, sustain proliferation, and metastasize. Intracellular Glucose-regulated-protein 78 (GRP78) is a key UPR regulator, which normally resides in the endoplasmic reticulum (ER). GRP78 is overexpressed and translocated to the cell surface in a broad range of solid tumors and hematological malignancies in response to elevated ER stress, making it an attractive target for immune-based therapies with T cells expressing chimeric antigen receptors (CARs). The goal of this project was to generate GRP78-CAR T cells and evaluate their effector function *in vitro* and *in vivo*. We designed a retroviral vector encoding a GRP78-CAR using a GRP78-specific peptide as the antigen recognition domain, and generated GRP78-CAR T cells by retroviral transduction of primary human T cells. Expression of GRP78-CARs in T cells did not induce fratricide, and the median transduction efficiency was 82% (± 5-8%, N=6). Immunophenotypic analysis of GRP78-CAR T cells showed a predominance of naïve and terminal effector memory subsets on day 7 after transduction (N=5). To initially determine the antigen specificity of GRP78-CAR T cells, we performed coculture assays *in vitro* with cell surface GRP78$^+$ MOLM13 (AML), A673 (Ewing's sarcoma), MDA-MB468 (triple negative breast cancer) or cell surface GRP78$^-$, non-transduced (NT T cells) targets. T cells expressing CARs specific for HER2-, CD19-, or a non-functional GRP78 (ΔGRP78)-CAR served as negative controls. GRP78-CAR T cells secreted significant amounts of IFNγ and IL-2 only in the presence of GRP78$^+$ target cells (N=3, $p<0.005$); in contrast, control CAR T cells did not. In addition, GRP78-CAR T cells only killed GRP78$^+$ target cells in standard cytotoxicity assays confirming specificity. Finally, we evaluated the antitumor activity of GRP78-CAR T cells in three xenograft models (MOLM13, A673, MDA-MB468). Tumor growth was either monitored by serial bioluminescence imaging or caliper measurements. In all three models (N=10 per group), a single intravenous dose of GRP78-CAR T cells induced tumor regression, which resulted in a significant ($p<0.001$) survival advantage in comparison to mice that had received control CAR T cells. In conclusion, we have successfully generated CAR T cells against the pan-cancer target GRP78. GRP78-CAR T cells had potent antitumor activity *in vitro* and *in vivo*, warranting further active exploration of our cell therapy approach to target a broad range of malignancies.

70. Faster, Higher, Stronger: Optimizing Fuel Selection to Improve CAR-T Cell Metabolic Fitness in Solid Tumors

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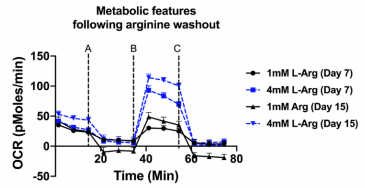
Introduction: The unprecedented success of CAR-T cell therapies in B-cell malignancies has not been reproduced in solid tumors. CAR-T cells traffic to solid tumors and undergo antigenic stimulation but the metabolic nature of the microenvironment impairs overall CAR-T durability. In solid tumors, heightened tumor cell metabolism and altered perfusion create a competition for nutrients. As the energy cost of proliferation and differentiation is high, CAR-T cell activity diminishes and persistence as well as effector function is impaired. We recognize that the current 9-11 day ex-vivo CAR-T cell expansion phase provides a valuable opportunity to condition the cell culture medium with factors conferring superior metabolic fitness; ultimately translating into enhanced engraftment and survival in hostile tumor environments. **Results:** A role for L-arginine has been identified in the metabolic and anti-tumor function of OT-1 murine T cells, although the mechanisms are poorly understood. We show that human CAR-T cells specific for CD19, expanded in medium conditioned with L-arginine, have enhanced anti-tumor function in Nalm-6 xenograft models of ALL. Similarly, L-arginine-treated anti-PSMA human CAR-T cells have enhanced durability and cytolytic activity in PC3-PSMA models of prostate cancer (Fig. 1A). Peripheral blood collection revealed an increased persistence of arginine-treated CAR-T cells; an effect likely supported by their distinct metabolic

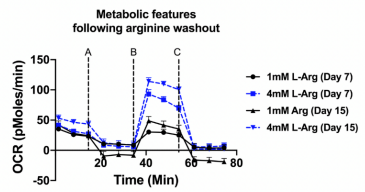
fate. Extracellular flux analyses revealed that short-term L-arginine treatment imprints CAR-T cells with a significantly increased respiratory capacity. This "built-in" contingency energy reserve is sustained several days after arginine washout (Fig. 2). Using a series of ^{13}C isotopically-labeled metabolic tracers, we show that enhanced arginine availability doesn't increase arginine metabolism directly. Instead, arginine-treated CAR-T cells have a reprogramed metabolic state where they selectively refuel the energy generating steps of proliferation and differentiation using branched chain amino acids (BCAA) leucine, isoleucine and valine. Based on the transcriptional profile and chromatin state we found that SLC7A5 (responsible for BCAA uptake), and BCAT1 (catalyzes the first step of BCAA metabolism), were upregulated in L-arginine-treated CAR-T cells. **Conclusion:** In summary, our findings explain how arginine confers superior metabolic attributes to CAR-T cells and provide the first evidence supporting a role of BCAA metabolism in CAR-T cell mitochondrial function. These findings are of immediate translational relevance given the recent data that select tumors favor BCAA metabolism over glucose to support their growth. For other tumors, these data position L-arginine and BCAA's as important fuel sources to support CAR-T cell activity, persistence and function in hostile tumor environments.

Fig. 2 L-arginine-treated primary human T cells retain superior mitochondrial function (spare respiratory capacity), long after arginine removal



Primary human T cells were stimulated with anti-CD3/CD28 dynabeads. Activated T cells were refed with medium conditioned with 4mM L-arginine on days 1, 3, and 5. The metabolic properties of control vs arginine-treated cells were analyzed using a standard "mito-stress" Seahorse assay on day 7 (9-10 technical replicates per condition). From days 7-15, the cells were washed and the medium was replaced with standard RPMI medium (1.15mM arginine). Values represent mean ± S.E.M. from 9-10 technical replicates. A: Oligomycin; B: FCCP; C: Rotenone & antimycin A

Fig. 2 L-arginine-treated primary human T cells retain superior mitochondrial function (spare respiratory capacity), long after arginine removal



Primary human T cells were stimulated with anti-CD3/CD28 dynabeads. Activated T cells were refed with medium conditioned with 4mM L-arginine on days 1, 3, and 5. The metabolic properties of control vs arginine-treated cells were analyzed using a standard "mito-stress" Seahorse assay on day 7 (9-10 technical replicates per condition). From days 7-15, the cells were washed and the medium was replaced with standard RPMI medium (1.15mM arginine). Values represent mean ± S.E.M. from 9-10 technical replicates. A: Oligomycin; B: FCCP; C: Rotenone & antimycin A

New Gene Editing Technologies and Approaches

71. Programmable C-to-G Transversions in Human Cells with Engineered CRISPR Base Editors

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Adenine base editors (ABE) and cytosine base editors (CBE) can efficiently install A-to-G and C-to-T transition edits, respectively. However, the field is currently lacking base editors to efficiently install C-to-G transversion edits in human cells in a programmable manner. We modified existing CBE architectures to repurpose them into new C-to-G base editors (CGBEs). Our optimized CGBE induced C-to-G alterations with efficiencies as high as ~70%, particularly in AT- rich sequence contexts. CGBE exhibited a narrow editing window within the target site (positions 5-7 in the SpCas9 spacer sequence) but we also showed that incorporation of SpCas9 PAM recognition variants that can recognize NG or NGA PAMs into CGBE are functional, thereby expanding the range of targetable sites. In addition, CGBE displayed favorable Cas9-dependent DNA off-target profiles compared to the BE4max C-to-T base editor. Finally, we found in side-by-side comparison that CGBE achieves higher efficiencies of C-to-G edits compared with the recently described PE2 and PE3 prime editing technologies at four different loci, although rates of prime editing might be further improved with additional optimization of the PE setup. Taken together, CGBE we describe here expands the editing capabilities of the base editor platform for both research and therapeutic applications.

72. Unconstrained Genome Targeting with Engineered Near-PAMless CRISPR-Cas Variants

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The ability to utilize CRISPR-Cas nucleases to manipulate DNA in a site-specific manner holds immense potential for the study and treatment of genetic disease. One limitation of CRISPR-Cas nucleases is that target recognition requires the presence of a protospacer adjacent motif (PAM), which for the commonly used *S.pyogenes* Cas9 (SpCas9) is NGG. The necessity of a PAM restricts the targeting range of various genome editing tools for applications that require high-resolution targeting, such as base editing, allele-specific editing, generating efficient HDR-mediated alterations, high-resolution tiling, etc. To eliminate the necessity of PAM recognition and thereby improve

genome accessibility for editing, we utilized a combination of rational directed engineering methods to generate novel SpCas9 variants. Initially, we sought to relax SpCas9 PAM preference from the canonical NGG to a more relaxed NGN motif. Using high-throughput design and screening approaches, we generated a highly active variant, named SpG, which can target NGN PAMs with greater activity than previously described xCas9 and SpCas9-NG variants. To enable even broader targeting, we then utilized SpG as a molecular scaffold for further engineering, leading to the generation of a novel SpCas9 variant named SpRY (for targeting NRN>NYN PAMs). We found that SpRY can efficiently target sites with NGN and NAN PAMs (collectively NRN), and also has detectable but reduced activity against sites harboring NCN and NTN PAMs (collectively NYN). In addition to their activities as nucleases, we demonstrate the broad utility of SpG ad SpRY as cytosine and adenine base editors (CBEs and ABEs, respectively). Furthermore, as a proof-of-concept of the expanded targeting range of these variants, we utilize SpG and SpRY CBEs to generate previously inaccessible single nucleotide variants associated with protecting individuals against complex genetic disease. Taken together, SpG and SpRY enable targeting of many sequences previously inaccessible to SpCas9 and other Cas9 orthologs, and should enable high-resolution targeting for a suite of genome-editing applications.

73. Efficient Multiplex Base Editing at Single Target Sites with a Dual-Deaminase Base Editor

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Adenine and cytosine base editors (ABEs and CBEs) are important CRISPR gene editing technologies that enable the programmable introduction of A-to-G and C-to-T point mutations, respectively. These editors require neither DNA double-strand breaks nor donor templates. Instead, they harbor an adenosine or cytidine deaminase that is fused to a catalytically impaired Cas9. This architecture allows for the targeted deamination of specific bases in DNA. However, the range of base editing outcomes induced by each platform remains limited to a single type of base substitution. Here we describe an approach to expand the portfolio of base editing outcomes in which we engineered and validated a dual-deaminase base editor (ddBE) that can efficiently introduce concurrent C-to-T and A-to-G edits in the same base editing window in human cells. We also show that these engineered ddBEs induce minimal RNA off-target editing. ddBE provides a useful technology for gene editing applications that require high-diversity outcomes, such as molecular recording or tiling screens and thereby broaden the research applications of the CRISPR base editing platform.

74. Rational Selection of CRISPR/Cas9 gRNAs to Maximize Homology-Directed Genome Editing

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Homology-directed repair (HDR) is the desired outcome for many applications of CRISPR/Cas9 genome editing. However, HDR editing is challenging in mammalian cells because not only is it in competition with other DNA repair pathways, such as NHEJ and MMEJ, but the absolute efficiency of HDR is currently not predictable. Consequently, panels of CRISPR/Cas9 gRNA and matched homology DNA templates must be generated and evaluated to identify optimal combinations. We demonstrate here that the indel signatures resulting from CRISPR/Cas9 editing in the absence of a homology template can be used to predict and strategically optimize gRNA selection to maximize HDR. To do this, we analyzed a panel of CRISPR/Cas9 gRNAs in both the K562 cell line and in therapeutically relevant primary human CD4+ T cells and hematopoietic stem cells. We confirmed previous studies that have reported that the pattern of indels resulting from a specific gRNA is conserved across cell types and extended this concept to primary cells. Importantly, we demonstrated for the first time that indels derived by MMEJ repair, characterized by deletions ≥3bp, can be used to predict a target site's potential for HDR and thereby guide the optimization of an HDR editing strategy. Additionally, by comparing the indel outcomes from *S. aureus* and *S. pyogenes* Cas9 gRNAs targeted to the same sequence, we add to the growing volume of data demonstrating that the targeted DNA sequence is a major factor governing the consistent and characteristic indel outcomes of Cas9-induced DSBs. Finally, we tested the predictive capacity of inDelphi, FORECasT, and Lindel, which are tools recently developed to predict the indel outcomes of Cas9-gRNA based solely on the targeted DNA sequence. In our system, Lindel best predicted gRNA indel signatures, and could be repurposed to predict the tendency of a gRNA target site to support HDR. In summary, we here propose a simple strategy for the rational selection and optimization of CRISPR/Cas9 gRNAs to optimize HDR editing.

75. dCas13-Mediated Therapeutic RNA Base Editing for *In Vivo* Gene Therapy

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The recently discovered microbial Cas13 proteins are RNA-guided RNA nucleases. The catalytically deficient version of Cas13 (dCas13) can serve as a platform for targeted RNA binding, and enable RNA base editing by coupling with an RNA-modifying enzyme. RNA base editing has several advantages as a therapeutic approach. First, it is independent of endogenous DNA repair mechanisms that limit some genome editing strategies such as homology-directed repair, and therefore can

function in a broader range of cells. Second, whereas DNA base editing involving other Cas effectors relies on a suitable protospacer-adjacent motif (PAM) in the target DNA, dCas13-mediated RNA base editing has a much more relaxed constraint on the target nucleic acid sequence context. Furthermore, RNA editing does not permanently alter the genetic information, and therefore has a potentially less concerning safety profile as a gene therapy approach. RNA editing tools comprising dCas13 fused with Adenosine Deaminase Acting on RNA deaminase domain (ADARdd) have been previously developed to convert an adenosine (A) to an inosine (I) that is functionally read as guanosine (G) by various cellular processes such as translation. A potentially broad therapeutic application is to correct pathogenic missense mutations and nonsense mutations (PTC; UAA, UAG, or UGA) caused by a G to A base change. The dCas13-ADARdd has been shown as an efficient RNA base editor in mammalian cell culture. However, the *in vivo* editing efficiency and therapeutic efficacy remain to be studied. In this study, we designed dCas13-ADARdd reagents that are amenable to AAV vector-mediated gene delivery, and examined their effectiveness in correcting a disease-associated mutation in mice. We first optimized several parameters for RNA editing efficiency using a reporter assay in HEK293FT cells, including Cas13 orthologs, ADARdd variants, fusion orientations, subcellular localization signals, gRNA designs, and promoters. We achieved robust editing with the reporter. Editing of endogenous transcripts and disease-related RNA targets in cell lines was also efficient. Selected editors were packaged into AAV vectors and systemically delivered to a mouse model of Hurler syndrome that harbors homozygous *Idua-W392X* mutation (UGG◊UAG) analogous to the most common mutation in patients. Five weeks later, liver total RNA was extracted and subjected to reverse transcription and PCR amplification. Deep sequencing of targeted *Idua* amplicon revealed that corrected transcript accounted for up to 15% of total *Idua* mRNA with minimal by-stander editing. The IDUA activity in liver lysate was restored to about 1% of the normal level, surpassing the targeted therapeutic threshold of 0.5%. Further assessment on the therapeutic efficacy and transcriptome-wide off-target editing is underway. *Co-corresponding authors.

76. CRISPR/Cas9-Mediated Gene Editing Corrects Disease-Causing Mutation in Crigler-Najjar Syndrome Mice

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AAV-mediated gene delivery for gene replacement therapy of monogenic liver disorders has demonstrated efficacy in several animal models and human clinical trials. However, since the therapeutic payload remains mostly episomal, the use of AAV vectors for liver-directed gene transfer during infancy or juvenile age is still limited in the long-term due to loss of vector DNA and efficacy, associated to hepatocyte duplication. Genome editing stands as a promising strategy for the treatment of genetic diseases, since it leads to a permanent and specific modification of the genome, that is transmitted to daughter cells upon proliferation.We previously demonstrated that promoterless genome targeting of the hUGT1A1 cDNA into the albumin locus (GeneRide) successfully rescued neonatal lethality in a mouse model of Crigler-Najjar syndrome. Therapeutic efficacy of GeneRide was subsequently increased by coupling the CRISPR/Cas9 platform. Here, we aimed at correcting the disease phenotype by editing a single base deletion present in the Ugt1a1 murine locus of Crigler-Najjar mice, responsible for the lethal phenotype. Neonatal mice were retro-orbital injected with two AAV8 vectors: one expressing the SaCas9 and the sgRNA, and the other one carrying the Ugt1a homology regions. Two sgRNAs were tested, Ex4-sgRNA, which targeted the point mutation, and NcoI-sgRNA, with a targeting site 120 bp upstream of the mutation. We observed 50% reduction in plasma bilirubin (TB) levels with the NcoI-sgRNA, which remained stable up to 5 months post-injection, while the correction with the Ex4-sgRNA was negligible. Next, we tested different Cas9:donor vector ratio combinations (1:1; 1:2; and 1:5), with the 1:5 ratio showing the higher efficacy in lowering TB levels. Finally, to further improve CRISPR/Cas9-mediated gene editing efficacy we used selected microRNAs regulating HR-mediated gene correction, identified by a robotic high-throughput approach in a library of 2,042 human microRNAs. These miRNAs markedly enhanced CRISPR/Cas9-AAV-based homologous recombination in the liver both in the albumin locus using eGFP reporter-system and in the mouse Ugt1a1 locus. In conclusion, we showed that CRISPR/Cas9 mediated gene editing in the Ugt1a1 locus was able to rescue the neonatal phenotype in Crigler-Najjar mice, and that HDR and therapeutic potential was further enhanced by selected miRNAs in the liver.

77. Development of a CRISPR/Cas9 System for Mitochondrial Diseases

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Mutations in mitochondrial DNA (mtDNA) are responsible for a substantial number of heritable mitochondrial diseases. mtDNA-associated mitochondrial diseases like neurogenic muscle weakness, ataxia, and retinitis (NARP) and Leigh syndrome are often debilitating and sometimes fatal. No cures currently exist for these diseases, and treatments can only manage symptoms. Targeting disease-associated mitochondrial mutations and destroying defective mtDNA would provide a long-term solution. However, such a strategy has proven to be difficult because it requires the efficient delivery of molecular components to membrane-encapsulated mtDNA. Additionally, mutations can often be point mutations, and an effective therapy would require the precise targeting of hundreds of affected mtDNA molecules in a cell. Site-specific gene editing tools like CRISPR/Cas would allow for such targeting. However, to date, no CRISPR/Cas system has been able to effectively target disease-relevant mtDNA mutations. We thus sought to develop a mitochondrial CRISPR/Cas system that can distinguish common mtDNA mutations and select for wild-type species. To create a mitochondrial CRISPR/Cas system, a Cas nuclease

and guide RNA both need to be imported into the mitochondrial matrix. By appending a mitochondrial targeting signal and removing nuclear localization sequences, we have relocalized Cas9 nuclease to mitochondria. We demonstrate that mitochondrial-targeted Cas9 (mitoCas9) is efficiently delivered to the mitochondrial matrix and enriched in mitochondrial fractions. To localize CRISPR guide RNAs to mitochondria, we have found effective methods using synthetic RNAs as well as the addition of mitochondrial-targeting moieties. Chemically modified guide RNAs are highly efficient at localizing to mitochondria under optimized delivery conditions, while guide RNAs with mitochondrial targeting sequences do not require such stringent conditions. Using mitoCas9 and a guide RNA targeting a NARP-associated ATP6 mutation, we demonstrate the functionality of a mitochondrial CRISPR/Cas9 system. Shifts in mutant-to-wildtype mtDNA ratios were quantified and monitored in NARP patient-derived cybrids. This study is an early demonstration of efficient Cas nuclease and guide RNA delivery to mitochondria. It not only shows that CRISPR/Cas can target non-nuclear DNA, but it also helps extend gene therapy to a large subset of inherited mitochondrial diseases.

CAR T and Other Engineered T Cells Targeting Hematological Malignancies

78. Exploiting CRISPR-Genome Editing and WT1-Specific T Cell Receptors to Redirect T Lymphocytes Against Acute Myeloid Leukemia

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Empowering T cells with new T cell receptors (TCR) can effectively redirect some of the most potent players of our immune system towards every surface and intracellular tumor target. TCR gene editing, the selective disruption of endogenous TCR genes coupled with the transfer of a tumor-specific receptor, has improved the potential for human therapeutics of TCR-engineered T cells. However, the identification of the exact tumor-specific TCR remains the major bottleneck for the wide-range exploitation of TCR-based therapies in clinical practice. In this study we set up a TCR hunting approach to build a library of receptors encompassing different human leukocyte antigen (HLA) restrictions and specifically recognizing Wilms Tumor 1 (WT1), a tumor-associated antigen overexpressed in a variety of hematological and solid tumors. WT1-specific T cells successfully expanded from 14 healthy donors by repetitive stimulation with autologous antigen-presenting cells loaded with a WT1 peptide pool, were co-cultured with antigen-expressing cells and primary leukemic blasts to assess their ability to recognize naturally processed epitopes and their on-target specificity. Over time tracking of the WT1-specific T cells in culture enabled the identification of 21 TCRαβ pairings recognizing a variety of WT1 peptides and restricted to different HLAs, including the highly frequent *HLA-A*02:01* allele. The anti-tumor activity of newly identified TCRs was validated through TCR gene editing in the context of acute myeloid leukemia (AML), an aggressive and still largely incurable hematological malignancy. Upon simultaneous disruption of the endogenous TCR α and β chains using CRISPR/Cas9 technology (knock-out efficiency >98%), we efficiently introduced WT1-specific TCR into T cells either by random integration into the genome using lentiviral vector (LV) transduction or by exploiting targeted integration into the TCR α-chain locus using an adeno-associated vector (AAV). TCR-redirected lymphocytes showed mainly a T stem cell memory phenotype. Edited T cells co-cultured with T2 cells pulsed with decreasing concentrations of WT1-derived peptides demonstrated a range of functional avidities, including some high affinity candidates. Log EC_{50} peptide concentration ranged from 0.5 to 8 ug/ml, depending on the TCR tested. In a Caspase-3 assay, edited T cells proved able to specifically and efficiently recognize HLA-matched primary AML blasts (efficiency up to 70% at an effector to target ratio of 5 to 1). Furthermore, using an alanine scanning approach, WT1 peptide residues critical for the TCR binding were clearly identified, thus determining the safety profile of the most promising TCR candidates. To verify the efficacy and specificity of the lead TCR edited T cells *in vivo*, NSG mice infused with human AML blasts were treated with edited T cells harboring the WT1-specific TCR (either upon LV or AAV delivery). A significant decrease in AML tumor burden was observed in mice treated with the WT1-specific T cells versus control mice ($p<0.0001$). Throughout the study, mice showed no signs of GvHD. In conclusion, we demonstrated that TCR-edited T cells can be effectively used to target WT1-expressing tumor cells in AML.

79. Co-Targeting CD38 with a Chimeric Costimulatory Receptor Enhances Adoptive T Cell Therapy for Hematological Malignancies

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Adoptive cell immunotherapy with genetically engineered T cells bearing a chimeric antigen receptor (CAR-T cells) or a tumor antigen-specific T cell receptor (TCR-T cells) has become a powerful strategy for the treatment of advanced hematological malignancies. Second generation CARs have induced dramatic responses in chemotherapy resistant B cell leukemias, lymphomas and multiple myeloma (MM). However, there is still a considerable proportion of patients who eventually experience tumor relapse. Clinical and experimental data of CD19-, BCMA-, and CD22- CAR-T cell use demonstrate that a critical mechanism of disease escape is the down regulation of antigen expression, to evade effector cell mediated killing. In addition, the

use of TCR-T cells has not met the clinical success of CAR-T cells, potentially due to inefficient T cell activation and persistence. We hypothesized that co-targeting of CD38, that is highly and uniformly expressed in all hematopoietic cells, with a costimulatory chimeric receptor (CCR) would enhance the antigen-recognition and killing capacity of CAR/TCR-T cells for hematologic malignancies and that the incorporation of a complimentary costimulation, with 4-1BB and CD28 signaling, would confer increased persistence. We found that double-targeting BCMACAR/CD38CCR T cells showed significantly increased cytotoxic capacity against $BCMA^{+}CD38^{+}$ MM cell lines and primary MM cells compared to the single-targeting BCMACAR-T cells. This effect was abrogated in the absence of CD38 expression while it was still observed when a truncated CD38 receptor was used, indicating that it is a result of CD38 engagement. Of note, the CD38CCR did not induce cell lysis of CD38 expressing targets. Using cell line-based models of low-antigen expression we showed that the use of CD38CCR could rescue the diminished lysis of BCMAlow K562 and CD19low Nalm-6 by BCMACAR-T and CD19CAR-T cells respectively. Similarly, CD38 co-targeting significantly improved the function of TCR-T cells since NYESO-1-TCR/38CCR T cells showed significantly increased cytotoxicity against a NYESO-$1^{+}CD38^{+}$ cell line. Furthermore, the engagement of the CD38CCR provided the CAR/TCR T cells with CD28 and 4-1BB mediated costimulation, resulting in markedly enhanced cytokine secretion, reduced PD-1 expression and optimal proliferative capacity. Most importantly, the costimulatory signaling could be also initiated in-trans through engagement of CD38 expressed on bystander accessory cells which remained intact while killing of tumor targets was maintained. In vivo, mice treated with BCMACAR/CD38CCR T cells showed significantly lower tumor growth and prolonged survival compared to mice treated with a second generation BCMACAR T cells. Importantly, BCMACAR/CD38CCR T cells persisted longer in vivo than BCMACAR T cells. In conclusion, we demonstrated that engagement of a CCR can increase the effector-to-target avidity and provide combinatorial costimulation to CAR/TCR T cells also in trans. As a result combination of a CAR/TCR and a CD38CCR against hematological malignancies results in enhanced antigen recognition, even for antigen-low variants, reduced exhaustion and improved in vivo anti-tumor function and persistence. Since no cytotoxicity is mediated through the CCR, the co-targeting of a highly expressed second antigen with a CCR is a potential powerful strategy to enhance anti-tumor function of CAR- and TCR-engineered T cells for many malignancies.

80. Generation of Human Memory Stem T Cells Specific for Tumor Antigens and Resistant to Inhibitory Signals by Genome Editing

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Chronically stimulated tumor specific T lymphocytes infiltrating solid and hematological tumors express several inhibitory receptors (IRs), such as PD-1, CTLA-4, Lag-3 and Tim-3. Cancer cells often up-regulate the ligands of these inhibitory molecules, thus fostering T cell exhaustion. Resulting exhausted T cells lose effector functions and the ability to effectively kill cancer cells. The blockade of the interaction between IRs and their ligands by monoclonal antibodies (e.g. anti-CTLA-4, anti-PD-1) rescues T cell exhaustion and this therapeutic approach produced substantial clinical results in several solid tumors. However, autoimmune-related adverse events often occur after immune checkpoint blockade, mainly due to the effect of these molecules on the entire T cell repertoire, rather than on tumor-specific T cells. Here, we aimed at generating innovative tumor-specific T cell products resistant to inhibitory signals and able to persist long-term in treated cancer patients. To reach these objectives we developed a strategy to simultaneously redirect T cell specificity by TCR gene editing and permanently disrupt IRs by CRISPR/Cas9 in long-living memory stem T cells (T_{SCM}).To avoid mispairing between the endogenous TCR and the tumor-specific TCR, we simultaneously inactivated the constant regions of α and βchain of the endogenous TCR genes by targeting *trac* and *trbc1/2* loci by ad hoc designed CRISPR/Cas9 nucleases. The inactivation of the endogenous TCR was combined with Tim-3 or LAG-3, in a multiplex approach. To redirect the T cell specificity, TCR-neg-IRs-neg cells were then transduced with a lentiviral vector encoding for a TCR specific for an HLA-A2 restricted peptide from the NY-ESO-1 tumor antigen. By this protocol, coupled with culture conditions to preserve early differentiated cells, we generated T_{SCM} cells redirected towards a tumor antigen and devoid of one inhibitory receptor. In co-culture assays, TCR-edited/LAG-3neg and TCR-edited/Tim-3neg T_{SCM} cells retained an effective and specific anti-tumor activity, thus indicating that IRs disruptions do not impact on the effector functions of T cells. When tested *in vivo* in preclinical models of multiple myeloma, in conditions of high tumor-antigen persistence limited doses of TCR-edited-LAG-3neg T cells displayed a reduced exhausted phenotype and a significantly enhanced anti-tumor activity compared to TCR-edited-LAG-3-competent cells. In the same model, Tim-3 disruption did not result in improved anti-tumor activity, suggesting a different role for this inhibitory molecule. Finally, when challenged for secondary responses, TCR-edited-LAG-3neg, but not

TCR-edited-IR-competent cells, prevented the engraftment of a second infusion of tumor cells, thus indicating an enhanced T cell fitness and resistance to exhaustion of TCR-edited-LAG-3neg cells. Overall, we exploited the versatility of multiplex gene editing by CRISPR/Cas9 to generate an innovative long-living tumor-specific cellular products resistant to inhibitory signals.

81. Pre-selected CAR $T_{N/SCM}$ Outperform CAR T_{BULK} in Driving Tumor Eradication in the Absence of Cytokine Release Syndrome and Neurotoxicity

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Capability of CAR T cells to expand and persist in patients emerged as a fundamental factor accounting for better outcome and durability of antitumor responses. These features inversely correlate with T-cell differentiation, suggesting that the enhanced T-cell fitness typical of early memory T cells may significantly improve CAR T cell therapeutic potential. Therefore, we sought to determine the role of pre-selected naive/stem memory T cells ($T_{N/SCM}$), as compared to total T cells (T_{BULK}), in enhancing CAR T cell anti-tumor responses. As expected, CAR $T_{N/SCM}$ were less lytic than CAR T_{BULK} and produced lower amounts of pro-inflammatory cytokines when stimulated with CD19+ targets *in vitro*, even though displaying a similar proliferative capacity. When challenged against tumor cells in HSC-humanized mice, limiting doses of CAR $T_{N/SCM}$ showed superior antitumor activity compared to CAR T_{BULK} and the unique ability to protect mice from leukemia re-challenge, together with higher *in vivo* expansion, persistence and a better CAR T cell fitness. Indeed, as evaluated by BH-SNE algorithm, after leukemia encounter CAR $T_{N/SCM}$ were characterized by prevalence of early memory T-cell subsets, together with the expression of multiple activation markers and a limited enrichment of inhibitory receptors. In contrast, CAR T_{BULK} were typified by the presence of terminally differentiated T cells, displaying an exhausted phenotype, as observed by co-expression of multiple inhibitory receptors in the absence of activation markers. Notably, at limiting doses and low tumor burdens no cases of severe CRS (sCRS) were reported. Conversely, when infusing high doses of CAR T cells in mice with high tumor burdens, detrimental CRS and neurotoxicity were only elicited by CAR T_{BULK}. In particular, more than 50% of mice receiving CAR T_{BULK} experienced a remarkably increased sCRS, characterized by drastic weight loss and increased serum elevation levels of IL-6 and SAA, culminating in the death of the treated mice. Moreover, 3 out of 6 CAR T_{BULK} treated mice showed multifocal brain hemorrhages, whereas in the group treated with CAR $T_{N/SCM}$ only one mouse presented with a small hemorrhagic focus. Taken together, these results indicate that CAR $T_{N/SCM}$ are intrinsically less prone than CAR T_{BULK} to trigger detrimental infusional toxicities, allowing deeper and more durable anti-tumor responses in the absence of sCRS and neurotoxicity, significantly widening the therapeutic index of current CAR T cell approaches.

82. Therapeutic Efficacy of CAR-T Cells Targeting gp350 in a Humanized Mouse Model of Epstein Barr Virus-Induced Lymphoproliferation and Prospects for Clinical Trials

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Objectives: Epstein-Barr virus (EBV) is an oncogenic herpesvirus widely spread in humans (>95%). Lymphoproliferative disease, development of B-cell lymphomas and occurrence of head and neck or gastric carcinomas have been associated with EBV oncogenesis. We previously reported that cord blood-derived T cells expressing chimeric antigen receptors (CARs) with the CD28/CD3ζ signaling domain and targeted against the EBV glycoprotein 350 (gp350) recognized and killed cell lines latently infected with EBV (Slabik et al, 2017 ASGCT poster presentation). For *in vivo* testing, EBV-fLuc strains were used to infect Nod.Rag.Gamma (NRG) immune deficient mice transplanted with human cord-blood derived $CD34^+$ hematopoietic stem cells (HSCs). We showed that prophylactic administration of either sorted $CD8^+/CD4^+$ or $CD8^+$ gp350-CAR-T cells lowered EBV-M81/fLuc spread, which was measured by full body bioluminescence optical imaging and determination of vector copies by PCR (Kalbarczyk et al, 2019 ASGCT, oral presentation). Here, we tested if administration of sorted $CD8^+$ gp350-CAR-T could result in therapeutic effects against pre-existing EBV and lymphoproliferation. Methods: Irradiated NRG mice were transplanted with $CD34^+$ HSCs and, seventeen weeks later, they were infected i.v. with EBV-M81/fLuc (10^6 infective units). In parallel, the $CD34^{neg}$ cryopreserved cord blood mononuclear cells were activated, transduced with γ-retroviral vectors expressing gp350-CAR/CD28/CD3ζ and expanded. Sorted $CD8^+$/gp350-CAR^+-T cells were sorted, briefly expanded and $2x10^6$ cells were administered i.v. at weeks 3 and 5 after EBV challenge. Experiments were performed in duplicates with cord blood and CAR-T cells obtained from two different donors. In total, 12 CAR-T treated EBV-infected mice were compared with 10 non-treated EBV-infected controls. Results: Long-term human hematopoietic reconstitution in humanized mice prior to EBV infection was confirmed by flow cytometry analyses of blood (huCD45: CTR 49.7±26.5%; gp350CAR-T 48.7±20.2%). EBV baseline infection was confirmed for all mice by optical imaging analyses, performed at 3 weeks post infection (wpi) and a day prior to CAR-T cell administration. From 4 to 6 wpi, all control and gp350CAR-T treated mice showed continuously detectable EBV infection. At 8 wpi, 1/10 mice in the control group died, 1/10 showed low EBV infection and 8/10 showed progressive disease (CTR optical imaging for the left full body side: 7.79e7 ± 1.2e8 Flux). For the gp350CAR-T cohort, all mice survived until 8 wpi. 9/12 mice showed a major response against EBV spread (6.11e5±3.64e5 Flux), while 3/12 mice showed no-response to therapy and EBV spread progressed(6.61e8 ± 9.3e8 Flux). Comparing the cohort of 9 mice alive in the control group with the cohort of 9 mice responding to gp350CAR-T therapy, the reduction of the bioluminescent signal at 8 wpi was statistically significant (p=0.0008). Remarkably, significantly higher frequencies of endogenous human $CD8^+$ T cells were detectable in the blood of non-treated controls (CTR 49.7±17.4% versus gp350CAR-T 34±11.9%; p=0.0356) indicating a

direct correlation between higher EBV spread and CTL expansion. EBER staining of tissues, PCR for detection of EBV copies and CAR detection, and analyses of cytokine biomarkers in plasma are ongoing. Conclusions and Outlook: This *in vivo* model demonstrated therapeutic potency of $CD8^+$/gp350-CAR^+-T against EBV. Currently, lentiviral vectors expressing gp350CAR are in production for validation of the cell manufacturing under good-manufacturing practices. Clinical trials for testing gp350CAR-T in EBV^+ associated malignancies are planned.

83. DNMT3A-Dependent Epigenetic Programs Constrain CAR T Cell Survival and Effector Function

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Background: T-cells expressing chimeric antigen receptors (CARs) have shown limited antitumor activity in early phase clinical studies for solid tumors and brain tumors. Lack of efficacy is most likely multifactorial, including limited CAR T-cell function, often termed exhaustion, which results in decreased expansion, persistence, and an inability to sequentially kill tumor cells. Here we report that exhaustion of human CAR T-cells occurs through an epigenetic repression of the CAR T-cell's multipotent developmental potential. **Methods:** Using CRISPR-Cas9 technology, we deleted the de novo DNA methyltransferase 3 alpha (DNMT3A) in CAR T-cells targeting different solid and brain tumor antigens. We next tested DNMT3A KO CAR T cell functionality in chronic antigen stimulation assays to determine their expansion, persistence and anti-tumor activity *in vitro*. In addition, we performed genome-wide DNA methylation profiling of DNMT3A KO CAR T cells to define epigenetic programs controlling CAR T-cell exhaustion. Finally, we evaluated DNMT3A KO CAR T cells' anti-tumor activity in multiple tumor models *in vivo*. **Results:** Here we demonstrate that we can successfully delete DNMT3A in CAR T cells without altering CAR transduction efficacy and T cell phenotype. In addition, we show that DNMT3A deletion preserved the cell's ability to proliferate and mount an anti-tumor effector response during tumor cell exposure for more than 10 weeks in DNMT3A KO CAR T-cells targeting tumor associated antigens, such as EphA2, HER2 and IL13Ra2. Importantly, DNMT3A KO CAR T-cell proliferation was strictly antigen dependent, demonstrating that DNMT3A deletion does not cause autonomous cell growth. *In vivo*, DNMT3A edited CAR T cells show improved tumor control which resulted in significantly better overall survival. Finally, whole genome DNA methylation analysis of the exhaustion-resistant DNMT3A KO CAR T-cells defined an atlas of genes targeted for epigenetic silencing. Using this atlas, we were able to determine a molecular definition of CAR T-cell exhaustion, which includes many transcription factors that regulate "stemness" of immune cells such as TCF7 and BACH2. To independently validate our findings, we took advantage of publicly available gene expression data of clinical CAR T-cell products and demonstrate that our developed DNMT3A-targeted gene expression score correlates with clinical outcome.**Conclusions:** Deletion of the epigenetic regulator DNMT3A improves CAR T-cell persistence and anti-tumor activity and prevents CAR T-cell exhaustion upon chronic antigen stimulation. Thus, epigenetic reprograming of CAR T-cells can improve current CAR T-cell therapies as well as offer a promising approach for cellular therapy in the context of solid tumors and brain tumors.

84. Insufficient Activation Limits the Efficacy of CAR T Cell Therapy in Chronic Lymphocytic Leukemia

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Background: Chronic Lymphocytic Leukemia (CLL) is a B cell malignancy that accounts for $1/3^{rd}$ of adult leukemia diagnoses in the Western world. Conventional chemo-immunotherapies initially control progression, but in the absence of curative options patients ultimately succumb to their disease. Chimeric Antigen Receptor (CAR) T cell therapy is potentially curative, but only 26% of CLL patients have a complete response. T cells stimulated with CLL cells have reduced effector functions including diminished proliferation, dysregulated immune synapse formation, decreased cytokine production, and poor cytotoxicity. However, systematic characterization of the mechanisms behind these defects has not been done; this study aims to address this gap. ***Findings:*** To study CLL-induced dysfunction we used an *in vitro* re-stimulation assay where we stimulate CAR T cells with primary CLL cells every five days for 3 stimulations; this assay recapitulated many of the T cell dysfunctions described above. We first hypothesized that CLL cells induce stable defects in CAR T cells. To determine whether dysfunction was a terminal state, we used an APC switching assay where we stimulated 1, 2, or 3 times with CLL cells and then switched to a positive control cell (aAPC) for a total of 3 stimulations. aAPCs potently stimulate CAR T cell effector responses. Interestingly, we found that the defects were not permanent, and function could be restored by switching to an aAPC stimulus. Flow cytometry showed that CLL-stimulated CAR T cells appear un-activated, suggesting that CLL cells fail to stimulate CAR T cells rather than rendering them non-functional. One mechanism that could dampen activation is immune suppression. We assessed this at a high level by stimulating CAR T cells with CLL cells and aAPCs mixed at known ratios. However, even cultures containing 75% CLL cells stimulated proliferation and cytokine production. CLL cell phenotyping showed that many CLLs expressed the IL-2 Receptor alpha chain, often at a high level. We therefore hypothesized that CLL cells sink IL-2 from the environment, blunting T cell activation. To test this, we supplemented IL-2 into CLL/CAR T cell co-cultures and showed that this rescued proliferation but only partially restored cytokine production, suggesting that IL-2 sinking explains some but not all of the functional defects. We next proposed that CLL cells may not express high enough levels of co-stimulatory and adhesion molecules to activate CAR T cells. Using flow cytometry we found that most CLL cells expressed low levels of co-stimulatory and

adhesion molecules (Fig. 1A); we hypothesized that up-regulating these molecules would enhance CAR T cell targeting of CLL cells. CLL cells were activated with CD40L and IL-4, which increased expression of CD54, CD58, CD80, and CD86. Stimulating CAR T cells with activated CLL cells enhanced CAR T cell proliferation (Fig. 1B) and induced clustering and cell conjugate formation, indicating cell activation (Fig. 1C). Therefore, improving CLL stimulatory capacity can rescue T cell dysfunctions. ***Conclusions:*** Together, these data show that CAR T cell "defects" in CLL are actually insufficient activation, and improving the stimulatory capacity of CLL cells may enable better clinical responses.

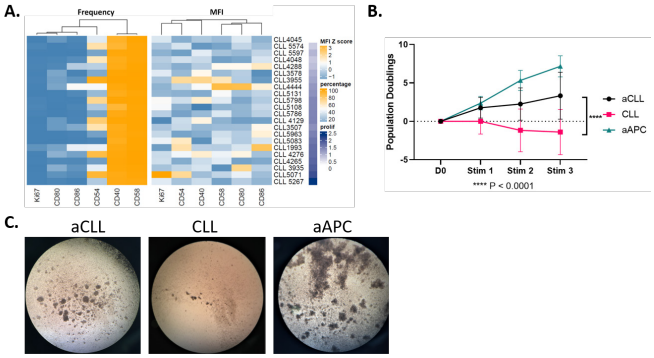

Figure 1: Activation of CLL cells Recues their Stimulatory Capacity
A: Primary CLL cells express co-stimulatory and adhesion molecules at both a low frequency (*left*) and at a low MFI (*right*).
B: Re-stimulation of CAR T cells with activated CLL cells partially rescues proliferation compared to the un-activated CLL control.
C: Activated CLL cells (aCLL) induce CAR T cell clustering and conjugate formation, indicating a productive activation response, more similar to the positive control (aAPC) than the CLL-stimulated cells (CLL).
**** < .0001, mixed effects analysis, Tukey Test.

AAV Vectors - Virology and Vectorology I

85. The Membrane-Associated Accessory Protein (MAAP) is Essential for Rapid Extracellular Secretion of Adeno-Associated Viruses

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Cellular egress of Adeno-Associated Viruses (AAV) likely plays a role in viral dissemination and spread. Recombinant AAV vectors belonging to different clades are known to be secreted into cell culture media during production with variable efficiency; however, the underlying viral or host cell mechanism(s) for this phenomenon are not well understood. A recent study (Ogden *et al.,* Science, 2019) revealed a novel +1 frameshifted open reading frame (ORF) in the VP1 region of the AAV Cap gene, which expresses a membrane-associated accessory protein (MAAP). This protein has been postulated to limit AAV production through competitive exclusion. Here, we further dissect the molecular function of MAAP in the context of recombinant AAV vector production. First, we mapped the conserved secondary structural elements of MAAP across multiple AAV serotypes and confirm through hemagluttinin/GFP tagged fusion constructs that MAAP from different serotypes localizes to the cell membrane. MAAP does not appear to directly interact with the intact AAV capsid, capsid protein monomers or the assembly-activating protein (AAP), which is expressed from a second frameshifted ORF in the Cap gene. Attenuation of MAAP expression by mutating the start codon of the alternate ORF does not impact the ultrastructure of AAV capsids as determined by transmission electron microscopy (TEM) or transduction efficiency of recombinant AAV particles *in vitro*. Strikingly, during production in HEK293 cells, the percentage of several AAV serotypes with mutated MAAP and retained within the cell was markedly increased compared to that secreted into the media. A subsequent time course study revealed a significant delay in extracellular secretion of AAV particles in the absence of MAAP. Further, we demonstrate that certain AAV serotypes with mutated MAAP are secreted more slowly than others, and that extracellular secretion kinetics of such AAV particles can be enhanced by *trans*-complementation of an MAAP from an AAV serotype displaying rapid extracellular secretion kinetics. Mechanistically, we demonstrate that ablation of MAAP expression results in a reduction of cellular exosome formation from HEK293 producer cells during viral production. Taken together, our study provides new functional insight into mechanisms that regulate extracellular secretion of AAV vectors during production and highlights the potential to exploit MAAP-mediated hypersecretion.

86. Dependency of AAV Entry on GPR108 Maps to Motif on VP1u That is Transferable Across AAV Serotypes

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High-throughput genetic screens have identified some host factors that play a role in the transduction of adeno-associated viruses (AAV), such as the well-known AAV receptor or AAVR. Previously, these screens also revealed GPR108 (G protein-coupled receptor 108), a 7-transmembrane receptor of unknown function that is located in the trans-Golgi network, as an essential entry factor for all serotypes but AAV5.We have shown that a specific motif within the VP1 unique (VP1u) region is required for the dependence to GPR108. VP1u however is folded inside the capsid and only exposed during endosomal acidification. Therefore, we speculate that GPR108 engagement occurs in the endosome or trans-golgi network only following VP1u extrusion due to endosomal acidification. In this work, we demonstrated that GPR108 dependence is transferrable between serotypes by generating capsid swap chimeras between AAV2, 8 and rh32.33 (GPR108 dependent) and AAV5 (GPR108 independent) and analyzing their transduction in Huh7 WT and GPR108 KO cells. Interestingly, GPR108 dependence is transferred in the two directions: we have generated GPR108 independent AAV2 and GPR108 dependent AAV5. This fact implies that AAV5 VP1u confers the ability to interact with an unknown host factor to bypass GPR108 usage. By constructing several chimeras this region has been narrowed down to the first 42 residues of the capsid, meaning that the swap of this region from AAV5 to AAV2 led to the creation of a new capsid that transduced at similar levels as AAV2 but independently of GPR108. In addition, swapping of AAV5 VP1u region conferred GPR108 independence to other serotypes, such as AAV8 and AAVrh32.33. The narrower swap of 42 residues in AAV8 capsid was not sufficient to break GPR108 dependence, suggesting that other regions or residues beyond may be involved in a structural motif that confers GPR108 dependence. We have identified

5 differential residues in VP1u region of AAV5, AAV2 and AAV8, which may potentially interact with the already mapped 42 residues. Our studies suggest that a very specific structure in VP1u is involved in GPR108 interaction, and speculatively, the very same region might be involved in the interaction with other entry factor to allow transduction of AAV5 and GPR108-independent chimeras. Further studies are being conducted to fine map the structural motif involved in GPR108 dependence and to validate the observed results in vivo in a GPR108 KO model.

87. USP36, A Nucleolar Enriched Deubiquitinating Enzyme, Promotes AAV Vector Production by Optimally Stabilizing AAP Levels

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Recombinant adeno-associated virus (AAV) is a promising *in vivo* gene delivery vector. However, the broader clinical application of AAV vectors is hindered by the difficulty of producing high titer AAV vectors and our limited knowledge of mechanisms underlying AAV virion assembly. We and others have found that AAV virion formation mechanisms vary across different AAV serotypes, further complicating the development of novel methods to improve AAV vector production. Assembly of AAV viral structural proteins, VP1, VP2, and VP3, is not an autonomous process and requires both the assembly-activating protein (AAP), a newly discovered AAV non-structural protein, and host cellular factors. It is now appreciated that AAP plays a multifaceted role in the AAV viral life cycle, including transporting VP proteins to the nucleus and the nucleolus, stabilizing VP proteins, assisting the capsid assembly process via ill-defined mechanisms, and beyond. In order to elucidate the process of AAV virion assembly, we employed a BioID proximity-based proteomics approach to identify host cellular proteins that participate in virion assembly and interact with AAV viral proteins. To date, we have identified USP36, a nucleolar-enriched deubiquitinating enzyme (DUB), as an AAP2-interacting protein. Here, we present preliminary experimental evidence in HEK293 cells indicating that USP36 promotes AAV virion formation by providing AAP with optimal stability through modulation of ubiquitin-mediated degradation of AAP, and show that both over-destabilization and over-stabilization of AAP result in decreased AAV virion production. USP36 knockdown by siRNA decreased AAP2 levels and AAV2VP3 virion production. Conversely, USP36 overexpression increased AAP2 levels. Although an increase in AAP2 by USP36 overexpression did not affect AAV2 VP3 virion production, these observations underscore a hitherto unknown regulation of AAP by ubiquitination. To further elucidate how USP36 regulates AAP, we fused USP36 to the N-terminus of AAP2. We hypothesized that ubiquitin tags on AAP2 would be removed more efficiently by in *cis* DUB activity than by DUB activity provided in *trans*. AAP2 and USP36-AAP2 were then transiently expressed in HEK293 cells and their stability was assessed by blocking protein translation with cycloheximide. This experiment demonstrated substantially increased stability of USP36-AAP2 compared to AAP2. Although USP36-AAP2 is more stable than AAP2, the capsid assembly-promoting function of USP36-AAP2 was significantly impaired compared to AAP2. Furthermore, we now have evidence that AAP2 is ubiquitinated. Taken together, our observations indicate that USP36 is a key regulator of AAP stability through its DUB activity and provides AAP with stability that is optimal for AAV virion formation. Considering that AAPs have degrons, motifs that promote degradation (please refer to the Abstract by Sairavi et al., presented separately), our results warrant further studies on coordinating roles of cellular factors and AAP degrons in achieving optimal spatio-temporal regulation of AAP stability that allows for effective AAV virion production.

88. Improved Genome Packaging Efficiency of AAV Vectors Using Rep Hybrids

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Recombinant adeno-associated viruses (rAAVs) are among the most commonly used vectors for a variety of gene therapy applications. While the last two decades have seen a lot of research focusing on the characterization and isolation of new *cap* genes resulting in hundreds of natural and engineered AAV capsid variants, the *rep* gene, the other major open reading frame, of the AAVs, has been largely ignored. The lack of research is because the *rep* gene from AAV serotype 2 enables the packaging of recombinant vector genomes into almost all utilized AAV serotypes or variant capsids. However, a major byproduct of all vector productions is empty AAV particles with higher percentages for non-AAV2 vector productions. Despite the packaging process being considered the rate-limiting step for rAAV production, none of the *rep* genes from the other AAV serotypes has been characterized for their packaging efficiency. Thus, the AAV2 *rep* gene was replaced with the *rep* gene of the same AAV serotype as the *cap* gene for AAV serotypes 1-8. While the packaging efficiency of vector genomes into capsids with the substituted *rep* genes appeared to improve, they also led to lower overall capsid protein expression relative to the standard AAV2-*rep* system. In further experiments, optimization of AAV vector packaging and production efficiency focused primarily on AAV1, AAV6, and AAV8 vectors. In a series of chimeras between the different AAV Rep proteins the 3' end of the AAV2 *rep* gene, which also contains the p40 promoter, was shown to be required for high capsid expression; whereas the DNA binding domain of the AAV1 and AAV8 Rep was shown to be responsible for improved vector genome packaging. AAV1, AAV6, and AAV8 vector produced from different Rep chimeras were purified, and analyzed by ELISA, qPCR, alkaline gel electrophoresis, and cryo electron microscopy. The results showed up to 5-times more genome-containing particles in the case of AAV1, 4-times in case of AAV8, and 2-times in the case of AAV6 vectors. These observations indicate that the utilization of these Rep chimeras could revolutionize rAAV production.

89. The Primate Selective Transduction of rAAV-LK03 Vectors is Unrelated to Variation in Double-Stranded Viral Genome Formation in the Nucleus Between Species

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Differential transduction of recombinant adeno-associated viral (rAAV) vectors between species has made it difficult to predict human outcomes from pre-clinical animal studies. One such vector, AAV-LK03 (Lisowski et al. Nature 2014) was shown to robustly transduce human but not mouse hepatocytes in a humanized mouse model. Furthermore, this capsid is currently being evaluated in several clinical trials. We aim to better understand the observed discordance between rAAV transduction amongst different species, not only to gain a better understanding of basic AAV virology but allow better means to predict more robust rAAV vectors for human gene therapy. Here we carried out AAV-LK03 transduction studies on a variety of different human and mouse cell lines and found a >20-100-fold higher level of transgene expression in human cells. To understand the striking difference in expression, we investigated each step of rAAV-LK03 transduction in various cells and found only small differences (<2-fold) between those derived from mice and humans in: 1) cell binding 2) internalization and 3) total and DNase resistant nuclear vector copy numbers of rAAV-LK03. These differences are not able to explain the differences in transduction based on transgene expression. In addition, a small molecule drug that may affect chromatin modifications was able to enhance AAV-LK03-mediated transgene expression specifically in mouse cells by 20-fold. Together, our data suggest that vector chromatin formation in the nucleus may be dictated in part by the capsid sequence and ultimately affect transgene expression in a species-specific manner. We are now investigating chromatin properties such as chromatin structure (ATAC-seq) and post-translational modification of histones (Cut&Run) of the proviral genome, to identify differences between human and mouse cells that will help us elucidate the mechanistic selectiveness of rAAV-LK03 transduction. Unraveling these new mechanisms will allow for better predictions of how to select optimal vectors for a given human application.

90. AAPs Harbor Degrons, a Protein Self-Degradation Motif, Important for Regulating Stability of AAP in the Process of AAV Capsid Assembly

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Assembly activating protein (AAP) is essential for effective AAV virion formation, but the roles of AAP in this process and how AAP and capsid VP proteins interact are poorly understood. AAP is known to transport capsid VP proteins to the nucleus and nucleolus, interact with VP proteins through its hydrophobic region near the N-terminus and promote assembly of VP proteins into a capsid composed of 60 VP subunits. It should be noted that we have found it very difficult to detect AAP by immunoblot in HEK293 cells where capsids have successfully formed possibly due to rapid degradation of AAP upon formation of AAV capsid. Given that persistent attachment of AAP to VP could cause structural hindrance of capsid assembly, there should be a mechanism allowing for effective degradation of VP-attached AAP in the process of capsid assembly. However, how AAP detaches from VP proteins and effectively degrade to allow for formation of functional AAV virions remains unknown. Here we present evidence that the hydrophobic region (HR) near the N-terminus and threonine/serine-rich region (TSR) in the middle of AAPs derived from many AAV serotypes contain degrons, a self-degradation motif that facilitates rapid disintegration of proteins. The degron hypothesis stem from our unpublished data showing rapid degradation of AAP upon formation of AAV capsid. To address this hypothesis, we searched regions of potential degrons within the AAP proteins based on known characteristics of degron motifs, and identified two putative regions, HR and TSR, which we experimentally characterized in this study. HR was chosen as it forms an amphipathic helix, a known characteristic of degron motifs, and TSR was chosen because it is enriched in putative phosphorylation targets, threonine and serine, that could mark the protein for degradation. To determine whether they are indeed degrons, we made GFP constructs fused with the putative degron region at the N-terminus of AAP (AAPxHR-GFP and AAPxTSR-GFP, x=serotype). These fusion constructs were then transiently transfected in HEK293 cells followed by GFP expression analyses through microscopy and immunoblots. GFP expression through microscopy was observed 24 hours and 48 hours post-transfection while immunoblot samples were collected 24 hours post-transfection. Analysis of the HR-GFP fusions for AAV serotypes 1 to 12 revealed degron activity in HR region of all serotypes except for AAP4HR and AAP11HR. Treatment of the AAPxHR-GFP-transfected cells with cycloheximide for 6, 12 and 24 hours revealed that the AAP4HR-GFP and AAP11HR-GFP are most resistant to protein degradation comparable to GFP by itself. Thus, the HR regions in the N-terminus of AAPs serve as a degron and regulate stability of AAPs. Comparison of the steady-state levels of protein expression of the N-terminal halves and C-terminal halves of AAPs by immunoblotting revealed the C-terminal halves to be more stable, supporting the notion that AAPxHR regions drive AAP degradation. Analysis of the TSR-GFP fusions for AAV2, 9 and 11 revealed that AAP2TSR and AAP9TSR but not of AAP11TSR drive AAP degradation. We are currently evaluating the TSR regions of AAPs from other serotypes and investigating whether these two regions, HR and TSR, harbor a potential bipartite function or they work individually to regulate AAP degradation. Thus, our observations provide new insights into the mechanisms of how the stability of AAPs is regulated, how it affects AAP-VP interactions during AAV capsid assembly, and biological significance of AAPs' possession of degrons that facilitate rapid self-degradation.

91. Cardiac Toxicity of AAV9 Vector Upon Heart-Specific Expression of an Immunogenic Non-Self Transgene

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Studies have demonstrated that AAV vectors can mediate long-term expression of non-self transgenes in mice through the induction of T- and B-cell tolerance. Notably, transduction of hepatic regulatory T cells and Kupffer cells plays a critical role in suppressing the cytotoxic T cell (CTL) response to the transgene product. Heart-targeted AAV vectors have emerged as a promising gene delivery platform for cardiac disease. However, the immunogenicity of AAV-delivered non-self transgenes when expression is excluded from tolerogenic liver-resident immune cells remains largely unknown. Here, we employed an AAV9 vector with a heart-specific TNT455 promoter to deliver firefly luciferase, a cytoplasmic protein with a well-defined T cell epitope for C57Bl/6 mice, and characterized immune responses and cardiac toxicity in this mouse strain. Intravenous administration of AAV9 resulted in heart-specific luciferase expression in C57Bl/6 mice. We observed robust luciferase epitope-specific CTL induction at 4 and 6 weeks post infection and AAV administration also led to the development of luciferase-specific antibodies at 4 and 6 weeks post infection. Notably, 3 out of 10 mice developed heart failure with pleural effusion and subsequently died at 4~6 weeks post infection. Necropsy of harvested hearts showed extensive immune cell infiltration. Lethality was dose-, antigen- and mouse genetic background-dependent, suggesting that all of these factors influence toxicity. Our data suggest that the immunogenicity risk of non-self transgenes expressed by AAV vectors may be elevated in cases where transgene expression is excluded from tolerogenic immune cells in the liver.

AAV Gene Delivery for CNS Disorders

92. A New Approach for Designing a Feedback-Enabled AAV Genome Improves Therapeutic Outcomes of MiniMeCP2 Gene Transfer in Mice Modeling Rett Syndrome (RTT)

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Rett syndrome (RTT) is an X-linked neurodevelopmental disorder caused by inactivating mutations in the dose-sensitive gene *MECP2*. AAV9/*MECP2* has been shown to extend the survival of *MECP2* KO mice, but causes side effects in WT and KO mice at high doses. We have shown that the more recently developed mini*MECP2* gene therapy can trigger the same dose-dependent side effects: aggravated phenotype severity scores and low weight. Additional adverse events observed for KO mice treated with AAV9/*MECP2* or AAV9/mini*MECP2* include lesions, self-amputation, and/or prolapses. Lesions and prolapses were also observed in some AAV9/mini*MECP2*-treated WT mice. As AAV9/*EGFP*-treated mice have been shown to be relatively healthy, these side effects are most likely linked to MeCP2 or miniMeCP2 overexpression. In other words, full-length or miniMeCP2 expression should be tightly regulated to correct the MeCP2 deficiency in KO mice without creating toxic overexpression-related phenotypes. Fortunately, the mini*MECP2* gene is small enough to permit insertion of additional regulatory elements into the self-complementary viral genome cassette. We combined high-throughput miRNA profiling and genome mining to create a novel miRNA target panel ("reg2") that - in theory - should permit feedback regulation to maintain safe miniMeCP2 expression levels in the brain. In this conceptual feedback loop, increased expression of total MeCP2 should upregulate endogenous MeCP2-responsive miRNAs that in turn reduce exogenous (mini)MeCP2 expression through RNA interference. We screened CNS RNA from WT and KO mice 2-3 weeks after they were treated with either saline or a toxic dose of intraCSF AAV9/*MECP2*. This approach allowed us to identify the baseline expression of CNS miRNAs as well as miRNAs upregulated in correlation with MeCP2. We then used the miRNA profile to filter and rank conserved miRNA targets from the 3' UTRs of nearly a dozen genes mediating intellectual disability as well as abnormal speech, seizures, microcephaly, and/or stereotypies during childhood. This two-pronged approach allowed us to create a compact target panel that is tailored for RTT gene therapy but may be applicable for other dose-sensitive genes. Within the context of mini*MECP2* gene transfer, reg2 permits widespread protein expression in the brain with robust regulation. After intrathecal administration in adolescent mice, AAV9/mini*MECP2*-reg2 provides a superior therapeutic profile to that of unregulated AAV9/mini*MECP2* as well as the published gold standard AAV9/*MECP2*. Specifically, AAV9/mini*MECP2*-reg2 extended KO survival and delayed the onset of severely abnormal gait among KO mice (vs. the gait of all other groups, and with equal or lower frequency of occurrence). AAV9/mini*MECP2*-reg2 also delayed the age of onset of severe clasping vs. that of AAV9/mini*MECP2*-treated KO mice. In WT mice, reg2 attenuated miniMeCP2-mediated weight loss and abnormal aggregate phenotype severity scores and protected the mice against acute miniMeCP2-mediated gait abnormalities. To date, we have not observed any self-amputations or prolapses among AAV9/mini*MECP2*-reg2-treated KO mice; nor have we observed any self-amputations, prolapses, lesions, or early deaths among AAV9/mini*MECP2*-reg2-treated WT mice. Thus far, reg2 also appears to decrease the frequency of lesions in virus-treated KO mice. Although the mechanistic underpinnings of reg2 have yet to be confirmed across cell types, our new approach for designing conditionally regulated viral genomes has yielded promising results and may extend to other gene dose-sensitive disorders.

93. Comprehensive Evaluation of a Barcoded AAV Library in Non-Human Primate Central Nervous System

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A general consensus is that non-human primates (NHP), the closest human Order, provide many similarities not found in other animal models, e.g. high DNA sequence homology and similar patterns of gene expression. There are primate-specific features of brain development that increase anatomical, cognitive, and behavioral complexity explaining why many human neurological diseases are not well modeled in rodents. It is why NHP are a preferred animal model for optimizing AAV-mediated CNS gene delivery protocols prior to clinical trials. However, in spite of its inherent appeal, it is challenging to compare different AAV capsid serotypes, delivery routes, disease indications, and control for animal-to-animal variability in a well-powered, comprehensive, multigroup NHP experiment. Recently, we have described the design and application of a new AAV-based vector encoding Luciferase, mApple, and a DNA barcode. By linking capsid variants to different barcoded versions of the vector and amplifying the barcode region from various tissue samples using barcoded primers, biodistribution and transgene expression of viral genomes can be analyzed with high accuracy in few animals saving time and resources. Here, we apply the multiplex barcode rAAV vector tracing strategy to a systemic analysis of 29 distinct wild-type AAV natural isolates and engineered capsids in the CNS of 8 macaques (*M. fascicularis*). The vectors were manufactured in HEK 293 cells, purified by double consecutive iodixanol gradient centrifugation, combined at equimolar titers in one single vector mix, and administered to 8 macaques by 4 different routes, 2 animals per each injection route: 1) intraparenchymal injection of the putamen (4.4e10 vg of each barcoded capsid in the mix); 2) intraventricular injection of the lateral ventricle (1.25e11 vg each); 3) injection of the *cisterna magna* (1.25e11 vg each); and 4) intrathecal injection (4.4e11 vg each). After 30-day survival period, animals were euthanized and the following brain structures were collected: cerebral cortex (prefrontal, frontal, parietal, occipital, and temporal lobes); telecephalic (caudate, putamen, and hippocampus); diencephalic (thalamus); brainstem (midbrain, pons, vermis of cerebellum, deep cerebellar nuclei, and medulla); spinal cord (pooled cervical, thoracic, lumbar, and sacral samples). Both total DNA and RNA samples were isolated from the tissues. To analyze the biodistribution and transgene expression of a panel of 29 different AAV capsids in 15 different areas of NHP CNS administered into 8 animals by 4 injection routes, we used a multiplexed barcoding approach: in addition to the internal barcode identifying AAV capsid, forward PCR primer barcode was an identifier for an animal and DNA/RNA sample while reverse barcode decoded a brain area. Sequencing of pooled NGS library was done by Illumina NextSeq 500 HC; custom Python scripts were used for the bioinformatics analysis and graphical visualization of the barcoded capsid vectors biodistribution and expression in all brain areas analyzed. By packaging barcoded rAAV cassettes into multiple of capsids, and utilizing 4 separate vector injection routes, we identify a set of capsids exhibiting efficient biodistribution and brain tissue transduction, for each injection route, and for each major brain area. The results from this multiplex screening approach will help the gene therapy scientific community determine appropriate AAV capsid candidate/s for a particular neurologic indication.

94. Gene Correction in Peripheral Organs May Contribute to the Treatment of Central Nervous System Diseases

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Central Nervous System (CNS) directed gene therapies are widely studied, but the contributions of peripheral organs in disease modulation are still poorly understood. Using Canavan Disease (CD) as a model, we hypothesize that peripheral organs significantly impact the therapeutic outcome of CNS-directed therapies. CD is caused by mutations in the aspartoacylase (ASPA) gene, leading to the characteristic elevation of N-acetylaspartate (NAA), resulting in spongy degeneration of white matter and ultimately lethality at very young ages. Previous therapeutic strategies aimed to restore ASPA functionality in oligodendrocytes, whose function is to hydrolyze NAA into L-aspartate and acetate, thus normalizing NAA levels. However, recent studies indicate that other tissues and cell-types may have a much greater role in rescuing the disease both phenotypically and epigenetically, suggesting novel, critical metabolic regulations along the peripheral organ-brain axis. To assess tissue/cell specific contributions in NAA metabolism, six tissue and cell specific ASPA expression cassettes were engineered into recombinant adeno-associated virus (rAAV), allowing for targeted expression in the CNS and periphery. Surprisingly, contrary to our hypothetical oligodendrocyte-centered approach, we found that restricted ASPA expression in astrocytes led to complete phenotypical and pathological rescue of CD. In the same study, we also found that CD mice treated with liver-restricted ASPA resulted in extended survival but lacked motor restoration and NAA normalization compared to that of wildtype (WT) mice and astrocyte-specific ASPA treated mice, thus raising into question the potential function peripheral organs could have in CNS diseases. We postulate that the partial recovery of CD mice treated with liver-restricted ASPA could be the result of metabolic crosstalk between the CNS and periphery, which might enable genetic regulations essential for therapeutic rescue. Using neuro-metabolomics, we identified 452 metabolites, several of which are associated with epigenetic regulation of gene expression, such as beta-hydroxybutyrate. Remarkably, upon tissue/cell specific gene therapy treatment, these metabolites were either decreased or completely normalized to WT levels. We also found that modifying NAA profiles by peripheral organ-restricted ASPA expression correlated with epigenetic modifications in the CNS, but not in the targeted peripheral organs. Most notably we identified certain histone modifications that suggest a connection between peripheral NAA metabolism and CNS gene expression regulation. This is intriguing because NAA cannot cross the blood brain barrier. This further suggests that metabolic cross-talking between the CNS and non-CNS organs might be critical for disease modification, as highlighted by the extended survival of

mice with liver-restricted ASPA expression. In ongoing experiments, we are exploring differential expression of genetic programs, which would allow for greater understanding of inter-organ communication, as well as the extent of dependence the CNS-directed gene therapy has on gene correction in peripheral organs. *Co-first authors

95. Evaluating the Efficacy and Safety of Cerebrospinal Fluid-Delivered Gene Therapy for Krabbe Disease in Murine and Canine Models

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Krabbe disease is a lysosomal storage disease caused by mutations in the gene encoding the enzyme galactosylceramidase (GALC), which is responsible for degrading galactosylceramide and galactosylsphingosine (psychosine). Toxic accumulation of psychosine results in demyelination within the central (CNS) and peripheral nervous systems (PNS). The most common presentation involves early infantile seizures with disease onset by six months of age and progression to death by two years of age. We previously demonstrated that administering pantropic AAV in the cerebrospinal fluid (CSF) of large animals via the cisterna magna shows efficient transduction of most motor neurons, sensory dorsal root ganglia neurons, and scattered cortical neurons. This method therefore provides enzyme sources to cross-correct both the CNS and PNS. We administered—through the CSF—AAVhu68 encoding human or canine GALC in the Twitcher mouse and Krabbe dog model, respectively. We established a dose-response in newborn Twitcher mice by determining that injections into the lateral ventricle led to a median survival of 130 days at the highest dose of $1x10^{11}$ gc ($6.7x10^{11}$ gc/g brain). GALC levels in the brain and serum were supraphysiological without toxicity. Furthermore, mice demonstrated improved neuromotor function and myelination in CNS and PNS. When newborn CSF administration was followed by bone-marrow transplants in postnatal day 10 conditioned mice, survival extended to >300 days without overt symptoms. In presymptomatic Krabbe dogs, a single cisterna magna injection of AAV.cGALC at $3x10^{13}$ gc provided phenotypic correction, survival increase, nerve conduction normalization, and improved brain magnetic resonance imaging and histopathology. This finding demonstrates the scalability of our approach and suggest it is viable for treating infants diagnosed with Krabbe disease.

96. Developing a CDKL5 Gene-Therapy Vector for a Mouse Model of CDKL5-Deficiency Disorder

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CDKL5-Deficiency Disorder (CDD) is a rare, severe neurodevelopmental disorder that affects children from infancy. Loss-of-function mutations in the X-linked Cyclin-Dependent Kinase-Like 5 (CDKL5) gene result in a lack of CDKL5 protein expression, which causes CDD. Characterized by early-onset seizures (i.e. epilepsy) and intellectual disability, other CDD symptoms include stereotypic hand movements, severe psychomotor retardation, and general hypotonia. There is currently no cure for CDD, with treatments typically focused on alleviating disease symptoms, although poorly controlled seizures are common in many cases. There is therefore an urgent medical need for novel therapeutic approaches to CDD. Gene therapy is a potentially promising curative approach in which the administration of an adeno-associated virus (AAV) encoding CDKL5 could restore CDKL5 expression in the central nervous systems (CNS) of affected children. In this study, we show that restoring CDKL5 expression in the CNS of two CDD mouse models significantly improved disease symptoms. We developed an AAV gene-therapy vector comprising the AAVhu68 capsid, an expression cassette with the human synapsin promoter, and a codon-optimized human CDKL5 transgene. When we administered the AAV-CDKL5 vector to Cdkl5 knock-out mice via neonatal injection into the lateral brain ventricle, we detected robust expression in up to 50% of neurons throughout the brain. Mice exhibited high tolerance for AAV administration and human CDKL5 expression. The human CDKL5 mostly localized to the cytoplasm. Protein expression and kinase activity persisted for over four months. We subjected cohorts of treated Cdkl5-ko mice to a battery of neurobehavioral tests and found that they performed significantly better than the untreated Cdkl5-ko mice. We then repeated the same study in a different CDD mouse model that carries a patient-derived frameshift mutation instead of the gene knock-out. We obtained very similar results, thus reiterating the therapeutic benefit of our CDKL5 gene-therapy vector. To test our AAV-CDKL5 gene-therapy vector in a larger animal, we conducted a study with rhesus macaques. We optimized infusion into the cerebrospinal fluid via the cisterna magna and achieved vector distribution throughout the entire brain at 0.1 to 1 vector copies per diploid genome. We observed much higher vector transduction in the dorsal root ganglia (DRG). Accordingly, *in situ* hybridization with probes specific for the human CDKL5 sequence showed abundant expression in DRG neurons but much sparser expression in cortical grey-matter neurons. All six rhesus macaques showed good tolerance for CDKL5 administration and expression at 60 days. However, we noticed mild axonopathy of spinal white matter tracts. In summary, we present promising pre-clinical evidence that CDKL5 gene therapy provides a lasting curative benefit to model mice and that non-human primates tolerate this therapy well. Further optimization of this approach may eventually offer an option for clinical intervention in children affected by CDD.

97. Intrathecal Delivery of Human Bicistronic Hexosaminidase Vector (TGTX-101) to Correct Sandhoff Disease in a Murine Model: A Dosage Study

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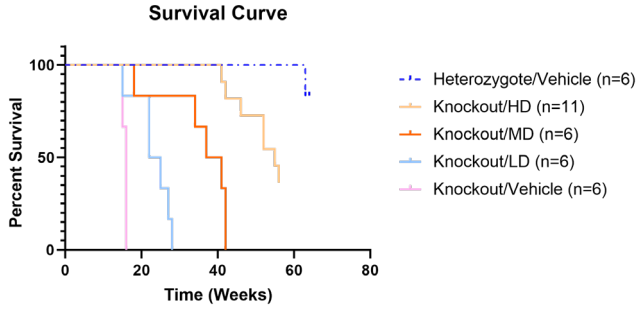
Sandhoff Disease (SD) is a severe metabolic disorder that results from pathological accumulation of GM2 gangliosides within the central nervous system. Normally, genes *HEXA* and *HEXB* encode the subunits of the enzyme responsible for GM2 degradation, known as β-hexosaminidase A (HexA). A mutation to either gene leads to production of a non-functional HexA, followed by GM2 storage. The GM2 load becomes overwhelming to the neuronal cell, promoting apoptosis and consequently widespread neurodegeneration. By 6 months of age, SD patients begin to experience rapid neurological decline and death by age 4, in its infantile form. Currently, there are no approved treatments to slow or stop the progression of SD. Instead, treatment consists of symptom management in the form of intense palliative care. According to recent preclinical data, the most promising therapy for SD may be gene therapy, which delivers the missing gene of interest (i.e *HEXB*) via viral vector to replenish the depleted enzyme (HexA). Previous efforts have established that an effective gene therapy for SD consists of an adeno-associated viral vector serotype 9 (AAV9) carrying both *HEXA* and *HEXB* (TGTX-101). When administered intravenously (IV), this bicistronic vector has proven to significantly reduce GM2 accumulation, increase HexA activity, and prolong survival of SD mice compared to controls. Although this treatment was effective in previous studies, IV administration poses several threats to human clinical translation due to the requirement for such a high dosage. This is due in part to the highly absorbent properties of the liver, which has a tendency to consume a large percentage of the delivered vector and degrade it upon systemic administration. Additionally, higher dosage of gene therapy has been associated with negative effects such as increased immune response and likelihood of oncogenic events. Therefore, the current study aims to identify an optimal dosage of therapy for clinical translation of the bicistronic vector. Here we administer the bicistronic vector via intrathecal lumbar puncture at varying dosages to a SD mouse model (*hexb*$^{-/-}$): high dose (2.5e11vg/mouse) (n=6), medium dose (1.25vg/mouse) (n=6), low dose (0.625e11vg/mouse) (n=6), and vehicle controls (n=6). We hypothesize CNS-directed delivery will decrease the necessary dosage by 1/10th relative to previous IV doses, and thus decrease the likelihood of harmful side effects. Preliminary results have suggested improvement in symptoms corresponds to increasing dosage of gene therapy. In fact, analysis of GM2 ganglioside storage in the brains of short-term vector-treated mice has illustrated a significant reduction in GM2 with the high ($p<0.0001$) and medium ($p=0.0014$) dose in comparison to vehicle controls. Vector biodistribution of short-term treated SD mice mimics the same pattern; the high dose exhibits the greatest increase in copy number, with the medium and low dose to follow. Additionally, as seen in Figure 1, survival of long-term high-dose treated mice has increased 3-fold compared to the vehicle control, with 4 high-dose mice remaining. As we move forward, we expect behavioural as well as HexA enzyme activity analysis to parallel this observed trend. Overall, this study is a major stepping-stone towards translation of our bicistronic vector from bench to bedside.



98. AAV9/*hCDKL5* Delivery to Cerebrospinal Fluid of Juvenile CDKL5-Deficient Mice Improves Learning and Memory and Motor Function in Adult Mice

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CDKL5 Deficiency Disorder (CDD) is a rare X-linked neurodevelopmental disorder characterized by early-onset seizures, global developmental delay with markedly impaired gross motor function, and intellectual disability. CDD is caused by pathologic mutations in the Cyclin- Dependent Kinase-Like 5 (*CDKL5*) gene that result in a loss of function of the CDKL5 protein. CDKL5 is a serine/threonine kinase that is expressed in neurons throughout the brain where it phosphorylates a number of different substrates including microtubule-binding proteins. Studies using CDKL5-deficient mice have uncovered a role for CDKL5 in dendritic spine growth and maturation, processes important for regulating synaptic function and enabling appropriate learning, memory and motor function. A significant unmet medical need exists for patients with CDD, as current management only addresses disease symptoms without targeting the underlying cause. Delivering a functional copy of the *CDKL5* gene to neurons throughout the central nervous system provides an opportunity to restore neuronal function by addressing the root cause of this disease. In this study, a single high dose (1.6e12 vg/mouse) of single-stranded adeno-associated virus serotype 9 (ssAAV9) vector carrying the human CDKL5 cDNA was injected into the cerebrospinal fluid of juvenile CDKL5-deficient male mice by intracerebroventricular (ICV) injection. Two weeks after dosing, RNAScope analysis revealed widespread distribution of human *CDKL5* mRNA in neurons across the brain. Increased phosphorylation of a known CDKL5 downstream target, microtubule-associated protein RP/EB family member 2 (EB2), provided evidence that the transgene-derived human CDKL5 was a functional kinase. A second cohort of both male and female CDKL5-deficient mice was injected between 3-5 weeks of age (early symptomatic) and underwent a battery of behavioral tests as adults at 2-3 months of age. Western blot analysis of microdissected tissue from multiple brain regions

demonstrated moderate but long-lasting increases in human CDKL5 protein across the brain (20-30% of WT levels in frontal cortex and brainstem, 35-70% in hippocampus) 3-months after dosing. Treated mice showed improvements in motor function and coordination, as well as in learning and memory. Importantly, our findings suggest that even moderate levels of functional CDKL5, delivered to juvenile symptomatic CDKL5-deficient mice, can lead to long-term expression and significantly improved brain function.

Oligonucleotide Therapies for Acquired Diseases

99. rAAV-Mediated Hepatocyte-Specific Expression of miR-375 Protects Against the Acetaminophen-Induced Acute Liver Failure in Mice

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Acetaminophen (APAP) overdose is the leading cause of acute liver failure (ALF) in many countries, including the United States. The high incidence of mortality associated with APAP-ALF can be attributed to its rapid onset and lack of an effective treatment. Intensive studies have suggested multiple microRNAs (miR) as potential biomarkers or therapeutic targets. Among these microRNAs, miR-375 transcription levels significantly correlate with severity of APAP-challenge in clinical samples, making it an attractive candidate. In a preclinical proof-of-concept study using APAP-ALF mouse model, we have exploited highly liver-tropic AAV8 for the TBG promoter-driven, hepatocyte-specific overexpression of miR-375, miR-122 and miR-125b. Following APAP challenge (350 mg/kg) and analyses of liver function (ALT), histology (H&E staining), mitochondrial function (the Seahorse assay) and transcriptome (RNA-seq), we found miR-375 completely blocked ALF. Interestingly, this protection was only observed in APAP overdose ALF model but not in Fas ligand-induced ALF model. Mechanistically, RNA-seq data from liver transduced with AAV8-miR-375 demonstrates that genes regulated by miR-375 are enriched in the cytochrome P450 system (CYP), APAP-metabolizing enzymes responsible for cytotoxic byproduct NAPQI (N-acetyl-p-benzoquinone imine) formation, as well as NAPQI-scavenging Glutathione-S-transferases (GST) and Sulfotransferases (SULT). The significantly decreased CYP2E1 and increased GSTM1 expression have been independently validated by RT-qPCR and Western blotting. Consistently, LC/MS-MS measurement revealed increased levels of liver glutathione (GSH) in the AAV-miR-375 treated mice before APAP dosing while NAPQI-protein adducts was significantly decreased after APAP challenge in miR-375 expressing liver. More strikingly, the overall APAP uptake in AAV-miR-375 transduced liver was significantly attenuated, suggesting miR-375 regulated active APAP transportation pathway which has not been reported. Furthermore, while AAV-amiRs-mediated *in vivo* silencing of each potential miR-375 target genes, Slc16a2, Cyb5b and Acsl5, partially mimics miR-375-mediated protection, complete recapitulation of the protective effects from miR-375 can be achieved in the combined administration of these three AAV-amiR vectors. Detailed mechanistic studies on these target genes are underway. Overall, our results identify miR-375 as a potential mRNA therapeutic target with potent anti-APAP-ALF effects, acting through complex, well-orchestrated mechanisms. [a]Co-corresponding authors.

100. Therapeutic saRNAstargeting CEBPA in Myeloid Cells. A potential Immunomodulatoryswitch for Anticancer Therapy

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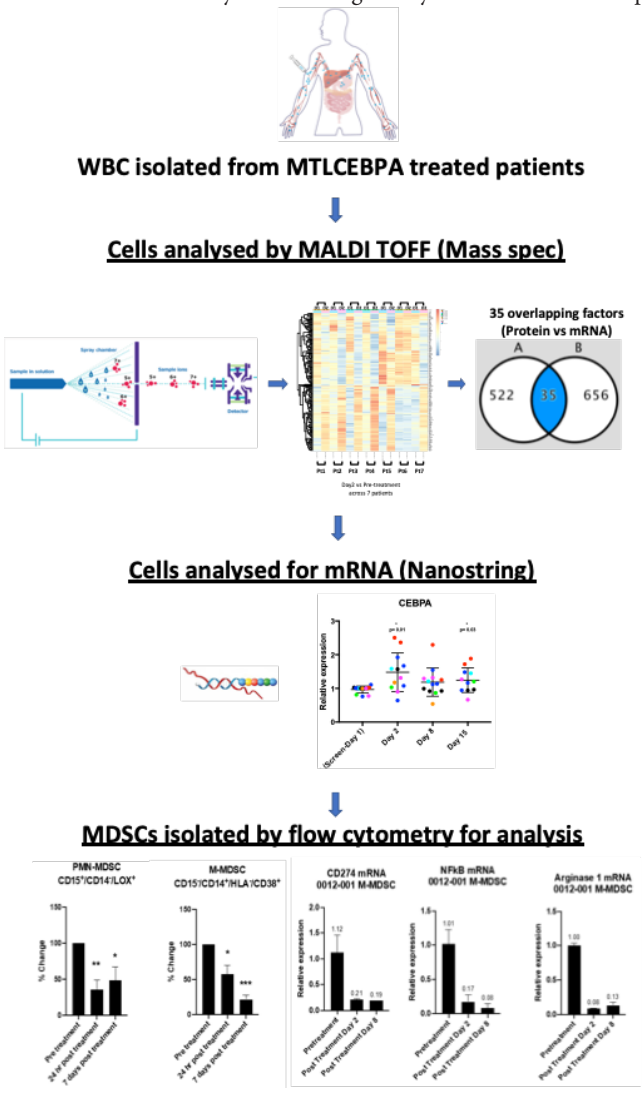
MTL-CEBPA is a first-in-class small activating RNA (saRNA) oligonucleotide which specifically up-regulates the myeloid cell master regulator C/EBP-α (CCAAT/enhancer-binding protein alpha). Here we present quantitative changes observed in circulating leukocytes of HCC patients treated with CEBPA-saRNA. MTL-CEBPA is currently in a Phase I, dose escalation / expansion trial in adults with HCC or secondary liver cancer. Patients received intravenous MTL-CEBPA at 28-160 mg/m^2 for 3 weeks either QW, BIW at d1 and d2, BIW at d1 and d3, or TIW at d1, d2, and d3 followed by a rest period of 1 week. Adverse events, serum PK and anti-tumor activity were assessed. Circulating white blood cells (WBC) were captured before treatment for baseline measurements and post-treatment at Day 2 , 8 and 15 for quantitative protein expression (MALDI-TOF Mass spectroscopy); gene expression (NANOSTRING) and flowcytometry. MTL-CEBPA demonstrated a good safety profile with several durable complete responders and partial responders observed. Treatment altered the expression of 522 protein and 656 genes in circulating WBC. The functional interpretation these factors were summarized using Ingenuity Pathway Analysis. An overlapping analysis revealed 35 key targets were affected in all of the patients. MTL-CEBPA treatment enhanced *CEBPA* expression in leukocytes affecting macrophage biology through regulation of *PU-1, ARG-1, BAX, CD14, CEBPB, ELANE, MAPK, IFN-γ* and *NFkB1*. Flow cytometry analysis of WBCs from treated patients demonstrated CEBPA upregulation caused a significant reduction in circulating

tumour-infiltrating polymorphonuclear and monocytic myeloid derived suppressor cells (MDSCs). MTLCEBPA can potentially act as an immuno-modulatory switch for regulatory T cells in cancer therapy.



101. Targeted Systemic Delivery of Small Nucleic Acids to Metastatic and Brain-Localized Triple-Negative Breast Cancer by HER3-Homing Nano-Capsids

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INTRODUCTION: The median survival of patients with metastatic breast cancer is 3 years, and patients with breast cancer metastases to the brain on average survive less than one year. These tumors tend to resist current therapies, including targeted therapies currently used in the clinic, thus improved alternatives are urgently needed. Increased cell surface levels of the human epidermal growth factor receptor subunit 3 (HER3) is associated with metastatic breast tumors, including those that spread to the brain. Increased HER3 is also associated with resistance to a number of clinically used targeted therapies including inhibitors of EGFR (lapatinib), HER2 (lapatinib, trastuzumab, T-DM1), HER2-3 (pertuzumab), and combination therapy. We have previously bioengineered a HER3-targeted tumor invading protein, HPK, that can home in on breast tumors resisting ErbB receptor family inhibitors. HPK is a recombinant ligand-mimicking biocarrier protein that assembles into cargo-containing particles displaying multivalent, neuregulin derived targeting ligands that enable stealth delivery into HER3+ tumors. We have previously shown that systemic HPK particles can direct the delivery of small nucleic acids, including siRNA, to HER3-expressing tumor xenografts in mice, and penetrate cell targets through a protein protonation and dismantling mechanism. **RESULTS AND METHODS:** In the present study we show that systemic HPK particles delivering 5' triphosphate-modified siRNA targeting the FOXP3 transcription factor reduce primary and metastatic tumor burden in an orthotopic syngeneic mouse model of triple-negative breast cancer (TNBC), which is highly invasive and expresses high levels of HER3. We show that a combination of HER3 homing, FOXP3 silencing and stimulation of tumor-originating inflammatory cytokines yielded a tumor-suicidal effect resulting in reduction of primary tumor growth and reduced metastasis to the lungs. We also found that HPK particles loaded with fluorescently tagged oligonucleotide cargo, or nucleoparticles, could home to TNBC tumors implanted in the brains of mice, leading us to discover that HER3 is expressed prominently on both mouse and human adult brain vasculature. Systemic delivery of HPK nucleoparticles in non-diseased mice exhibited localization at the brain endothelium where HER3 is expressed and in extravascular brain parenchymal regions where HER3 is not expressed. Brain localization of HPK particles declined over time in the absence of continued systemic treatment. Recapitulation of the human blood-brain barrier (BBB) using a human induced pluripotent stem cell (iPSC)-derived organ-on-a-chip model (BBB chip) showed robust levels of HER3 present on the endothelial lumen. Flow of HPK particles through the BBB chip lumen yielded initial particle localization with the endothelial HER3 and subsequent emergence into the overlaying neuronal-astrocyte layer, in contrast to non-targeted oligonucleotide probes. **CONCLUSIONS:** Taken together, our findings indicate that systemically delivered HPK particles can use HER3 homing to target and reduce TNBC metastasis, as well as mediate nucleic acid delivery across the BBB and into HER3+ intracranial tumors. The highly invasive nature of TNBC and the association of HER3 with resistance and metastasis, including brain metastasis, highlights the potential for HER3-homing nanocarriers such as HPK to improve the therapeutic targeting of HER3+ metastatic tumors, including those that spread to the brain.

102. Synthetic mRNA Nanomedicine for Tumor Suppressor Restoration and Cancer Immunotherapy

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Loss/mutation of tumor suppressor genes is a dominant force in tumor development and progression, along with the gain of pro-tumorigenic functions. Recent clinical results have also suggested that loss of tumor suppressor PTEN may correlate with the poor-response/resistance of different cancers (e.g., melanoma) to immune checkpoint blockade (ICB) therapy. Nevertheless, it is largely unknown if restoration of functional tumor suppressors may revert the tumor's sensitivity to ICB therapy. Recently, we have successfully demonstrated the feasibility of using synthetic mRNA nanoparticles to reconstitute tumor suppressors, such as PTEN (*Nature BME 2018, 2:850-864*) and p53 (*Sci Transl Med 2019, 11:eaaw1565*), for cancer treatment. Herein, we present our new studies that explicitly address two previously unexplored questions: i) can PTEN reactivation induce anti-tumor immunity and ii) can PTEN mRNA nanoparticles improve the tumor's sensitivity to ICB therapy? We demonstrate that synthetic mRNA nanoparticles can effectively reactivate PTEN in *PTEN*-null or mutated murine tumor cells and induce immunogenic cell death. In addition, the PTEN mRNA NPs can promote CD8+ T cell infiltration to tumor tissues and reverse the immunosuppressive tumor microenvironment. The combination of PTEN mRNA nanomedicine with anti-PD-1 antibody further leads to a highly potent anti-tumor effect in different animal models of melanoma and prostate cancer. Our new study suggests that synthetic mRNA nanomedicine may provide a potent novel immunotherapy strategy for different malignancies along with ICB.

103. Abstract Withdrawn

104. A Novel Approach to Potentially Treat Influenza: Selective Induction of Apoptosis in Infected Cells by Hijacking the Virus Machinery

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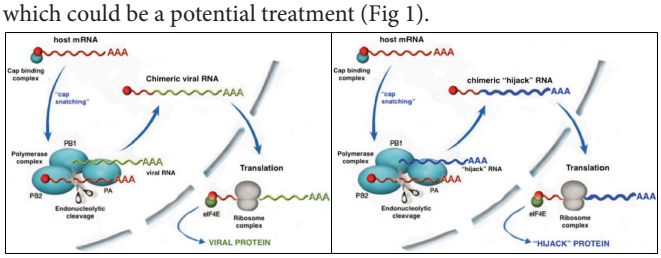
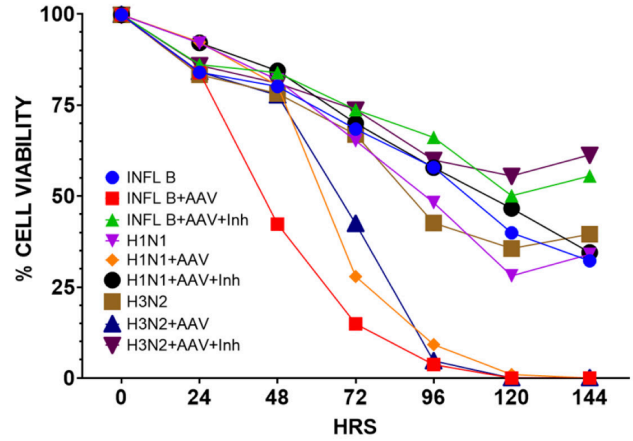
Background: Annual epidemics of influenza result in ~1 billion infections, 3-5 million cases of severe illness and about 500,000 deaths. Current antiviral treatments are mainly designed to interfere with the virus life cycle to suppress the functions of viral replication machinery. Based on the fact that influenza virus is a single-stranded negative-sense RNA virus that utilizes influenza polymerase complex to express viral proteins or replicate its viral genome, we developed a vector construct expressing a negative-sense non-coding (nc)RNA that is designed to engage with influenza polymerase. The ncRNA "hijacks" the virus machinery to induce apoptosis specifically in infected cells, which could be a potential treatment (Fig 1).



Methods: Adeno-associated virus (AAV) was packaged with a novel vector expressing our ncRNA, aka influenza "hijack RNA", that transcribes the reverse complementary strand of zsGreen marker (AAV.infv.rcZsGreen) or caspase-9 (casp9) gene (AAV.infv.rcCasp9) between influenza genomic RNA non-coding sequence (NCS) regions that are highly conserved across several influenza virus strains. Madin-Darby Canine Kidney (MDCK) cells were infected with influenza A H1N1 or H3N2, or influenza B virus at 0.1 MOI. Influenza infected and uninfected MDCK cells were transduced with AAV.infv.rcZsGreen vector, and with AAV.infv.rcCasp9 in the presence and absence of casp9 inhibitor Z-LEHD-FMK to determine the functionality of the hijack vector. Flow cytometry was used to determine zsGreen expression. Cell viability and proliferation were evaluated daily by FACS, Annexin assay, and automated cell count. **Results:** All AAV.infv.rcZsGreen vector transduced influenza-infected cells successfully produced zsGreen protein, confirming that the hijack RNA was recognized and transcribed by the polymerase complex in each influenza strain. Influenza infection killed 60%-68% of the untreated cells by day 6. 91%-95% of infected cells treated with casp9 hijack vector at 24h post-influenza infection died on day 3 post-treatment. Casp-9 inhibition in the culture salvaged vector induced cell death and extended the lifespan of infected cells to 5-6 days (Fig 2). There was no significant cell death in uninfected control groups.



Conclusions: A vector delivered in trans to engage with influenza polymerase hijacks the virus machinery to induce death in influenza infected cells 40% more rapidly than untreated infected cells. This effect was seen across viral strains, likely due to conserved nature of polymerase. This novel approach could be used to develop an effective treatment for influenza.

105. Systemic Senolysis in Naturally Aged Mice Using a Fusogenix FAST-LNP Gene Therapy Approach

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Therapeutic approaches to eliminate senescent cells (SCs) *in vivo* using transgenic mouse models have demonstrated significant improvements in lifespan, reduction of cancer and amelioration of age-related degeneration. Unfortunately, this approach requires that the organism be genetically engineered from the embryo and thus cannot be implemented in humans. Furthermore, previous studies have shown the need for repetitive dosing over the course of the organism's lifespan, which means that a gene delivery approach using viruses will be ineffective over time. We therefore sought to develop a clinically viable gene therapy to selectively target SCs *in vivo* with fusogenic lipid nanoparticles (LNPs). These LNPs employ fusion-associated small transmembrane (FAST) proteins that can efficiently transduce a wide range of cells *in vivo* and can be administered repeatedly without inducing a deleterious immune response. The expression of a potent pro-apoptotic suicide gene is driven by a senescence-associated promoter such as $p16^{Ink4A}$ or p53 and SCs are selectively cleared following administration of a small molecule chemical inducer of dimerization. We have developed fusogenic formulations targeting $p16^{Ink4A}$+, p53+, or p16+/p53+ cells and demonstrated their efficacy *in vitro* and *in vivo*. *In vitro*, treatment of irradiated cells leads to a significant decrease in the amount of SA-β-Gal+ p16+ cells, demonstrating effective ablation of senescent cells. Non-senescent cells are unaffected by treatment. These effects were transferable *in vivo*, where systemically administered LNPs targeting $p16^{Ink4A}$+ or p53+ cells were associated with a decrease in senescent cell burden in aged animals. Treatment was also associated with improvements in various health indicators such as microvascular perfusion in the brain and bone density. We hypothesized that these improvements in organ function would be associated with an improvement in overall lifespan. To test this, we treated a cohort of naturally aged C57BL/6 mice (105 week-old at start of trial) at monthly intervals until death, and observed significant improvements in median lifespan. Experiments analyzing the systemic expression of senescence-associated secretory phenotype in treated animals are ongoing. We have developed a metabolomic signature characterizing aged mice and are currently using this signature to investigate the correlation between aged and young mice in our treatment cohort. Finally, we have demonstrated the safety of this senolytic in a non-human primate study. We also observed extensive systemic distribution of the transgene in all organs tested, demonstrating potential to alleviate organ decline in myriad of tissues. In summary, this approach represents a first-in-class therapeutic that targets cells based on transcriptional activity, rather than surface markers or metabolism, and represents a viable strategy for the systemic ablation of senescent cells to positively impact human healthspan and lifespan.

HSPC Gene Therapies for Blood and Immune Disorders

106. Durable Therapeutic Restoration of Immunity in the Scid-X1 Canine Model via In-Vivo Delivery of Cocal Pseudotyped Lentivirus Vector Carrying Il2R-Γc

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Ex-vivo gene therapy with hematopoietic stem and progenitor cells (HSPC) using lentiviral vectors has been shown to cure a number of genetic diseases affecting the hematopoietic system including . X-linked severe combined immunodeficiency (SCID-X1). However, the use of this platform requires sophisticated facilities to manufacture the therapeutic product (lentivirus vector and purification of stem cells) and presents enormous challenges when considering the utility and feasibility of this approach in the global perspective. Additionally, the need for toxic conditioning regimens to facilitate the engraftment of *ex-vivo* gene-modified HSPCs adds to the toxicity of this procedure. An alternative to *ex-vivo* HSPC gene therapy is *in-vivo* gene therapy (IVGT) whereby direct injection of viral vector carrying the therapeutic cDNA without conditioning regimen will abolish the need to *ex-vivo* manipulation of the stem cells. This far-reaching approach will be easier to disseminate and could be instrumental while treating genetic disorders in the most prevalent areas of worldwide population. On that note, we have previously established an IVGT platform with foamyviral vector that have shown excellent clinical benefit in treating the SCID-X1 in canine model. Considering the clinical translatability of the viral vector platform, in the current study, we utilized a lentiviral vector pseudotyped with cocal envelope (cocal-LV-hPGK-γC). Cocal envelope provides several advantages over the conventional VSVG envelope including lower immunogenic potential, higher transduction ability of CD34+ HSPC's and ease of large-scale production due to availability of stable cell line. Intravenous injection of cocal-LV-PGK-γC post stem cell mobilization in SCID-X1 neonatal canines achieved long-term therapeutic immune-reconstitution with no prior conditioning. Long-term (14 months) monitoring of two treated SCID-X1 dogs demonstrated therapeutic levels of CD3+ T-cells along with normal levels of CD4+ and CD8+ T cells. Within the T-cell population, gene correction with cocal-LV-hPGK-γC stabilized at ~90% within 45 days post injection (Figure 1). Furthermore, the persistence of normal T cell receptor excision circles (TREC) levels and CD45RA+ naïve T cells over a year post treatment suggest a continuous maturation of thymic-derived cells. Validation of T-cell functionality using spectratyping analysis exhibited a diverse repertoire of T cell receptor gene rearrangement. Moreover, retroviral integration site analysis (RIS) demonstrated polyclonal contribution to the reconstituted T-cells and safety of cocal-LV IVGT approach. The gene-corrected canines exhibit equivalent health and physical attributes to normal littermate controls and were able to clear bacterial and protozoal infection. In summary, our data demonstrated that cocal-LV vector deliver durable therapeutic gene correction in a large-animal model for SCID-X1 gene therapy without prior conditioning. Most importantly, these results indicate that cocal-LV-hPGK-γC IVGT is

a viable and accessible option for rebuilding long-term immunity in SCID-X1 human clinical trials or could be used as a surrogate measure in patients where allogeneic BMT is not feasible and ex-vivo HSPC gene therapy failed.

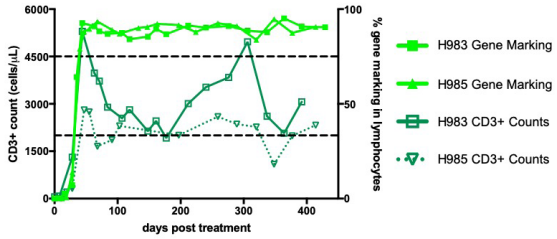

Figure 1: Kinetics of gene marking based on fluorophore expression (right Y axis) and absolute number of CD3+T cell counts (left Y axis) within circulating lymphocytes from dogs treated with Intravenous administration of cocal pseudotyped lentiviral vector (Cocal-LV-hPGK-γC) expressing IL2r-γ chain under human phosphoglycerate kinase promoter (hPGK).

107. Efficient, Specific and Universal Therapeutic Gene Editing of *ELANE* for Severe Congenital Neutropenia in Human Hematopoietic Stem Cells

Shuquan Rao[1], Josias Brito-Frazao[2,3], Qiuming Yao[1,4], Anna V. Serbin[5], Kevin Luk[3], Yuxuan Wu[1], Jing Zeng[1], Chunyan Ren[1], Anne H. Shen[1], Ruth Watkinson[6], Myriam Armant[1], Luca Pinello[4], Scot A. Wolfe[3], Benhur Lee[6], Roberto Chiarle[7], Akiko Shimamura[1], Peter Newburger[2], Daniel E. Bauer[1]

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Severe congenital neutropenia (SCN) is a life-threatening disorder of circulating granulocyte deficiency, requiring lifelong G-CSF pharmacotherapy, with ongoing predisposition to myelodysplasia and acute myeloid leukemia. The most common etiology of SCN is germline monoallelic *ELANE* mutation. Although misfolded or mislocalized neutrophil elastase appears to cause cell death of granulocyte precursors, neutrophil elastase itself appears dispensable for neutrophil maturation and redundant for host defense functions. In our previous work, we found that targeting premature termination codons to early exons of *ELANE* could induce nonsense-mediated decay and bypass neutrophil maturation arrest of *in vitro* derived hematopoietic stem and progenitor cell (HSPC) derived neutrophil maturation culture. Here we extend the work to a novel in vivo xenograft model of SCN. Using three human donors, we demonstrate that targeting frameshift mutations to *ELANE* late exons mimics naturally occurring SCN-associated mutations, and produces promyelocyte/myelocyte stage neutrophil maturation arrest in the bone marrow of xenografted NBSGW mice. In contrast, targeting frameshift mutations to *ELANE* early exons to produce nonsense mediated decay results in comparable human bone marrow chimerism and multilineage reconstitution as neutral locus targeting, measured 16 weeks after infusion **(Figure 1)**. We identified RNP electroporation conditions which could achieve 97.3% indels in the input cell product. We found 89.0% indels persisting in the 16 week engrafting bone marrow, with 90.6% indels in $SSC^{high}CD16^{+}$ human granulocytes. We evaluated the off-target potential of this potentially therapeutic RNP at 48 predicted off-target sites with 3 or fewer mismatches. We found only 1 of 48 sites with any indels detected by amplicon deep sequencing, at a noncoding sequence of no predicted regulatory function. Editing with an attenuated variant form of SpCas9 (HiFi), produced undetectable indels at this off-target site while the on-target indel frequency remained 88.5%, suggesting favorable specificity potential. Using CD34+ HSPCs from four *ELANE* mutant congenital neutropenia patient donors, we demonstrated that *ELANE* exon 2 targeting could efficiently restore neutrophil maturation *in vitro*. Together these results support the development of *ELANE* early exon targeting by template-free gene disruption as a highly efficient, specific, and universal therapy for *ELANE* mutant SCN.

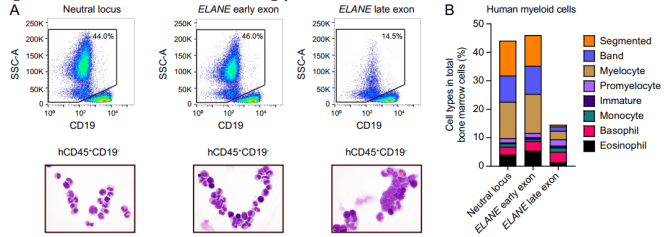

Figure 1 Impaired granulopoiesis in engraftment of *ELANE* late exon edited CD34⁺ HSPCs in NBSGW immunodeficient mice
CD34⁺ HSPCs from three healthy donors were edited with RNP, coupling with sgRNAs targeting neutral locus, *ELANE* early exon or late exon, and transplanted into NBSGW mice. A total of 0.8-1 million cells were infused per mouse. Mice bone marrow (BM) was subject to immunophenotyping and Wright-Giemsa staining 16 weeks after transplantation.
(A) Representative flow cytometry of mice BM xenografted with neutral locus (left), *ELANE* early exon (middle) and late exon (right) edited CD34⁺ HSPCs. The lower panel shows Wright-Giemsa staining of hCD45⁺CD19⁻ from each group.
(B) Statistical summary of different cell types from mice BM.

108. A Genomic Editing-Based Therapeutic Approach for RAG2 Deficiency

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Recombination-activating gene 2 (RAG2) deficiency is classified as a severe combined immunodeficiency disorder (SCID), where the adaptive immune cells are unable to properly assemble functional antigen-specific receptors of the immunoglobulin (BCR) and T-cell (TCR). RAG2 functions as part of the complex required for V(D)J-recombination activity, which is essential during lymphocytes development and antigen-recognition. Less than 1% V(D)J-recombination activity leads to typical RAG2-SCID, with a complete absence of circulating T and B cells ($T^{-}B^{-}NK^{+}$), where > 1% V(D)J-activity leads to atypical RAG2-SCID classified as Omenn syndrome (OS), expansion of γδ-T cells and, in some patients, granulomatous inflammation and/or autoimmunity. Hematopoietic

stem cell transplantation is a curative therapy for patients with typical RAG2-SCID and OS. However, in the absence of an HLA-matched donor, high incidence of graft failure and poor immune reconstitution limits the therapeutic success. Although conventional approaches to gene therapy are being considered for RAG2 deficiency, they carry theoretical risks of genomic instability associated with dysregulated expression of the gene. To address these issues, we report a CRISPR/Cas9 based proof-of-concept genome editing approach designed to correct all pathogenic mutations in the *RAG2* gene. Our approach uses adeno-associated viral vector of serotype 6 (AAV-6) to deliver a codon-optimized *RAG2* (RAG2co) therapeutic transgene at the endogenous *RAG2* gene translation initiation site in human hematopoietic stem and progenitor cells (HSPCs). RAGco bulk allele genome targeting (GT) analysis shows a median 40.5% (range 21.0% - 53.3% n=7), while single cell analysis confirms 71% alleles targeting efficiency (30/42 clones) with 30.9% mono-allelic and 40.5% bi-allelic GT. RAG2co GT into healthy donor-derived HSPCs engrafted (median=15.8%, bone marrow, BM) into immunodeficient NSG mice (n=11) at no statistical difference from control cells (n=5 mice per condition). 18 weeks post engraftment analysis of sorted human cells derived from RAG2co GT HSPCs, showed a median bulk allele GT level of 51.0% (range 16.6% - 70%, n=4 mice) and 34.6% (range 12.3% -64.0%, n=7 mice) in BM and spleen of mice, respectively. Human sorted T-cells (n=4 mice) derived from BM of mice engrafted with RAG2co GT HSPCs showed a median of 52.3% (range 40.0% - 70.0%) bulk allele GT, while spleen-derived and sorted T-cells and B-cells had a median of 44.5% (range 16.6% -64.0%, n=4 mice) and 25% (range 12.3% - 46.5%, n=3 mice), respectively. Engraftment analysis of the RAG2co GT HSPCs confirmed multi-lineage reconstitution. Lastly, we report 40% bulk allele GT in one RAG2 patient (c.296 >A; c1242C >A) frozen mobilized $CD34^+$ HSPCs. *In vivo* studies are currently underway to assess the engraftment potential and functional rescue of patient-derived RAG2 HSPCs. Off-target analysis by next generation sequencing (NGS) of RAG2 patient GT HSPCs (RNP condition), identified insertion and deletions (INDELs) in only 2 out of the 48 COSMID predicted sites, at levels < 0.2% located at an intergenic (> 38kb from nearby gene) and intronic (MIR-383) loci. Our current preclinical data demonstrates a robust and precise next generation gene therapy treatment for RAG2 deficiency.

109. A Phase 1/2 Study of Lentiviral-Mediated *Ex-Vivo* Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I): Initial Results from the First Treated Patient

Donald Kohn[1], Gayatri Rao[2], Elena Almarza[3,4], Dayna Terrazas[1], Eileen Nicoletti[2], Augustine Fernandes[1], Caroline Kuo[1], Satiro De Oliveira[1], Theodore Moore[1], Ken Law[2], Brian Beard[2], Julian Sevilla[5], Cristina Mesa-Núñez[3,4], Juan Bueren[3,4], Jonathan Schwartz[2]

[1]University of California, Los Angeles, Los Angeles, CA,[2]Rocket Pharmaceuticals, Inc., New York, NY,[3]Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[4]Advanced Therapies Unit, Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD/UAM), Madrid, Spain,[5]Pediatric Oncology Hematology and Stem Cell Transplant Department, FIB Hospital Infantil Universitario Niño Jesús, and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain

Introduction: LAD-I is a rare inherited disorder of leukocyte (primarily neutrophil) adhesion to endothelial cell surfaces, migration, and chemotaxis resulting from *ITGB2* gene mutations encoding for the β2-integrin component, CD18. Severe LAD-I (i.e., CD18 expression on <2% of PMNs) is characterized by recurrent severe infections, impaired wound healing, and childhood mortality. Although allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative, its utilization and efficacy are limited by HLA-matched donor availability and risk of graft-versus-host disease (GVHD). RP-L201-0318 (clinical trials.gov # NCT03812263) is a phase 1/2 open-label clinical trial evaluating the safety and efficacy of autologous CD34+ cells transduced with a lentiviral vector (LV) carrying the *ITGB2* gene encoding for CD18 (Chim-CD18-WPRE) in severe LAD-I. **Methods:** Pediatric patients ≥ 3 months old with severe LAD-I (demonstrated by CD18 expression on <2% PMNs and at least 1 prior significant bacterial or fungal infection) are eligible. Peripheral blood (PB) HSCs are collected via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and Plerixafor. CD34+ HSPCs are selected, transduced with Chim-CD18-WPRE LV, and cryopreserved. Myeloablative conditioning with busulfan (with therapeutic drug monitoring (TDM) to adjust dosing to enable target area under the curve (AUC)) is administered, followed by infusion of the investigational drug product (RP-L201). Patients are followed for safety assessments (i.e., replication competent lentivirus (RCL) and insertion site analysis (ISA)), and efficacy -- survival to age 2 and at least 1-year post-infusion without alloHSCT, increase in neutrophil CD18 expression, PB vector copy number (VCN), decrease in infections and/or hospitalizations, and resolution of skin or periodontal abnormalities. **Results:** An initial LAD-I patient (age 9) with recurrent severe infections and documented *ITGB2* mutations has been treated as of January 2020. Baseline CD18, CD11a, and CD11b expression were < 1%. Mobilization and apheresis procedures were performed successfully and busulfan was administered at the target AUC. Investigational product was comprised of $4.2x10^6$ CD34+ cells/kg with VCN of 3.8 copies/cell (liquid culture) and infused without complications. No serious treatment-emergent adverse events were

reported. Neutrophil engraftment was observed 18 days post-infusion. PB PMN CD18 expression 3 months post-treatment was 44.9% with comparable CD11a and CD11b expression; PB CD15 (myeloid) VCN at 2.5 months was 1.5. Safety and efficacy data 6 months post-treatment will be available at the time of presentation. **Conclusion:** Preliminary evidence demonstrates that RP-L201 enables *ITGB2* genetic correction with robust CD18/CD11 neutrophil expression in this frequently fatal primary immunodeficiency.

110. Updated Results of a European Gene Therapy Trial in Fanconi Anemia Patients, Subtype A

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A phase I/II gene therapy trial in FA-A patients has been conducted using HSCs mobilized with filgrastim and plerixafor. $CD34^+$ cells collected from 2-3 apheresis cycles and purified with the Clinimacs system were pre-stimulated and transduced during a total period of 20-24h with the PGK-FANCA.Wpre* lentiviral vector and infused into patients in the absence of any pre-conditioning treatment. We have previously shown the successful engraftment of gene corrected cells in the first four treated patients that were followed for 18-30 months post-infusion (Rio *et al* Nat Med 2019). Insertion site analyses in patients' PB and BM cells revealed the absence of genotoxic events. Currently we have concluded the recruitment of this phase I/II trial, with a total of 9 treated patients age 3-7 years and infused with $7.3x10^4$ to $1.9x10^6$ $CD34^+$ cells/kg. Most procedure-related adverse events were mild and have resolved completely at the time of analysis. One patient developed bacteremia after infusion of the medicinal product which was treated with antimicrobial therapy. Analyses of vector copy numbers per cell (VCN/cell) in colonies generated from aliquots of the cell manufacturing product ranged from 0.2 to 0.9 copies /cells. Analyses performed in PB samples from seven evaluable patients at 6 months post-infusion showed a dose dependent engraftment of gene-corrected cells. Additionally, a progressive engraftment of corrected cells was confirmed in the first four treated patients after 24-36 months post-infusion. This observation was associated with progressive increases in the survival of the progenitor cells exposed to mitomycin-C, and also with progressive decreases in the proportion of PB T cells with diepoxybutane-induced chromosomal breaks. Stabilized PB cell counts have been observed in patients with higher levels of gene corrected cells, suggesting that gene therapy of non-conditioned FA patients has potential to stabilize or prevent FA-related bone marrow failure. Our results demonstrate that the procedure is safe and have facilitated the activation of a phase II clinical trial currently underway, focused on the consistent infusion of higher numbers of corrected $CD34^+$ cells and prevention of bone marrow failure.

111. Long-Term Follow-Up Study after Lentiviral Hematopoietic Stem/Progenitor Cell Gene Therapy for Wiskott-Aldrich Syndrome

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Wiskott Aldrich syndrome (WAS) is a rare primary immunodeficiency associated with thrombocytopenia, eczema, infectious and autoimmune complications, and lymphomas. Patients lacking an HLA-matched donor may benefit from an alternative therapeutic approach based on autologous gene corrected CD34+ cells. We previously reported a non-randomised, open-label, phase 1/2 clinical study,lentiviral vector based gene therapy (GT) protocol in 7 paediatric patients with severe WAS (score ≥ 3/5). One patient died 7 months after GT because of pre-existing severe infections, as reported. Two additional patients have been treated since that initial report. We here present a comprehensive long-term study on 8 patients with a follow-up from 4.2 to 8.9 years. The safety and efficacy of the approach is thoroughly investigated, with a particular focus on the correction of thrombocytopenia and autoimmunity. A stable engraftment of genetically and functionally corrected lymphoid and myeloid cells was reached in all patients with lack of severe adverse events or clonal expansion. Corrected lymphoid cells displayed a selective advantage over time with increasing vector copy number (VCN) level. T cell differentiation and function normalized, as shown by TRECs level, TCR repertoire diversity and immunological synapse organization. Although platelet counts remained below normal range, patients did not develop hemorrhagic syndrome. Following GT, platelets were found to express sub-normal levels of WAS protein (WASP) and to

partially augment their size. Platelet function studies indicated a partial correction of thiscompartment after GT, which may be sufficient to prevent occurrence of the hemorrhagic symptoms typical of WAS. The number of transduced B cells as well as WASP expression in this compartment progressively increased after GT, reaching normal absolute B cell counts. B cell function, KRECs and immunoglobulin (Ig) production improved over time and Ig replacement therapy could be discontinued in 2 patients. Regarding autoimmunity, autoantibody titers dropped, reaching normal background levels. The clinical manifestations after GT included mild persistence of a severe low-limb vasculitis prior to GT, responsive to Ig administration, a single episode of arthritis of the knee for a second patient, fully recovering after conventional anti inflammatory treatment, and severe nephrotic syndrome in a third patient, possibly related to conditioning or immune dysregulation. This long-term follow-up study provides evidence that GT is a valuable alternative treatment option for WAS patients lacking anHLA-matched donor. Overall clinical remission was observed in our patients despite severe disease scores before GT. Our data highlight that however thrombocytopenia or autoimmunity may not be fully resolved by the current approach. Further optimization of the procedure could be considered to provide additional clinical benefit especially regarding thrombocytopenia

112. Results from a Phase I/II Clinical Trial for X Linked Chronic Granulomatous Disease (CGD): Possible Impact of Inflammation on Gene Therapy Efficacy

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X-linked chronic granulomatous disease (X-CGD) is a rare, inherited, primary immunodeficiency characterized by defective microbicidal activity in phagocytes caused by mutations in the gp91phox subunit of the NADPH-oxidase. This leads to increased susceptibility to recurrent, life-threatening bacterial and fungal infections, but also to a sterile, chronic, granulomatous inflammation.Patients lacking an HLA-matched donor may benefit from an alternative therapeutic approach based on the infusion of autologous gene corrected CD34+ cells. Because of the lack of selective advantage of gene-corrected cells and and the inflammatory context, genetic engineering in CGD remains challenging. Here we report the results from a Phase I/II monocentric clinical trial based on a self-inactivating lentiviral vector, G1XCGD, driving myeloid-specific gp91phox expression. We treated 4 patients lacking an HLA-compatible donor, after a busulfan myeloablative-conditioning regimen, with a follow up of 5 months (m) to 3 years (y). We obtained satisfying levels of gene correction in the drug products (median vector copy number (VCN): 1.26, range 0.6-1.77). Except for patient P1, we optimized the transduction process with prostaglandin E2 (PGE2), which was validated on patent. Among the 4 treated patients, two showed a clinical and biological benefit after GT. In particular patient P4 (2y follow up) had a stable engraftment of gene corrected cells (neutrophils VCN=1), with a complete remission and off treatment after GT. He recovered to a normal life from a life-threatening lung aspergillosis resistant to anti-mycotic treatments; without any supportive treatment including prophylactic. The other patient presenting a clinical benefit is patient P1 with severe pulmonary disease (3.4 y follow up); he presented a partial engraftment of gene corrected cells after GT (neutrophils VCN=0.1), sufficient to provide a stable clinical status without any treatment.For P2 and P5 patients (15m and 5m follow up, respectively) we observed a decreased in the level of gene corrected cells shortly after GT infusion (neutrophils VCN<0.01) and the patients got back to previous prophylactic treatment. For these two patients, their clinical history was in both cases characterized by a long lasting inflammatory disease, resistant to multiple treatments. In order to understand further the heterogeneity in the long-term engraftment of gene-corrected cells, we undertook a transcriptomic analysis of CGD HSPC. This analysis revealed an enrichment in inflammatory and interferon signatures in CGD patients, which was reduced in the presence of PGE2. However even in the presence of PGE2 results are still heterogeneous in term of sustained engraftment of gene-corrected cells. We are exploring the inter-individual variability, that could further clarify differences in patient's clinical recovery and outcome.Altogether these results demonstrated that this GT approach for X-CGD can provide significant clinical benefit but also highlight the critical impact of the underlying disease (especially inflammation) that can hinder efficient engraftment. In this context, transcriptomic analysis of CGD HSPC could provide with new markers to help in better selecting future patients to be treated by GT trials for CGD.

Antiviral Immunotherapy

113. In Situ Analysis of Follicular Targeting Antiviral CD4-MBL CAR/CXCR5 T Cells in SIV Infected Rhesus Macaques

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Virus-specific CD8$^+$ T cells play a key role in the control of HIV and simian immunodeficiency virus (SIV) infection. However, they are unable to fully suppress viral replication. This is likely due to the fact that the majority of HIV-1 and SIV replication is concentrated within B cell follicles of secondary lymphoid tissue (SLT), where virus-specific CD8$^+$ T cells tend to be largely excluded. In addition, increased levels of follicular SIV-specific CD8$^+$ T cells are associated with decreased levels of follicular viral replication. Taken together, these findings support the development of a functional cure for HIV that targets antiviral CD8 T cells to follicles. With this aim, we engineered

T cells that co-express the B cell follicle homing molecule CXCR5 and a bispecific SIV-targeting chimeric antigen receptor (CD4-MBL CAR). We hypothesize that CD4-MBL CAR/CXCR5 (CAR/CXCR5) T cells accumulate in B cell follicles, interact with virus-infected cells, proliferate and kill virus-infected cells resulting in better control of viremia. In order to test these hypotheses, we infused CAR/CXCR5 T cells into SIV-infected rhesus macaques and evaluated the location, abundance, and persistence of these cells and viral RNA in tissues using RNAscope and immunohistochemisty. First, we treated a chronically SIV-infected rhesus macaque with the CAR/CXCR5 T cells and tissues were evaluated 2-days post-treatment. Then we did a 6-animals pilot study in which, 3 antiretroviral therapy (ART)-suppressed SIV-infected rhesus macaques were infused with autologous CAR/CXCR5 T cells on the same day that ART was stopped. Three untreated ART-suppressed SIV-infected animals were used as a control group. In the first study, at 2 days-post infusion, CAR/CXCR5 T cells successfully homed to B cell follicles of lymphoid tissues, where they showed evidence of in vivo expansion and direct interaction with virus-infected cells. In the pilot study, the three treated animals showed evidence of an early viral control with two of them maintaining long term control. The CAR/CXCR5 T cells were most abundant during the first week post-treatment and were detected in 90-100% of B cell follicles examined. The CAR/CXCR5 T cells persisted for at least 28 days in one animal. Interestingly, levels of CAR/CXCR5 T cells in B cell follicles declined faster in the treated animal that lost control compared to the two treated animals that maintained long term control. Moreover, the two controlling animals had a lower level of follicular SIV-RNA$^+$ cells and a lower percentage of follicles with free virions trapped by the follicular dendritic cells network than the treated animal that lost control, and than the untreated control animals at 28 days post-treatment. These findings show successful accumulation of the CAR/CXCR5 T cells in B cell follicles, direct interaction of CAR/CXCR5 T cells with virally infected cells in follicular areas, and association of CAR/CXCR5 T cells with a reduction in both virus-RNA$^+$ cells and free virions trapped by the follicular dendritic cell network in vivo. These results support developing CD4-MBL CAR/CXCR5 T cell immunotherapy to treat HIV infections.

114. Targeting HIV Using Anti-HIV duoCAR-T Cell Therapy

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Adoptive immunotherapy using second-generation chimeric antigen receptor (CAR) gene-modified T cells has shown remarkable success for the treatment of refractory B-cell malignancies. Past attempts using first-generation anti-HIV CAR-T cells for the treatment of HIV showed only modest efficacy in early clinical trials despite the long-term persistence of gene-modified T cells in HIV-infected individuals. Here, we hypothesized that development of HIV-1-based lentiviral vectors encoding novel anti-HIV CAR molecules targeting multiple highly conserved sites on the HIV-1 envelope (Env) glycoprotein would improve CAR potency, breadth, and resistance to HIV infection. To test this hypothesis, we engineered anti-HIV CARs with up to three Env targeting domains (mD1.22, m36.4, and C46) using a unique two-molecule CAR architecture, termed duoCAR, to facilitate effectual targeting and destruction of HIV-infected cells. To evaluate anti-HIV CAR-T cell efficacy *in vitro* and *in vivo*, we challenged duoCAR-T cells or single-molecule CAR (monoCAR) T cells with donor-matched PBMCs infected with replication-competent infectious molecular clones (IMC) of HIV encoding different *env* genes and a *Renilla* luciferase reporter (Env-IMC-LucR) to allow for a highly sensitive and quantitative assessment of HIV infection in primary cells. We show that transduction with HIV-1-based lentiviral vectors encoding multispecific anti-HIV duoCARs redirected autologous T cells to potently suppress HIV infection in primary cell cultures infected with broad strains of Env-IMC-LucR viruses (up to 99%) while simultaneously protecting CAR-T cells from HIV infection. Moreover, anti-HIV duoCAR-T cell therapy abated HIV persistence, demonstrated long-term control of HIV infection, and mitigated human CD4$^+$ T cell depletion in a humanized mouse model of bNAb-resistant HIV infection. Finally, we present new data on the optimization of the final anti-HIV duoCAR vector selected for clinical translation and demonstrate its exceptional anti-HIV efficacy *in vitro* and *in vivo*. We conclude that anti-HIV duoCAR-T cell therapy is a broadly-reactive and highly-efficacious new therapy for the treatment of HIV that warrants further clinical investigation.

115. Genome Editing the Immunoglobulin Locus with Single-Domain Antibodies to Create HIV-Specific B Cells

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Engineering B cells to express pre-determined antibody sequences could allow adaptive immune responses with characteristics that are not elicited by vaccination. Towards that goal, genome editing of the immunoglobulin (Ig) locus is especially attractive since this could result in the expression of both the secreted antibody and the surface B cell receptor (BCR) that responds to antigen. Recent studies have targeted the Ig locus to express antibodies against HIV and other viruses, for example by inserting both heavy (H) and light (L) chains at a single H chain locus in a linked cassette. However, unless editing is also performed at a separate locus to inactivate the endogenous L chain, these strategies are susceptible to the formation of unwanted H and L chain combinations. To address these challenges, we have developed a simplified engineering strategy based on the unique features of single-domain antibodies (sdAbs), that bypasses the interaction with the endogenous L chain. sdAbs occur naturally in camelids and comprise a single antigen-specific VHH domain linked to a shortened IgG constant region that is unable to pair with and functions independently of an L chain. To recapitulate this structure using site-specific genome editing, we insert a promoter-VHH cassette within an intron of the human IGHG1 gene, which encodes the constant region of IgG1. Transcription driven by the internal promoter allows splicing of the VHH domain to the remaining exons of IgG1, and endogenous splicing allows the

production of both membrane-bound and secreted forms of the engineered sdAb. A panel of spCas9 single guide (sg) RNAs, targeted to the IGHG1 intron, were screened for on- and off-target activity, and assessed for their ability to precisely insert GFP at the IGHG1 intron when combined with customized homology donor constructs. For the most effective sgRNA, homology donors were also constructed containing the B cell-specific EEK promoter, a VHH domain, and a splice donor sequence, to enable sdAb generation. VHH domains were chosen from two previously published HIV-specific sdAbs (VHH-A6 and VHH-J3) with distinct virus neutralization profiles. B cell lines (Raji and Ramos cells) were genome edited using these reagents and flow cytometry confirmed surface expression of the engineered BCRs. Site-specific insertion was also confirmed by in-out PCR and Sanger sequencing. Cells edited with VHH-A6 or VHH-J3, but not GFP, secreted sdAbs that neutralized HIV infection *in vitro*. Quantification by IC50 showed that the anti-HIV activity per molecule was similar whether sdAbs were produced by engineered B cells, or by transient transfection of 293T cells with a full-length sdAb cassette, confirming that the sdAbs produced by gene editing were fully functional. In summary, we have established a strategy for reprogramming of B cells with sdAbs by site-specific gene insertion into the IGHG1 gene. The hybrid engineered cassette generates both the membrane and secreted forms of an antibody necessary for B cell function, and secreted sdAbs exhibited robust anti-HIV activity. Studies are underway to characterize this approach in primary human B cells.

116. Lentiviral-Mediated Expression of Monoclonal Antibodies in the Lung to Protect Against Influenza

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Protein based therapeutics, such as monoclonal antibodies and enzyme replacement therapies, have had dramatic clinical impact in chronic diseases, but these therapies can be associated with high treatment burden (e.g. multiple inhalations or injections). Delivery of lentiviral and AAV vectors to organs such as the liver and muscle can generate 'protein factories' in the body. The secretion of therapeutic proteins into the circulation from a single administration of vector can result in persistent, stable levels without the fluctuating 'peak and trough' concentrations observed with multiple (often weekly) injections. We are investigating the expression of monoclonal antibodies in the lung to protect against respiratory pathogens, including broadly neutralising antibodies for long-lasting passive immunity to widely divergent and emerging strains of influenza. Our preferred vector is based on a third-generation, self-inactivating simian immunodeficiency virus (SIV), which has been pseudotyped with the F and HN proteins from Sendai virus (rSIV.F/HN) for efficient cell targeting in the lungs (Thorax 2017;72:137-147). We constructed rSIV.F/HN vectors using the hCEF promoter to express luciferase and EGFP reporter genes, as well as vectors expressing the broadly neutralizing antibody T1-3B, which was isolated from vaccinated volunteers (V_H1-69 germline family), and is able to cross-react with multiple group 1 influenza A strains. Intranasal delivery of rSIV.F/HN hCEF Lux (5E7 Transducing Units (TU)) to BALB/c mice (n=4) resulted in robust luciferase expression in both the nose (~1E6 p/s/cm^2/sr) and lung (~5E5 p/s/cm^2/sr) that was maintained for at least 150 days post-delivery ($p>0.15$). Visualisation of lung sections from mice dosed with rSIV.F/HN expressing EGFP (1E8 TU), showed fluorescence in multiple cell types including airway epithelial and parenchymal cells, as confirmed with co-localisation of β-tubulin (ciliated) or Surfactant protein C (Alveolar Type II) cell markers with native EGFP. Intranasal delivery of rSIV.F/HN expressing T1-3B (5E7 TU) resulted in expression of antibody in both the serum (~0.1 μg/mL) and Bronchoalveolar Lavage Fluid (BALF) (~0.1 μg/mL) that was significantly higher than naive or irrelevant vector treatment ($p<0.05$ for all). Using the murine influenza challenge model, we showed that intranasal delivery of 1E8 and 2.7E8 TU of the vector could protect mice against the highly lethal influenza strain A/PR/8 H1N1 (10 LD50), resulting in 83% ($p<0.01$) and 100% survival ($p<0.01$), respectively. We have also shown protection in mice against the 2009 pandemic H1N1 virus (A/California/7/2009) (10 LD50), in which 1E7 TU and 1E8 TU of the vector resulted in 67% ($p<0.001$) and 100% ($p<0.001$) survival, respectively. We speculate that during the next human influenza pandemic, prophylaxis provided by lung gene transfer may be more cost-effective and time-responsive than traditional, vaccines or parenteral administration of therapeutic antibody, offering an important first line of defence for essential health workers.

117. Multi-Virus Specific T Cells Dominate and Persist in the Patient Immune Repertoire Post Allogeneic Hematopoietic Stem Cell Transplant

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Viral infections remain a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplant (HSCT). The risk of viral reactivation is greatest in the first three months after transplant before the reconstitution of anti-viral T cell responses transferred within the graft. Adoptive cell transfer (ACT) of *ex vivo* generated donor multi-virus-specific T cell products (VST) has been established as an effective treatment of reactivating cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (AdV) post-HSCT and it is possible that early prophylactic infusion of VST cells after transplant might prevent reactivations. However, it is unclear if infusions of VST enhance the immune competence of donor T cells already transferred at the time of transplant. To examine the contribution of adoptively transferred VSTs to the overall immune reconstitution post HSCT we analyzed recovery of viral specific immunity in patients receiving multi-virus specific T cell product (MVST) early post graft (n=6) in a Phase 1 clinical trial (NIH 14-H-0182) with comparable transplant recipients not receiving MVST infusions as controls (n=4). Clinical grade donor-derived MVSTs were manufactured by stimulating donor derived peripheral blood lymphocytes (PBLs) with dendritic cells (DCs) pulsed with 15mer overlapping peptide libraries containing CMV (pp65 and IE1), EBV (BZLF1 and EBNA1), BK virus (VP1 and Large T antigen), and AdV (Ad5) used as immunogens. At the end of 14 day culture, MVST products were tested for release criteria and cryopreserved. MVSTs

were administered to HLA-matched sibling HSCT recipients early post-transplant (target day +14). Comparable donor derived MVST products were manufactured for control patients, but not administered. Contribution of the infused MVST cells to the immune repertoire post-HSCT was examined using high throughput T cell receptor CDR3 sequencing at D+60, 100 and 180 and compared with the CDR3 repertoires in peripheral blood of untreated (control) HSCT recipients. In MVST patients, 3.50-8.40% of unique TCR sequences were derived from the MVST product at day 100, but accounted for 22.5-76.2% of the overall repertoire, suggesting robust contribution to the post-HSCT repertoire. At six months post-transplant, 5/6 MVST patient and 1/4 control patient peripheral cell repertoires contained greater than 50% CDR3 sequences originally present in ex vivo generated MVST cell product, however there was no correlation between dominant clones persisting post-ACT and the clones most frequently found in the infused MVSTs. These data demonstrate that adoptive transfer of MVST cell products rapidly and robustly reconstituted anti-viral immunity after HSCT compared to transplant recipients not receiving MVST.

118. Protection Against Crimean-Congo Hemorrhagic Fever Virus in a Cynomolgus Macaque Disease Model by Genetic Vaccination

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There is currently no specific prophylaxis or vaccine against Crimean-Congo hemorrhagic fever virus (CCHFV). Crimean-Congo hemorrhagic fever (CCHF) is a severe hemorrhagic fever transmitted in endemic areas by Hyalomma ticks through handling of infected livestock or care of infected patients. We developed a DNA-based vaccine delivered by in vivo electroporation (EP) and evaluated protection in an CCHFV-mediated disease in a non-human primate disease model. Cynomolgus macaques were vaccinated thrice with a DNA-based vaccine containing two plasmids encoding the glycoprotein precursor (GPC) and the nucleoprotein (NP) of CCHFV. Following two to three vaccination we could detect potent antibody and T-cell responses. All macaques were challenged with CCHFV three weeks after the last vaccination. All control vaccinated animals developed viremia, high tissue viral loads and CCHF-induced disease, whereas the NP + GPC vaccinated animals showed significant protection. In conclusion, this is the first evidence of a vaccine that can protect against CCHFV-induced disease in a non-human primate model. This supports clinical development of the vaccine to protect groups at risk for contracting the infection.This work was supported, in part, by the Intramural Research Program of the NIH/NIAID.

119. Superior Hematopoietic Stem Cell based CAR-T Cell Therapy for HIV Infection

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p.p1 {margin: 0.0px 0.0px 0.0px 0.0px; font: 12.0px Arial} Due to the durability and persistence of reservoirs of HIV infected cells, combined antiretroviral therapy (ART) is insufficient in eradicating HIV-1 from the body. Achieving HIV-1 cure or remission without ART treatment will require the enhancement and persistence of effective antiviral immune responses. Chimeric Antigen Receptor (CAR) T-cells have emerged as a powerful immunotherapy for various forms of cancer and show promise in treating HIV-1 infection. Previously, we showed successful long-term engraftment and production of anti-HIV CAR T cells with a CD4-based CAR (CD4CAR) in modified hematopoietic stem cells (HSCs) in vivo. Here we report development and *in vivo* testing of novel second generation CD4-based CARs against HIV infection. We found that a modified, truncated CD4CAR does not mediate HIV infection but maintains similar CTL activity as compared to full length CAR. In addition, we found that the novel truncated CAR allows better CAR-T cell differentiation from gene modified hematopoetic stem cells as compared to full length CD4CAR. Interestingly, the co-stimulatory molecule 4-1BB, but not CD28, allows successful hematopoietic differentiation and improved anti-viral function of CAR T cells from CAR modified HSCs. In contrast to CD4-based CARs, we found that broad neutralizing antibody based CARs that contain IgG4-Fc linker, failed to engraftment in the humanized mice bone marrow. Lastly, as compared to peripheral CAR T cell therapy, the HSC based CAR T cell therapy shows drastically superior long- term persistence and anti-viral responses. Our data suggests that HSC-based CAR therapy stands as a feasible, long lived, and potentially more efficacious therapy for treating HIV infection.

Hematopoietic Cell Therapies

120. MGTA-456, A Cell Therapy Utilizing an Aryl Hydrocarbon Receptor Antagonist (AHRa) Culture, Promotes Expansion of $CD34^+CD90^+$Cord Blood (CB) Hematopoietic Stem Cells (HSC), Resulting in Rapid Hematopoietic Recovery, Uniform Engraftment and Better HLA Matched Grafts for Larger Recipients

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Background. $CD34^+CD90^+$ expression identifies a rare subpopulation of hematopoietic cells enriched for HSC capable of long-term

engraftment. Expansion of $CD34^+CD90^+$ cells would be desirable in settings where limiting numbers of HSC are available, e.g. after gene modification or in the context of allogeneic CB transplant. It has previously been shown that SCID-repopulating cells almost exclusively reside in the $CD34^+CD90^+$ population of MGTA-456, the cell therapy product composed of expanded CB CD34+ cells, cultured in SCF, Flt-3L, IL6 and TPO in the presence of an AHRa and its companion cryopreserved $CD34^{neg}$ fraction. Based on encouraging expansion characteristics and transplant outcomes in the first 36 recipients receiving freshly expanded MGTA-456, the aim of this study (NCT03674411) was to determine a) the impact of lowering the cell dose threshold of the CB unit starting material from the standard 3.0 x 10^7 to 1.0 x 10^7 total nucleated cells/kg to improve HLA match, and b) the safety and efficacy of fully cryopreserved MGTA-456. *Methods*: 17 patients aged 2-47 yrs (median weight 61 kg; r, 12-159) with high-risk hematologic malignancy were enrolled with 12 transplanted on-study to date. Conditioning consisted of cyclophosphamide 120 mg/kg, fludarabine 75 mg/m^2 and total body irradiation 1320 cGy in 10 patients with a busulfan-based regimen in two children aged 2 yrs. GVHD prophylaxis was cyclosporine and mycophenolate mofetil, and G-CSF was started on d+1 after MGTA-456 infusion until neutrophils exceeded 2500/uL for 3d. *Results*: Per protocol, all patients received >10 x10^6 nucleated cells/kg in the expanded product. Median CD34+ cell fold-expansion was 421-fold (r, 219-1476). Patients received a median CD34+ and CD34+CD90+ cell dose of 2.6 x 10^7/kg (r, 0.9-13.5 x 10^7/kg) and 1.3 x 10^6/kg (r, 0.5-7.0 x 10^6/kg), respectively. Excluding 1 patient recently transplanted (d+10), neutrophil recovery occurred in 100% of patients at a median of 13 d (r, 8-31) vs 25 d in prior recipients of unmodified CB ($p<0.01$). Similarly, platelet recovery and RBC transfusion independence occurred in 100% at a median of 36 d (r, 30-56) and 48 d (r, 13-196), respectively. Time to neutrophil and platelet recovery strongly correlated with CD34+CD90+ dose (Figure). Incidences of grades 2-4 and 3-4 GVHD were 20% (CI, 0-45%) and 0%, respectively, with no extensive chronic GVHD or non-relapse mortality. Of note, lowering the cell dose threshold from 3.0 to 1.0 x 10^7 TNC/kg permitted the selection of better HLA matched CB units in 6 of 9 patients weighing >60kg. With a median f/u of 7.7 mos (r, 0.3-11.6), 11/12 are alive with 1 death from relapse.

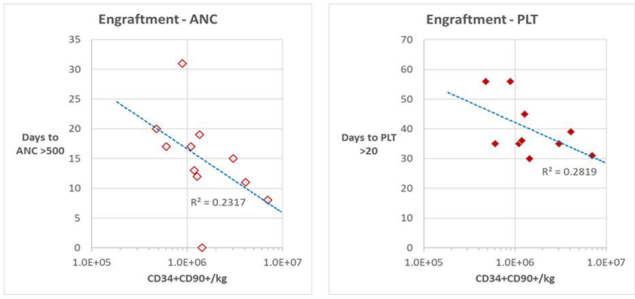

Figure: Correlation between CD34+CD90+ cells in the graft and time to neutrophil (ANC) and platelet recovery (PLT).

Conclusion: MGTA-456 markedly expands the number of CD34+CD90+ cells leading to rapid and sustained hematopoietic recovery, complete engraftment in all subjects, and improved HLA match in most patients >60 kg. As CB units with lower cell doses can now be considered, the opportunity of finding a better HLA matched unit for adolescents and adults may account for the low incidence of GVHD and TRM observed thus far in recipients of MGTA-456.

121. Exchange of Alveolar Macrophages Restores Pulmonary Immunity by Niche Specific Adaption of *Ex Vivo* Generated Macrophages

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During the last years macrophages (MΦ) have become an increasingly attractive cell type for cell therapy due to the discovery that many tissue resident MΦ (I) are of embryonic origin, (II) are long lived and (III) have the ability to self-renew. Given the importance of MΦ in various disease entities, we aimed to develop a MΦ transfer strategy to harness their therapeutic potential and replace dysfunctional MΦ populations by swapping the pulmonary MΦ pool with *ex vivo* generated MΦ. To achieve this we employed clodronate liposomes to deplete endogenous alveolar MΦ (AMs) to open up the lung niche. After intra-pulmonary clodronate administration we observed efficient depletion of endogenous $CD45^+CD11c^+SiglecF^+$ AMs after 5-7 days, whereas MΦ numbers in other organs e.g. the spleen remained unchanged. AMs started to repopulate the lung after 14 days. Using day 7 post-depletion as an optimal time point, we transferred *ex vivo* generated bone marrow (BM) derived MΦ directly into the lungs of the depleted animals. This led to a robust engraftment between 10-15 % of total $CD45^+$ cells in the bronchoalveolar lavage fluid. Analysis of engrafted cells revealed a stepwise adaption of the MΦ toward the lung environment. 14 days after the transfer, *ex vivo* generated MΦ had adapted a surface marker profile similar to AMs ($CD45^+CD11c^+SiglecF^+CD11b^-$). Similarly, we performed single cell RNA sequencing during the adaption process at days 3, 7 and 14 after the pulmonary transfer and compared these cells to both the in-going BM-derived MΦ and wild type AMs. We found that the transferred MΦ clustered away from the BM-derived MΦ and showed a stepwise approximation towards AMs. Moreover we found a gradual downregulation of BM MΦ defining genes and an upregulation of AM associated genes over the observed time span. Interestingly in both the AM and the MΦ 14 days post-transfer we found an exclusive subset of cells that expressed proliferation markers such as Ki67, indicating first adaption, followed by local proliferations. As a therapeutic application of the adoptive MΦ transfer, we used $Ifngr1^{-/-}$ mice as a mouse model of Mendelian Susceptibility to Mycobacterial Disease (MSMD), a rare congenital disease that is characterized by dysfunctional MΦ due to mutations in the IFN-γ-IL-12/23-loop and a high susceptibility to mycobacterial infection. Swapping the dysfunctional AMs with healthy MΦ in $Ifngr1^{-/-}$ mice led to engraftment levels of around 10 %, similar as in the wild type

context. Of note, this therapeutic intervention protected the mice from subsequent intra-pulmonary infection with Bacillus Calmette-Guérin and resulted in an average 3-fold lower number of colony forming units in the spleen. Of note, 14 days after infection the cells were still present in the lung of the animals and showed a typical AM phenotype. In summary, we here show that MΦ are a highly attractive cell type for novel cell therapy approaches. We developed an adoptive pulmonary transfer scheme for *ex vivo* generated MΦ that results in the engraftment and adaption of the cells to the lung. Transferring this scheme to a disease model led to clinical benefit and could be transferred also to other diseases and organs.

122. Engineered B Cells Undergo Antigen Induced Activation to Allow Memory Retention, Class Switch Recombination and Clonal Selection in Mice

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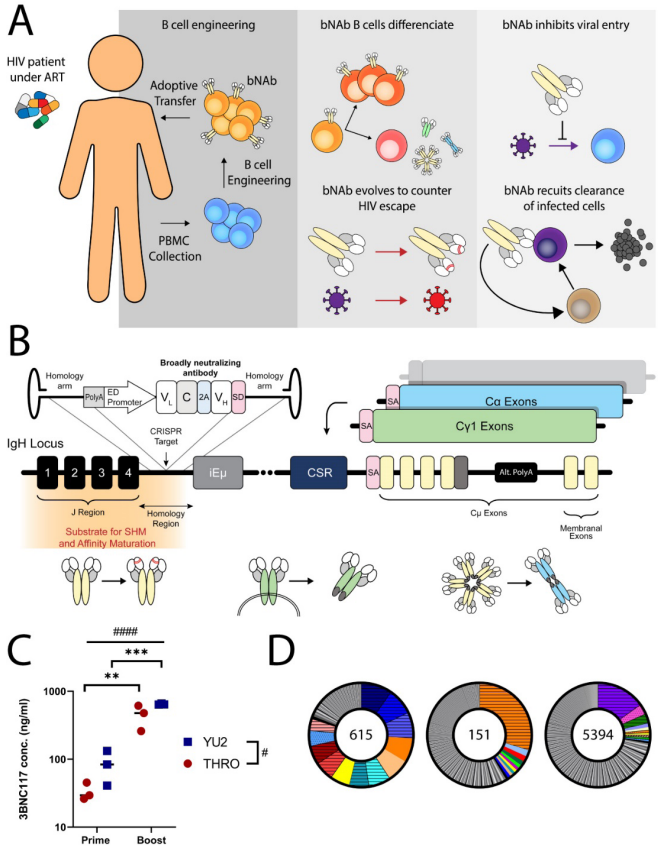
HIV viremia can be controlled by chronic Antiretroviral Therapy (ART). However, treatment tolerability, polypharmacy and adherence remain a challenge. Combination therapies of broadly neutralizing antibodies (bNAbs) can suppress viremia for longer periods of time. However, chronic injections and higher cost of administration limit clinical utility. bNAbs may be constitutively expressed by various tissues following AAV transduction, but anti-drug antibodies may develop, the virus may escape and the expression of the antibody may be poorly regulated. In contrast, bNAb genes integrated at the Immunoglobulin Heavy chain (IgH) locus of B cells may allow for a potent, long-term and adaptive response to the frequent mutations of the virus. Indeed, in immunocompetent mice, adoptive transfer of B cells engineered to express HIV-bNAbs facilitated the production of HIV-neutralizing antibody titers. Integration of single-chain anti-RSV antibodies into the IgH locus further allowed protection from lethal infection in immunocompromised mice. However, in all previous *in vivo* studies, the engineered B cells allowed no immunological memory and demonstrated no clonal selection possibly due to the exhaustive *ex-vivo* manipulations. These limitations may significantly hinder clinical application of the technology against the highly diverse and rapidly evolving HIV.To overcome anergy in engineered B cells, we combine *ex-vivo* activation and expansion of B cells using Toll-like receptors and *in-vivo* immunizations. In particular, we use CRISPR/Cas9 and AAV as donors to introduce a cassette coding for the anti-HIV bNAb 3BNC117 into the IgH locus. Using this protocol, we reached up to 74% engineering efficiency in primary murine splenic B cells, and we have developed a similar protocol for human blood B cells. Following adoptive transfer of the cells and immunization of immunocompetent mice with the HIV antigen gp120, engineered B cells home to Germinal Centers (GCs). Higher rates of engineered B cells were found in GCs, compared to the rates of non-engineered B cells, a strong indication of antigen induced activation. Furthermore, the engineered B cell response monopolized the gp120 specific response, demonstrating immunodominance of the engineered cells over the low-affinity, rarely neutralizing, endogenous response. The immunizations allowed for differentiation of the engineered B cells into memory and plasma cells. Indeed, serum bNAb concentrations were increased following boost immunizations, reaching 1ug/ml, concentrations known to allow broad HIV neutralization. Multiple isotypes off 3BNC117 were detected, signifying Class Switch Recombination (CSR), a key factor in allowing both systemic and mucosal protection. Finally, antibody genes of engineered B cells in GCs undergo Somatic Hypermutation (SHM) and clonal selection, which may be important in counteracting HIV escape. In conclusion, uniquely, our method enables *in-vivo* antigen-induced activation of engineered B cells, followed by memory retention, CSR, SHM and clonal expansion. In the human setting, this method may constitute a single-shot alternative to chronic ART therapy.



(A) Premise of the proposed therapy. (B) Targeting scheme. (C) bNAb concentrations, as measured by ELISA, following immunizations with the gp120 antigen coming from the HIV strains YU2.DG and THRO4156.18. (D) Pie charts representing clonal distributions of 3BNC117 mutant alleles from mice immunized with gp120 YU2.DG. Top 10 clones are colored. Numbers represent the number of different clones.

123. MGTA-145, in Combination with Plerixafor, Rapidly Mobilizes Large Numbers of HSCs in Humans That Can Be Gene Edited with CRISPR/Cas9 and Mediate Superior Engraftment to Standard-of-Care

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Background. An optimal dose of hematopoietic stem cells (HSCs) is critical for successful cell/gene therapy outcomes. This can be achieved by a rapid, robust, and reliable HSC mobilization regimen. While G-CSF and/or plerixafor are used to mobilize HSCs for gene therapy applications, cell dose can be suboptimal, and G-CSF is contraindicated in some indications, like sickle cell disease. MGTA-145, a CXCR2 agonist, when used in combination with plerixafor, a CXCR4 inhibitor, rapidly mobilizes HSCs in mice and nonhuman primates (Hoggatt et al Cell 2018; Goncalves et al Blood 2018). Here, we show for the first time that a single day mobilization with MGTA-145 and plerixafor leads to higher numbers of NSG-engrafting human HSCs than standard-of-care and that these cells can be gene-modified. ***Results.*** In mice, a single dose of MGTA-145/plerixafor mobilized an equivalent number of Lin-Sca1+cKit+CD150+CD48- long-term HSCs compared to a multi-day dosing regimen of G-CSF, but led to >10-fold higher engraftment at 20 weeks after transplant at limit dilution ($p<0.001$, n=5-8 mice/dose). In secondary transplants, MGTA-145/plerixafor cells showed >50-fold higher engraftment compared to either plerixafor, G-CSF or G-CSF/plerixafor at 20 weeks, suggesting that MGTA-145/plerixafor rapidly mobilizes LT-HSCs with superior engraftment potential. In a Phase 1 healthy-volunteer study (n=107 donors), a median of 40 CD34+ cells/μL was achieved at the optimal dose of MGTA-145/plerixafor (n=12 donors). 11 of 12 (92%) donors with MGTA-145/plerixafor mobilized >20 CD34+ cells/μL with single day dosing compared to 8 of 14 (57%) donors mobilized with plerixafor alone. MGTA-145 was well tolerated as monotherapy and with plerixafor. 4 subjects were treated with a single dose of MGTA-145/plerixafor and apheresed on the same day. A median of $4.3x10^6$ CD34+ cells/kg were obtained and 33% of these cells were CD90+CD45RA-, a cell type enriched for HSCs, compared to 10% with G-CSF. Mechanistically, MGTA-145 engaged CXCR2 on neutrophils and led to an increased plasma concentration of the protease, MMP-9, to mobilize CD34+ cells. Markers of neutrophil activation, which is associated with vaso-occlusion following administration of G-CSF to sickle cell disease patients (Falanga et al. Blood 1999) were not significantly elevated (<2-fold vs baseline, n=12-14 donors). To assess engraftment capabilities of human cells, we transplanted MGTA-145/plerixafor or G-CSF CD34+ cells at limit dilution (n=2-3 donors) into NSG mice. MGTA-145/plerixafor CD34+ cells showed a >5-fold increase in long-term, multilineage engraftment (SCID-repopulating cell number) compared to G-CSF mobilized CD34+ cells ($p<0.001$, n=8 mice/dose). These data demonstrate that MGTA-145/plerixafor CD34+ cells have superior engraftment capabilities compared to cells isolated by conventional mobilization regimens. To determine whether these cells could be gene-modified, CD34+ cells from MGTA-145/plerixafor mobilized healthy donors (n=2 donors) were edited with CRISPR/Cas9 targeting beta-2-microglobulin. 90% editing was achieved in CD34+ and CD34+CD90+CD45RA- cells and these cells engrafted in NSG mice (n=8 mice per group), demonstrating that MGTA-145/plerixafor mobilized blood cells can be efficiently gene-modified for therapeutic applications. ***Conclusions***. These data demonstrate that MGTA-145/plerixafor is a rapid, reliable, efficient, and G-CSF free method to obtain high numbers of wild-type and gene-modified HSCs with robust and durable engraftment potential. This single-day mobilization/collection regimen could therefore improve cell collection protocols and autologous gene therapy outcomes for a variety of diseases.

124. "Cerberus" T Cells: A Single Glucocorticoid-Resistant T Cell Product to Simultaneously Target Multiple Pathogens in Immunocompromised Patients

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Adoptive immunotherapy (AI) with pathogen-specific T cells is a promising alternative to pharmacotherapy for the treatment of opportunistic infections after allogeneic hematopoietic stem cell transplantation (allo-HSCT) or solid organ transplantation. However, clinical implementation of AI is limited to patients receiving either low dose or no steroids, a prerequisite for optimal T-cell function, thus practically excluding the most susceptible to infections patients from the benefits of AI. We developed a CRISPR/Cas9 system to genetically disrupt the glucocorticoid receptor (GR) in T-lymphocytes and confer resistance to steroids. Ten guide RNAs (gRNAs) were prepared to target genomic sequences corresponding to various domains of the GR (a transcription start site, and exons 2, 3, 4 and 5) and delivered separately into the T2 lymphoblastic cell line by lentiviral vectors. Transduced T2 were subsequently incubated in the presence or absence of dexamethasone (DEX). Cells transduced with an "empty" viral vector expressing Cas9 but no gRNA were used as negative control. T2 cells edited with 7/10 single gRNAs, presented normal proliferation on DEX treatment as contrasted to their untreated counterparts and the empty vector-transduced cells, suggesting functional DEX-resistance. The on-target GR inactivation in T2 cells was confirmed by western blotting and the T7 assay. On the basis of high GR disruption efficiency and low off-target activity, the optimal gRNA among those gRNAs that proved functional, was selected to disrupt GR by Cas9/gRNA ribonucleoprotein (RNP) electroporation in pentavalent pathogen-specific T cells targeting 4 viruses [adenovirus, cytomegalovirus (CMV), Epstein Barr virus (EBV) and BK virus] and the fungus Aspergillus fumigatus. The generated steroid-resistant pathogen-specific T-cells were called "Cerberus" T cells (Cb-STs)

from the monstrous three-headed dog of Greek mythology, due to their triple potential of specificity against viruses, specificity against fungi and resistance to glucocorticoids. In ongoing studies, Cb-STs are assessed as regards their phenotype, functionality in vitro, Dex-resistance, specificity of "on target" or/and "off target" cleavage and safety in a xeno-GvHD mouse model. Overall, we provide a series of gRNAs for CRISPR/Cas9-disruption of the GR using non-viral genome engineering to generate steroid-resistant, pentavalent pathogen-specific T cells. We anticipate that like "Cerberus" who guarded the gates of the underworld, Cb-STs will serve as a powerful guard system against multiple pathogens in transplanted patients, even under the unfavorable condition of intense immunosuppression.

125. Restored Macrophage Function Ameliorates Disease Pathophysiology in a Mouse Model for Very Early Onset Inflammatory Bowel Disease (VEO-IBD)

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Under physiological conditions, the intestinal immune system governs the tight balance between pro-inflammatory responses required for control of pathogens and anti-inflammatory responses for the prevention of unintended injuries to the intestinal mucosa. Key players within this process are intestinal macrophages, which, in crosstalk with regulatory T cells and other immune cells, critically shape gut immune responses. However, if this finely balanced network is disturbed, excessive inflammatory responses can lead to inflammatory bowel disease (IBD). IL-10 signaling is a major regulator of immune tolerance in the intestinal mucosa, and mutations in IL-10 or the IL10-receptor lead to very early onset (VEO) IBD, a life-threatening disease which is often unresponsive to conventional medication. Recent studies demonstrated that defective IL-10 receptor signaling on innate immune cells is a key driver of severe intestinal inflammation in VEO-IBD. These data suggest that the correction of the macrophage defect alone may have therapeutic implications. Here we evaluated the potential of hematopoietic stem cell gene therapy in an *IL10rb*$^{-/-}$ VEO-IBD mouse model and demonstrate that the therapeutic responses closely correlate with gene correction in intestinal macrophages, but not with lymphoid cell gene marking. To further explore the role of macrophages in VEO-IBD, we investigated the therapeutic efficacy of a local intraperitoneal macrophage administration. A 6-week therapy employing a combination of (i) clodronate depletion of endogenous hyperinflammatory macrophages followed by (ii) administration of wild-type macrophages significantly reduced colitis as shown by decreased frequency of inflammatory macrophages in the lamina propria (24.6±4.9 vs 15.4±3.9, p=0.003) as well as improved histology scores of colon sections (6.2±2.3 vs 3.25±1.5, p=0.019) in the *IL10rb*$^{-/-}$ VEO-IBD mouse model (both mean±SD, n=8). Transplant derived, wild-type macrophages were detected in the lamina propria and the intraperitoneal cavity at the end of the observation period, and macrophages re-isolated from the peritoneal cavity of these animals showed a strong reduction of proinflammatory cytokine secretion after LPS stimulation compared to non-treated animals. Moreover, we observed a normalization of peripheral blood values in animals treated with the combination therapy. The increased myelopoiesis commonly associated with inflammation in *Il10rb*$^{-/-}$ IBD mice was significantly reduced in animals only when treated with a combination therapy of macrophages and clodronate administration (p=0.0024 for T-cells and p=0.0117 for granulocytes), indicating a systemic anti-inflammatory effect after macrophage transfer. Interestingly, these local and systemic therapeutic effects could not be seen in control groups receiving clodronate liposomes or macrophages as a monotherapy. In summary, we show that a restoring macrophage function by adoptive wildtype macrophage transfer combined with depletion of endogenous hyperinflammatory macrophages can ameliorate colitis in an *Il10rb*$^{-/-}$ mouse model for VEO IBD and potentially represent a novel cell-based treatment approach for VEO-IBD patients.

126. Natural Killer Cells Generated from Human Induced Pluripotent Stem Cells Under Defined Conditions, Engineered for Immunotherapy of Solid Tumors

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Although many of the molecular mechanisms underpinning cancer have been characterized and more aggressive treatment options are currently available, cancer still remains a major cause of mortality throughout the world. More specifically, targeted immunotherapy with engineered natural killer (NK) cells has recently been proven to be a promising and efficient approach for the treatment of solid tumors and blood cancers, alike, with fewer side effects and a potentially superior clinical outcome than traditional chemotherapies. However, NK cells are difficult to source from patients, exhibit a low ex vivo expansion, and are often dysfunctional when harvested from cancer patients, altogether slowing the development of immunotherapies for solid tumors. Therefore, an alternative source of NK cells, derived from induced pluripotent stem cells (iPSCs) can be harnessed to overcome the challenges of existing NK cell-based immunotherapy treatments. We have successfully generated "off-the-shelf" NK cells, differentiated from a fully authenticated iPS cell source, using a streamlined, and efficient feeder- and serum-free cell culture protocol. Our streamlined protocol consists of a two stage differentiation process, wherein CD34^{+} hematopoietic progenitors are generated from embryoid bodies over the first 11 days using serum-free APEL2 medium and initiating cytokines SCF, BMP4, and VEGF. Following embryoid body formation, progenitor cells are differentiated into NK cells over a subsequent 28 days by the time-specific induction of cytokines including FLT-3L, SCF, IL-3, IL-7, and IL-15. These cytokines are added to mimic the in vivo development of NK cells in the bone marrow. iPS-derived NK cells were characterized and determined to be phenotypically mature expressing activating - NKp30, NKp44, NKp46, NKG2D, and DNAM-1 - and inhibitory - NKG2A/CD94, KIRs and CD19 - receptors present

on mature peripheral blood-derived NK cells, as well as CD16 which can mediate antibody-dependent cellular cytotoxicity (ADCC), as measured via flow cytometry. Furthermore, these iPS-derived NK cells were functionally active against a number of solid tumor targets (A549 lung carcinoma cells, GBM43 glioblastoma cells, and PC3 prostate cancer carcinoma cells) in terms of cancer lysis as well as cytokine (IFN-γ) secretion and degranulation potential, performing similarly to or better than peripheral blood derived NK cells in all cases. We have also shown that iPSC-NK cells express TIGIT, an inhibitory receptor which signals by binding to CD155 on cancer cells to inhibit NK cell anti-tumor immunity and are responsive to an anti-TIGIT and anti-CD73 co-blockade against primary models of glioblastoma (GBM43), further validating these cells as a platform for NK cell immunotherapy. We have shown that phenotypically- and functionally-mature NK cells can be efficiently generated from iPSCs using our novel and robust protocol. These cells are equivalent or superior to peripheral blood-derived NK cells in response to a variety of tumor cell types. We are also exploring the genetic engineering of these iPSC-NK cells to co-target TIGIT and CD73-induced immunosuppression in solid tumors using novel, responsive genetic constructs in our lab. These cells represent a safe, allogeneic platform that can be easily engineered, allowing improved cancer targeting over traditional chimeric antigen receptor-NK therapies.

Vector and Cell Engineering, Production or Manufacturing II

127. Enhanced Lentiviral Vector Transduction of CD34+ Hematopoietic Stem and Progenitor Cells by Targeting Anti-Viral Restriction Factors

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Ex vivo lentiviral gene transfer into CD34⁺ hematopoietic stem and progenitor cells (HSPCs) has demonstrated remarkable clinical success in gene therapy for monogenic hematopoietic disorders. However, the elevated cost of clinical grade lentiviral vectors (LVs) and the limited number of clinical grade LV manufacturers remain two of the most critical limitations for broader application of this therapeutic approach. Innovative approaches to reducing the amount of LV needed for the manufacture of HSPC gene therapy products are therefore a priority. This can be achieved using transduction enhancers, molecules of different chemical nature that enhance vector transduction and increase gene delivery. While most of these transduction enhancers facilitate attachment of the vectors to the cells, others target intracellular pathways that are relevant for the lentiviral vector life cycle. HSPC have been shown to express high levels of antiviral proteins that can restrict viral infection. We recently reported that the antiviral protein IFITM3 reduces LV infectivity and therefore gene delivery in HSPC. IFITM3 restriction can be overcome using naturally occurring cyclosporine molecules to enhance transduction. Here we have synthesized novel cyclosporine-like molecules with enhanced potency against IFITM3 in cell line models. The most potent cyclosporine-like molecules were tested for their ability to enhance LV transduction of CD34+ HSCPs. The levels of transduction were determined both by measuring the expression of the GFP reporter protein and by quantifying the number of integrated proviral copies by qPCR. Significant enhancement at different levels (up to 10-fold) was observed in the presence of these compounds without any effect in cell viability. Further enhancement was achieved when these compounds were combined with a combination of entry transduction enhancers that is currently used in our gene therapy clinical trials (LentiBOOST™ and Protamine sulphate).

128. Establishing cGMP Manufacturing of CRISPR/Cas9-Edited Human CAR T Cells

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Chimeric antigen receptors (CARs) are synthetic receptors that redirect and reprogram T cells to mediate tumor rejection. CD19 targeted CAR T cells have showed remarkable efficacy for chemorefractory/relapsed B cell malignancies. Recombinant retroviral vectors mediated- CAR gene transfer is currently the standard method to generate cGMP grade CAR T cells. However, CAR gene expression is highly variable, owing to position effects and vector incorporation variations. CAR T cells engineered in this manner are capable of tumor eradication, but are prone to tonic signaling and accelerated exhaustion. We have developed a novel genetic engineering strategy to insert CAR genes into a precise genomic location in human peripheral blood T cells. T cells are electroporated with Cas9 mRNA or Cas9 protein and a guide RNA, followed by transduction with a recombinant Adeno-Associated Virus encoding the CAR sequence to facilitate the insertion of the CAR gene upstream of the constant region of TCR alpha chain. This results in the endogenous TCR promoter (TRAC) controlled CAR expression and abrogation of TCR surface expression. This strategy not only allows uniform CAR expression, but also delays T-cell differentiation and exhaustion, leading to enhanced T cell function and anti-tumor efficacy. The edited cells vastly outperformes retrovirally modified CAR T cells in a pre-B ALL NALM6 mouse model. The targeting of CARs to the TCR locus also provides a safer therapeutic T cell by minimizing the risks of insertional oncogenesis, and TCR-induced autoimmunity and alloreactivity (thus spanning both autologous and allogeneic T cell applications). In addition, we have incorporated in the CAR a mutant CD3z chain encoding a single immunoreceptor tyrosine-based activation motif that improves the balance between effector and memory T cells composition.

We are in the process of translating this novel approach into the clinical setting by establishing cGMP conditions and protocols for the manufacturing of TRAC-CAR T cells. Using the 4D-LV electroporator, we have demonstrated that we can knockout the TCR at large scale (100 E06 CD3+ T cells) with high efficiency (70-80%) as that obtained at small scale. We have evaluated AAV6 to deliver the CAR transgene in the TCR locus. Data will be presented on optimizing the manufacturing of the TRAC-CAR T cells and on evaluating their anti-tumor efficacy *in vivo*.

129. Abstract Withdrawn

130. Validation of cGMP-Generation of Genetically Engineered T Cells on the CliniMACS Prodigy™

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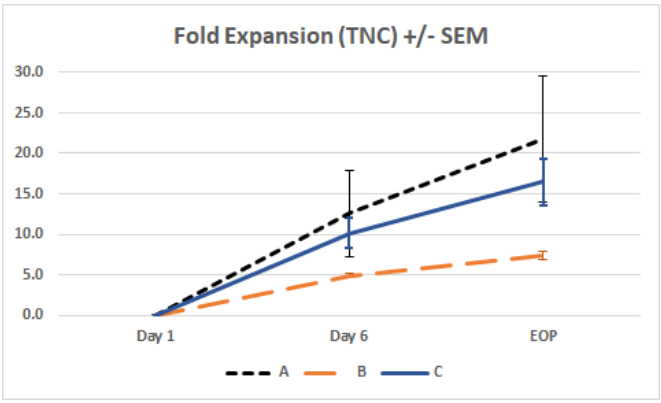
Genetically modified T cells are being developed for different cancers and individual patients using a variety of manufacturing approaches. These include chimeric antigen receptor (CAR) and engineered T-cell receptor (eTCR) T cells. We present validation of 3 engineered T-cell procedures using CliniMACS Prodigy™ as a single manufacturing platform. Two CAR processes used the standard T-cell Transduction (TCT) program; 1 eTCR process used a customized application (CAP) program. Starting materials were aphereses from healthy donors. Fourteen validation runs are summarized (**Table**). Four CAR runs used the TCT program for 7 days (**A**); 6 CAR runs used the TCT program for 8 days (**B**), and 4 eTCR runs used the CAP program for 8 days (**C**). Initial aphereses contained 56.2% T cells (CD45+/CD3+; range, 31-71). **A** and **B** started processing with 3 x 10^9 T cells for CD4+ and CD8+ selection using magnetic beads conjugated to anti-CD4 and CD8 antibodies. **C** started with 1.5 x 10^9 TNC (total nucleated cells) for proprietary T-cell subset selection. Target cell purity of selected products was 93.5% (range, 87-99). Target cell recovery was 51.0% (range, 24-75). Selected target fractions were activated with TransAct™ beads, transduced, and expanded in TexMACS™ medium containing human AB serum supplemented with cytokines. Transduction with a different CAR or eTCR-encoding lentiviral vector for each process occurred 18 to 24 hours after activation - using both research and clinical grade vector lots. Cells were harvested after 6 or 7 days of expansion. Overall end-of-process (EOP) T-cell purity was 97.1% (range, 91-100). Expansion of T cells was impacted more by viral vector than duration of culture (**Figure**). Transduction efficiency (% CAR/eTCR expression) at EOP was ≥ 50% in 8 of 14 runs (average 57.5%; range, 14-93). All EOP cells tested negative for endotoxin, mycoplasma and bacteria. We performed 14 comparable validation runs on CliniMACS Prodigy™ to support 3 different clinical trials. We demonstrated robust feasibility of GMP-compliant manufacturing of CAR/eTCR T-cell products in this closed, automated system using 3 lentiviral vectors and 3 expansion protocols.

Process	A: TCT 7-day process (n=4) Mean (range)	B: TCT 8-day process (n=6)	C: CAP 8-day process (n=4)	Combined (n=14)
Starting cell #	3 x 10^9 T cells	3 x 10^9 T cells	1.5 x 10^9 TNC	NA
Target cell for selection	CD4+/CD8+	CD4+/CD8+	Proprietary T-cell subset	NA
Post-selection				
% Target cell purity	93.2 (91-94) (CD45+/CD3+)	92.4 (87-97) (CD45+/CD3+)	95.5 (93-99) (T-cell subset)	93.5
% Target cell recovery	59.9 (51-75)	47.3 (24-59)	47.6 (37-61)	51.0
% CD4 (lymphs)	65.3 (29-80)	66.9 (57-71)	67.3 (55-78)	66.6
% CD8 (lymphs)	33.8 (18-71)	30.4 (28-34)	27.7 (19-36)	30.6
Log depln B-cells (CD19 or 20+)	2.4 (2.2-2.7)	2.5 (1.2-3.5)	1.6 (0.9-2.5)	2.2
Log depln NK cells (CD3-CD56+)	1.1 (0.7-1.3)	1.1 (0.9-1.4)	2.3 (1.7-2.8)	1.4
Culture medium - plus TexMACS™	HAB[1] + IL-2	HAB + IL-2	HAB + IL-7, IL-5	NA
TransAct™ bead activation (hr)	20-24	18-20	24	NA
Lentivirus MOI	1-4	2.5-3.2	2-40	NA
EOP[2]				
% T-cell purity (CD45+/CD3+)	98.5 (95-100)	95.0 (91-99)	98.8 (98-99)	97.1
% CD4 (lymphs)	69.6 (50-83)	59.5 (41-76)	48.7 (34-66)	59.3
% CD8 (lymphs)	30.7 (18-51)	41.7 (24-59)	48.4 (30-64)	40.4
Fold T-cell expansion EOP	23.1 (16-36)	7.6 (7-8)	16.9 (14-21)	14.7
% CAR/eTCR expression (T cells)	61.6 (14-93)	41.7 (27-64)	71.8 (56-80)	57.5



131. The Influence of Cytokines and Seeding-Density in a Scalable Feeder-Free System for Natural Killer Cell Expansion

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Bio-Techne, Woburn, MA

A challenge for Chimeric Antigen Receptor Natural Killer cell (CAR-NK) therapies is generating clinical doses of natural killer (NK) cells. NK cells compose approximately 5-15% of peripheral blood mononuclear cells (PBMCs) and current manufacturing methods to expand NK cells include culturing irradiated feeder cells with the PBMCs. However, these feeder-cell based methods are restrictive due to high costs, scale-up difficulties, and licensing restrictions. In this study, we investigated a scalable feeder-free system to expand NK cells for NK therapies. Specifically, we studied the impact of cell activation methods, cell seeding density, and cell culture vessels on NK expansion and purity. Human peripheral blood mononuclear cells (PBMCs) were cultured for 10-days in either T25 Flasks, G-Rex®6 or G-Rex6M plates. Prior to expansion, PBMCs were analyzed using a flow cytometer for $CD3^-/CD56^+$ NK cells to determine NK seeding density. The Cloudz™ NK Cell Expansion Kit or Miltenyi MACS iBeads were used to activate & expand NK cells. A 1/2 media change was performed every 3 days and cytokines were refreshed at the following concentrations: 27 ng/mL IL-2 and 10 ng/mL each of IL-12, IL-18, & IL-21. After 10 days in culture the cells were analyzed for $CD3^-/CD56^+$ NK cell expansion and purity, defined as % $CD3^-/CD56^+$ cells relative to the total population. We applied a systematic approach to develop a manufacturing protocol for NK cells from PBMCs in the scalable G-Rex6M Platform. First, the cytokine scheme was developed using T25 Flasks. We found that the Cloudz NK Cell Expansion Kit produced 318-fold NK cell expansion and 85% purity after 10-days when used with a cytokine combination of IL-2/IL-12/IL-18/IL-21. For comparison, MACS iBeads produced an average of 177-fold expansion and 64.5% purity. Second, these conditions were translated to a G-Rex6 platform to determine the seeding desnity. In the T25 culture vessels, a seeding density of 4,800 NK cells/cm^2 yielded the highest growth and purity after 10-days. However, in the G-Rex6 platform, increasing the seeding density to 30,000 NK cells/cm^2 yielded the highest growth and purity after 10 days. Third, we applied these conditions to the scalable G-Rex6M plates. PBMCs were cultured in the G-Rex6M platform using the cytokine scheme and the seeding density determined previously. In the G-Rex6M platform, NK cells expanded from approximately 270,000 NK cells and 8% purity to 150,000,000 NK cells and 92% purity in 10-days -a 582-fold expansion (Figure 1). The results in the G-Rex6M are scalable up to multi-liter batches. Furthermore, since the G-Rex6M plates do not require media exchanges, we tested whether it was necessary to refresh the cytokines every 3-days. Our results suggest that similar NK purity and expansion can be achieved when cytokines are only added at the time of seeding. This finding decreases the amount of cytokines required, reduces the need for manipulating the cells, and lowers the risk of potential contamination during expansion. Overall, it was determined that the seeding density is an important variable to consider when culturing NK cells under new conditions and that cytokine combinations and dosing schedules play an important role growing NK cells in scalable feeder-free cell systems. These data together demonstrate the promise of using the Cloudz™ NK Cell Expansion Kit and the G-Rex bioreactor systems for clinical scale NK cell manufacturing to support CAR-NK therapies.

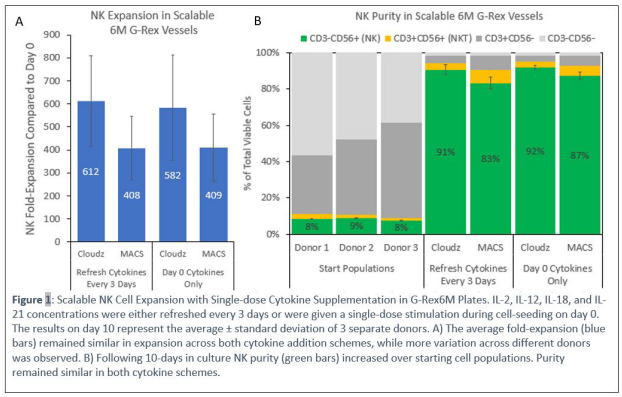

Figure 1: Scalable NK Cell Expansion with Single-dose Cytokine Supplementation in G-Rex6M Plates. IL-2, IL-12, IL-18, and IL-21 concentrations were either refreshed every 3 days or were given a single-dose stimulation during cell-seeding on day 0. The results on day 10 represent the average ± standard deviation of 3 separate donors. A) The average fold-expansion (blue bars) remained similar in expansion across both cytokine addition schemes, while more variation across different donors was observed. B) Following 10-days in culture NK purity (green bars) increased over starting cell populations. Purity remained similar in both cytokine schemes.

132. Clinical Production of Ex-Vivo Gene Corrected Hematopoietic Stem and Progenitor Cells Using a cGMP-Compliant Semi-Closed Manufacturing Process

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The ex-vivo HDR-mediated gene correction of CD34+ hematopoietic stem and progenitor cells (HSPCs) using CRISPR/Cas9 and AAV has emerged as a novel strategy for treating monogenic diseases such as Sickle Cell disease (SCD). The critical technical challenge of efficient gene correction at optimal levels is achieved using the co-delivery of gRNA and Cas9 as a ribonucleoprotein (RNP) followed by the transduction of cells with a recombinant Adeno-associated virus-6 (AAV6) for the targeted delivery of the HBB gene that lacks the SCD causing E6V mutation. The therapeutic use of such ex-vivo gene corrected cell products relies on robust manufacturing processes that consistently produce HSPCs with optimal levels of gene correction for desired efficacy. We have developed a large scale semi-closed manufacturing process for the cGMP-compliant production of ex-

vivo gene corrected HSPCs at clinical scale enabling the clinical translation of a first-in-human gene correction strategy for SCD. Our scalable GMP process involves a two-phase manufacturing process - 1) Selection and banking of SCD-patient HSPCs collected by Plerixafor mobilized apheresis (HPC-A) and 2) Cell expansion and correction of the E6V mutation. The semi-closed, large scale manufacturing process using culture bags and GMP reagents enables the culture of >300x10^6 cells and mitigates the risk of cell loss during manipulations, while achieving optimal doses of cell products with >70% viability and up to 57% gene correction assessed by ddPCR that meet the release criteria. Cell product manufactured with the developed process demonstrates retention of corrected HBB alleles *in vivo*. Product characteristics such as safety, identity, purity and potency of the gene corrected cell product manufactured using the novel semi-closed cGMP-compliant system will be discussed. We expect this new approach for clinical scale production of ex-vivo gene corrected HSPCs to enable early phase clinical trials.

133. Chromatin (DNA) Removal from Harvest Before an AAV Capture Step Greatly Improves Robustness, Purity, and Yield of the Overall Downstream Process

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The most critical tasks for an AAV purification process are recovery of vector capsids, removal of DNA, removal of host-cell protein, and separation of empty and full capsids. The process development group at BIA receives AAV harvests from many sources produced in both mammalian and insect cultures that have been harvested under a myriad of conditions. Due to the extreme variability of the crude-vector harvests received, developing a tool-box of methods for the up-front removal of DNA in the form of chromatin and chromatin/protein agglomerates before the capture step is crucial. Removal of chromatin dramatically improves robustness of the capture step and purification of the vector by reducing the multimodal nature of the capture step induced by binding of chromatin to the capture column. Pretreatment of harvest prior to loading the capture column can greatly improve the robustness and efficiency of the vector capture step and later downstream unit operations. Various combinations of high-salt, low pH, flocculating agents, solid-phase extraction, nuclease treatment, and tangential flow filtration (TFF) were explored for multiple upstream feeds and AAV serotypes. Vector purity as assessed by SDS-PAGE showed the classic VP1, 2, and 3 bands of AAV with virtually no contaminating proteins in the captured peak fraction from multiple harvests with various harvest pretreatment methods. Analytical size-exclusion chromatography (SEC) combined with multi-angle light scatter (MALS), protein fluorescence (tryptophan), DNA fluorescence (picogreen), and UV absorption (260 and 280 nm) detection showed that extensive chromatin reduction prior to the vector capture step correlated with high purity of the peak vector fraction by SDS-PAGE. TFF combined with nuclease digestion produced a cumulative yield of 43% from clarified harvest to purified, full AAV8 capsids (88% full capsids) in four unit-operations [TFF with nuclease, CEX (CIMmultusTH SO3), buffer exchange, and AEX (CIMmultus^{TM} QA)]. Additional processing sequences with different vector harvests will be presented.

AAV Vectors Preclinical and Proof-of-Concept Studies in Optimizing the Toolbox

134. A Novel Lung Tropic AAV Vector Restores Surfactant Homeostasis and Improves Survival in a Mouse Model of Inherited Surfactant B Deficiency

Martin Kang[1], Laura P. Van Lieshout[2], Liqun Xu[1], Jakob M. Domm[2], Arul Vadivel[1], Laurent Renesme[1], Christian Mühlfeld[3], Maria Hurskainen[1], Ivana Mizikova[1], Yanlong Pei[1], Jacob P. Van Vloten[2], Sylvia P. Thomas[2], Claudia Milazzo[1], Chanele Cyr-Depauw[1], Jeffrey A. Whitsett[4], Larry M. Nogee[5], Sarah K. Wootton[2], Bernard Thébaud[1]

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Surfactant protein disorders such as surfactant protein B (SP-B) deficiency (OMIM#265120) are genetic lung diseases that impair pulmonary surfactant homeostasis resulting in alveolar collapse and lethal respiratory distress in neonatal patients. The only treatment that currently exists is lung transplantation, and without this procedure affected infants will die within months of birth. SP-B is expressed by surface alveolar type 2 (AT2) epithelial cells, and the expression of this protein is crucial for surfactant homeostasis. A persuasive argument exists for using gene therapy to treat this disease, as *de novo* protein synthesis of SP-B within these cells is required for proper surfactant production. However, the challenges in delivering transgenes to AT2 cells have been formidable due to obstacles such as the immune response, the requirement of cell-surface receptors for vector entry, and the barrier properties of respiratory mucus and alveolar fluid. Adeno-associated virus (AAV) has demonstrated some of the most promising and clinically translatable results in the treatment of genetic diseases. Here, we utilize a novel, rationally designed AAV6 capsid genetically engineered to increase heparin binding and inhibit ubiquitination, to efficiently transduce lung epithelial cells based on flow cytometry and imaging analysis. Intratracheal administration of this vector delivering murine or human SP-B cDNA into a SP-B deficient mouse model restored surfactant homeostasis, prevented lung injury, and improved lung function. Untreated SP-B deficient mice developed fatal respiratory distress within two to seven days. AAV-mediated gene therapy resulted in an unprecedented improvement in median survival to more than six months. The clinical relevancy of this vector

is further supported by the rapid expression of SP-B within days of administration, long term therapeutic expression of transgenes for up to nine months, therapeutic efficacy and safety in neonatal mice, and no signs of adverse effects as observed by increases in body weight and the lack of an inflammatory cytokine profile in treated mice. This vector is also able to transduce precision cut lung slices from human lung tissue, and establishes its potential as a feasible therapeutic strategy for this lethal disease.

135. Quantitative PET/CT Based Pharmacokinetic Study of AAV9 Administered to the Cerebrospinal Fluid of Non-Human Primates

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The system of perivascular channels in the CNS that communicates with the cerebro-spinal fluid (CSF) may provide a robust avenue for gene delivery to CNS targets. However, the properties of the vector as well as the administration region may affect the vector access to a particular target. We investigated by quantitative PET/CT the transport of AAV9 labeled with I-124 and Zr-89 after administration of 10^{13} vector particles to either cerebral (cisterna magna) or spinal (thecal sac) CSF. The vector was packaged with an expression cassette containing GFP under a CAG promoter. The vector was administered to cynomolgus monkeys via direct needle injection. The spinal injection was followed by a 0.5 ml/kg body weight flush. Full body images were acquired at 0.5, 1, 1.5, 2.5, 3, 4, 24 and 48 hours after the administration of AAV using a PET/CT imaging system consisting of MicroPET Focus 220 and Ceretom NL3000 scanners, respectively. The data were reconstructed by iterative OSEM2D/MAP algorithm. The label content in the tissues was measured in manually drawn regions of interest. After intracisternal administration, the dose was found to spread throughout the cerebro-cervical CSF and penetrate into the fissures and major perivascular spaces within the first 30 minutes. Intracisternal administration resulted in the dose spread over the cerebro-cervical CSF without significant penetration into thoracic and lumbar segments of the spine. The high-volume lumbar dose placement resulted in AAV delivery to both the spinal and cerebral CSF. PET imaging of the dose placement was also found to be instrumental to detect and measure the fraction of the dose that leaked out of the CSF to the epidural space or soft tissues through the needle canal, which in some animals was found to be significant. In the brain, the maximal vector concentration in the areas supplied by the smaller perivascular channels was reached by 3.5±1 hours. AAV9 remained mobile in the CSF and cleared from it with a rate approximately equal to the CSF replacement rate ($t_{1/2}$=3.5±1 hours). The residual fraction of the dose remaining in the brain was approximately 1.2% in the gray and 0.2% in the white matter at 24 hours, as measured with Zr-89. Our PET data demonstrates that AAV9 delivered through administration into the CSF remains in the liquid phase (is not entrapped in the meningeal structures) and has access to the perivascular channels leading to the full depth of the CNS.

136. Building a Toolbox of Compact Enhancers for Cell Type-Specific Gene Expression from AAVs in the Primate Brain

John K. Mich[1], Bryan B. Gore[1], Refugio A. Martinez[1], Lucas T. Graybuck[1], Joseph T. Mahoney[1], Jeremy A. Miller[1], Shenqin Yao[1], Darren Bertagnolli[1], Jeff Goldy[1], Rebecca D. Hodge[1], Trygve E. Bakken[1], Zizhen Yao[1], Nick Dee[1], Kimberly A. Smith[1], Ryder P. Gwinn[2], Charles Cobbs[2], Andrew L. Ko[3], Jeffrey G. Ojemann[3], C. Dirk Keene[4], Daniel L. Silbergeld[3], Viviana Gradinaru[5], Hongkui Zeng[1], Bosiljka Tasic[1], Ed S. Lein[1], Jonathan T. Ting[1], Boaz P. Levi[1]

[1]Allen Institute for Brain Science, Seattle, WA,[2]Swedish Neuroscience Institute, Seattle, WA,[3]Neurological Surgery, University of Washington School of Medicine, Seattle, WA,[4]Pathology, University of Washington School of Medicine, Seattle, WA,[5]Biology and Biological Engineering, California Institute of Technology, Pasadena, CA

AAV gene therapy has tremendous potential to transform the treatment of brain diseases. Tissue and cell class-selective transgene expression could improve the safety and efficacy of AAV-based therapeutics for brain circuit-related disorders compared to ubiquitous transgene expression. Unfortunately, there are few known compact genetic regulatory elements that limit transgene expression to specific cell populations. We undertook a systematic screen to find enhancer elements capable of targeting AAV expression to all major neocortical cell populations. Our strategy leverages multi-modal transcriptomic and epigenetic analyses of brain cells from mouse and human, followed by brain-wide *in vivo* testing in mouse and ex vivo testing in NHP and human. These studies generated a diverse collection of enhancer-AAV vectors that specifically target neocortical neuronal and glial cell populations across species. Furthermore, we show endogenous enhancers can be rationally optimized to increase expression levels for preclinical lead optimization studies. These studies yield a growing toolbox of enhancer elements for cell type-specific targeting in the human brain that will enable more precise gene therapy.

137. Rapalog-Mediated Regulation of an AAV miRNA Targeting *Huntingtin* in a HD Transgenic Mouse Model

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AAV-mediated RNA interference-based gene therapy is a promising approach to treating a variety of diseases, including Huntington's Disease (HD). Studies in HD mouse and sheep models have shown

safe and efficient targeting of human *HTT* using AAVs expressing artificial miRNAs. For some indications the ability to regulate the silencing with a small molecule after AAV delivery would be desirable. In these studies, we used HD mice as a model in which to determine if peripheral administration of small molecule would be able to cross the blood brain barrier and regulate AAV-miRNA expression in the CNS. We created a regulatory AAV system where AAV expression and subsequent miRNA expression is controlled pharmacologically, with the use of an exogenous drug. Several regulatory systems have been developed using the small molecule rapamycin, which is FDA-approved, or non-immunosuppressive rapamycin analogs (rapalogs). In these systems, the small molecule or "dimerizer" causes heterodimerization of two proteins comprising an activation domain and a DNA binding domain. The heterodimerization of these proteins leads to reconstitution of an active transcription factor, which induces expression of the AAV-miRNA. Due to the packaging restrictions of AAV, most rapamycin-controlled systems require two AAV viral vectors. However, due to their small size, artificial miRNAs can be incorporated into a single vector system. With this technology we have developed a rapalog regulated AAV-miR^{HTT}. Using YAC128-HD mice, which express full length human *HTT*, we show that rapalog dosing causes a significant reduction in human HTT protein expression in the brain. In contrast, in the absence of rapalog, the AAV is inactive and HTT protein levels in the brain are unchanged. This work is proof-of-concept that an artificial miRNA targeting huntingtin can be regulated *in vivo.* It provides a tool for further investigation of *HTT* gene silencing and could be used to mitigate concerns about long-term *huntingtin* silencing.

138. A Discovery Platform that Screens Thousands of Gene Therapy Candidates in a Single Animal

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Gene therapy has had remarkable success in treating rare, monogenic diseases. As a modality, it holds potential for precise manipulation of both molecular targets and tissues. Realizing this potential for diseases without a clear genetic cause will rely on new methods for identifying optimal molecular targets with demonstrated efficacy. Complex diseases often involve the interplay of different cell types and contributions from the systemic environment, meaning that therapeutic efficacy is ultimately only testable *in vivo*. The time and effort required for mammalian experiments has meant that only a small fraction of the therapeutic target space has been explored for complex diseases, and thus a more scalable way of identifying targets is needed. We have developed a platform, XaNDER, that allows thousands of candidate gene therapies to be tested simultaneously, in a single animal. XaNDER relies on high-throughput single-cell genomics to measure biomarkers of disease in individual cells from diseased tissues, each affected by a different therapeutic candidate. This shifts the crucial *in vivo* tests to the beginning of the therapeutic discovery process, while scaling throughput to allow perturbation of the majority of potential targets. We demonstrate controlled delivery of single viral payloads to individual cells using adeno-associated virus, resulting in robust expression of marker genes and therapeutic candidates. Expression is maintained for multiple months from individual episomal genomes, allowing us to measure progressive therapeutic effects. The specificity of XaNDER was validated using two genetic disease models, Hunter syndrome and Progeria, where the correct therapeutic targets are known. These positive control therapeutic candidates were included alongside other interventions in an *in vivo* screen, and phenotypic readouts identified clinically validated targets as hits of the screen. The effect of the therapeutic gene therapies was compared to systemic administration of clinical drugs, and validated in multiple tissues. We also used the platform to model the relative effect size of each candidate. This platform offers multiple advantages over traditional high-throughput screening: Because experimental cells are surrounded by unperturbed diseased tissue, XaNDER can probe disease pathways involving cell-cell communication. Biological variability can largely be separated from therapeutic effects because each individual animal contains an entire screen, including internal controls within the screen. Moreover, being able to conduct a large screen in a single animal enables the use of the most relevant disease models, including spontaneous and/or progressive diseases in non-rodent species that most closely match relevant human physiology.

139. Combined Transgene and Intron-Derived miRNA Therapy for the Treatment of SCA1

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Spinocerebellar ataxia type I (SCA1) is an autosomal dominant neurodegenerative disease caused by a (CAG) repeat expansion within the *ataxin 1* (*ATXN1*) coding region. We have previously shown that viral-based delivery of RNA interference (RNAi) molecules targeting human *ATXN1* can both prevent and reverse disease phenotypes in a mouse model of SCA1. Additionally, we have demonstrated that overexpression of the highly conserved ATXN1 paralog, ataxin 1-like (ATXN1L) improves disease readouts when delivered to SCA1 mice pre-symptomatically. In order to reduce the viral dose required for effective therapy, we combined these approaches into a single transgene and tested its ability to reverse disease symptoms in SCA1 mice. Dual expression was achieved by incorporating a miRNA targeting human ATXN1 (miS1) into an upstream intron of a human ATXN1L (hATXN1L) minigene (AAV.hATXN1L int2 miS1). Early symptomatic SCA1 animals underwent initial motor assay by rotarod at 11 weeks of age and received direct deep cerebellar nuclei injection of varying doses of vector one week later. Mice underwent a second rotarod analysis at 22 weeks of age, after which tissues were harvested and analyzed for transgene expression and RNA processing. Here we show that our dual expression transgene is effectively processed into mature hATXN1L transcripts and functional miS1 guides. Interestingly, these vectors achieve greater knockdown and higher transgene expression than our previous separate constructs when delivered at similar doses and may significantly reduce the virus load required for therapeutic benefit.

140. A Highly-Evolved Novel AAV Gene Therapy Directly Addresses Fabry Disease Pathology In Vivo by Cell Autonomous Expression in the Heart and Other Target Organs

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Introduction Fabry disease (FD) is an X-linked disorder caused by mutations in the *GLA* gene that encodes α-galactosidase A (AGA). Mutations in *GLA* that cause diminished or absent AGA activity result in accumulation of intracellular globotriaosylceramide (Gb3), thought to be cytotoxic to several cell types including cardiomyocytes and vascular endothelial cells. Chronic biweekly infusions of enzyme replacement therapy (ERT) result in Gb3 reduction in endothelial cells and may slow some aspects of disease progression. Poor cellular uptake (heart), likely underlies the unmet medical need: premature death from cardiomyopathy, arrhythmia, renal failure and stroke. We are developing a gene therapy that efficiently expresses AGA in a cell-autonomous manner in FD-affected tissues, as well as systemically, with the potential to fully address disease pathology and improve clinical outcomes. **Methods** We applied industrialized directed evolution ("Therapeutic Vector Evolution") in non-human primates (NHP) *in vivo* and human target cells *in vitro* to identify and characterize a novel AAV capsid variant with a vector profile targeted to tissues implicated in FD, including specifically the heart. We explored the ability of this capsid variant, expressing the human *GLA* transgene (4D-310), to produce intracellular functional AGA in human Fabry disease iPSC-derived cardiomyocytes and endothelial cells. We performed dose-ranging studies in wildtype mice to explore the potency of 4D-310 to increase AGA activity in plasma and target tissues. We then treated a *GLA* knockout FD mouse model to further explore the effect of 4D-310 on tissue and plasma AGA activity as well as elimination of intracellular Gb3. In both wildtype and knockout mouse studies, we performed immunohistochemical analyses to examine AGA expression in FD target organs and cell types (cardiomyocytes, podocytes and vascular smooth muscle cells). Finally, we conducted a comprehensive GLP-compliant toxicology and biodistribution evaluation. **Results** A single low dose IV infusion of the novel 4D-310 AAV capsid localized to the heart, kidney, skeletal muscle and liver in NHPs; heart targeting was superior to AAV8/9. 4D-310 showed high transduction efficiency and dose-dependent increases in intracellular AGA in human iPSC-derived FD cardiomyocytes and vascular endothelial cells. In wildtype and FD mice, 4D-310 produced sustained supranormal AGA activity in plasma, heart and kidney at low doses highly suitable for clinical translation. Compared to placebo-treated animals, 4D-310 treatment led to plasma lyso-Gb3 reduction by > 95% percent in GLA knockout mice. Immunohistochemical analysis showed robust AGA in all FD target tissues and cell types (including cardiomyocytes). No toxicity was seen at any dose. **Conclusion** This is the first use of directed evolution to identify a vector engineered in primates to efficiently transduce Fabry disease target organs, including the heart. The tropism profile of this novel AAV vector represents a significant advance over existing AAV serotypes and is well suited for the development of gene therapies for other diseases involving the heart including Lysosomal Storage Diseases (e.g. Pompe) and Muscular Dystrophies (e.g. Duchenne). A single low IV dose of 4D-310 results in sustained AGA activity in key organs, specifically the heart, as well as the plasma, with no observed toxicity. 4D-310 shows the potential of overcoming the limitations of cellular uptake that attenuate ERT effectiveness by leveraging cell-autonomous production in the context of exquisite tissue targeting plus high plasma enzyme levels.

AAV Vectors - Virology and Vectorology

141. Abstract Withdrawn

142. Effects of Altering HSPG Binding and Capsid Hydrophilicity on Retinal Transduction by AAV

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PURPOSE: Retinal transduction and cell tropism of AAV is dependent on a variety of factors including delivery route and biological interactions between capsid surface and environment. For example, outer retina is more efficiently transduced by subretinally injected (SRI) capsids that do not utilize heparan sulfate proteoglycan (HSPG) as a receptor (i.e. AAV5, AAV8, AAVrh.8, etc.). Inner retina is more efficiently transduced by intravitreally injected (IVI) capsids that bind HSPG (i.e. AAV2 and AAV6 derivatives). The purpose of this study was to further interrogate the impact of HSPG affinity and capsid hydrophilicity on retinal transduction. **METHODS:** Starting with AAV2(Y444F), we created variants predicted to have increased hydrophilicity by altering residues in variable regions that do not overlap with previously identified receptor binding footprints for AAV2. Next, we ablated HSPG binding (ΔHS) by these variants via R585S and R588T mutations. In separate experiments, starting with AAV2ΔHS we created a series of variants containing increasing numbers of hydrophobic Y-F and/or T-V substitutions. All variants were packaged with self-complementary smCBA-mCherry and tested in Nrl-GFP mice following SRI (2E8 or 2E9 vg) and IVI (2E9 vg). Fundoscopy and flow-cytometry were performed to quantify mCherry expression. Heparin binding assays and differential scanning fluorometry (DSF) were performed on a subset of variants. Constructs containing CBA or hGRK1 promoters driving GFP were packaged into AAV2(4pMut)ΔHS and AAV5. Seven macaques received SRI at 1E11 vg. In 4 eyes, two 60μL blebs were made (subfoveal, and peripheral). In 3 eyes, three 30μL extrafoveal blebs were created. GFP expression was assessed by confocal scanning laser ophthalmoscopy (cSLO). Rod/cone transduction was quantified in retinal sections. **RESULTS:** Addition of hydrophilic residues uniformly ablated

retinal transduction in mice following IVI and, in all but one variant, AAV2(Y444F+L583R) following SRI. All variants had decreased capsid stability relative to AAV2(Y444F) except AAV2(Y444F+E530K). Removal of HSPG binding residues on AAV2(Y444F+W502H)ΔHS resulted in a variant with high transduction efficiency following SRI, similar to AAV2(Y444F)ΔHS. AAV2(Y444F+V387R)ΔHS and AAV2(Y444F+E530K)ΔHS had negligible or low transduction following SRI. When AAV2ΔHS was made progressively more hydrophobic, a concomitant increase in retinal transduction following SRI was observed, with AAV2(4pMut)ΔHS being the top performer. In macaque, AAV2(4pMut)ΔHS- mediated GFP expression was observed well beyond the SRI bleb margins. Extrafoveal injection of AAV2(4pMut)ΔHS led to transduction of 98% of foveal cones and strong expression in RPE. **CONCLUSIONS:** Addition of hydrophilic mutations onto an AAV2-based capsid ablated vector transduction via IVI in mice without diminishing HSPG affinity. In the absence of canonical HSPG binding, the W502H mutation is tolerated, suggesting that this residue competes with the canonical HSPG footprint on AAV2, but not the receptor footprint utilized by AAV2ΔHS. AAV2(4pMut) ΔHS exhibits increased retinal transduction and enhanced lateral spread following SRI in mouse and macaque relative to AAV5. AAV2(4pMut)ΔHS efficiently transduces foveal cones without the requirement of foveal detachment. This capsid will be useful for treating diseases of the central retina, but detachment of the area is counter indicated, or in diseases where transduction of large areas of retina is beneficial.

143. 4D-C102, a Novel Muscle-Tropic AAV Variant Demonstrates Superior Gene Delivery in Cardiac and Skeletal Muscle Tissues Versus Wild-Type AAV in Human Cells and Non-Human Primates

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Introduction To unlock the full potential of gene therapy, there is a need to identify novel vectors that show enhanced tropism for target tissues when delivered by clinically relevant routes of administration at commercially feasible doses. Gene replacement strategies leveraging adeno-associated virus (AAV) vectors with high tropism for affected organs may directly address the underlying pathophysiology of neuromuscular diseases, and lysosomal storage diseases, through a combination of cell autonomous correction in target tissues and, when necessary, restoring serum enzyme levels by expression from other key organs, such as heart and skeletal muscle. **Methods** An industrialized directed evolution approach ("Therapeutic Vector Evolution") was employed in the most relevant animal species (non-human primate; NHP) to discover novel adeno-associated virus (AAV) capsid variants capable of efficient and preferential gene delivery to and robust gene expression in cardiac and skeletal muscle following a single intravenous (IV) administration. The lead candidate from this discovery program, 4D-C102, was characterized using a reporter transgene in human cardiomyocytes and skeletal myofibers *in vitro*, and in NHP *in vivo*. Human embryonic stem cell-derived cardiomyocytes and human primary skeletal muscle cells were transduced using varying multiplicity of infections (MOIs) of wild-type control vectors (AAV1,8,9) or variant 4D-C102. Groups of NHP (n=3 each) received 1 x 10^{13} vg/kg of wild-type control vectors AAV8 and AAV9 or variant 4D-C102. After approximately seven weeks, systemic tissues were harvested for analyses, including histopathology, viral genome localization, protein expression, and immunofluorescence. **Results** 4D-C102 exhibited dose-dependent transduction efficiency of both human cardiomyocytes and skeletal muscle cells and was capable of highly efficient gene delivery at very low MOI in human cardiomyocytes. At all MOIs explored, 4D-C102 transduced a significantly higher percentage of cells compared to wild-type AAVs. In NHP, a single low dose IV infusion of 4D-C102 was well-tolerated and resulted in robust delivery of viral genomes and protein expression within all regions of the heart (30 of 30 genome samples) and multiple skeletal muscle groups (81 of 81 skeletal muscle samples). Genome delivery in NHP with 4D-C102 was superior to heart and muscle tissues compared to the wild-type AAVs tested. There were no histopathological or clinical pathology findings of toxicity with 4D-C102 at 1 x 10^{13} vg/kg in contrast to AAV9. **Conclusions** This is the first use of directed evolution in NHP to identify a vector engineered to efficiently transduce multiple muscle tissues by IV administration. The lead variant, 4D-C102, demonstrated superior gene delivery in both human cells *in vitro* and NHP *in vivo*. The tropism profile of this novel AAV vector represents a significant advance over existing AAV serotypes and is well suited for the development of gene therapies for neuromuscular conditions, lysosomal storage diseases involving muscle tissues, and muscular dystrophies.

144. Systematic Pharmacokinetic Profiling of AAV Capsids Through Deep Sequencing of Barcoded Capsid Libraries

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Previous studies have leveraged barcode-based sequencing approaches to interrogate the clearance rates of AAV from blood in both mice and non-human primates. Here we extend these approaches by studying a library of 2048 capsid variants, drawn from an ancestral sequence reconstruction of the most recent common ancestor of AAVs 1- 3, and 6-9 (BC-Anc80Lib). This library (along with a number of barcoded AAV controls) was systemically administered to mice and non-human primates. Blood was drawn at different timepoints and viral barcodes were subsequently isolated and sequenced from the sera of these animals. Comparisons were then made to uncover residues within our BC-Anc80Lib which may modulate persistence in both species. Preliminary data shows that a single amino acid change at two different sites within the library contributes to increased serum persistence in mice. Moreover, we present evidence that one of those two states might have an opposite effect on serum persistence in non-human primates. Furthermore, we analyzed livers and spleens

from these animals to investigate whether serum clearance correlated with barcode abundances in either of these tissues. Consistent with published work, we observed that increased AAV genome abundance in liver is associated with a decreased abundance in spleen and vice-versa in both mice and non-human primates. However, increased genome abundance in the liver was positively correlated with serum persistence in our murine model, but the opposite correlation was found in non-human primates.

145. A Systematic Capsid Evolution Approach Performed In Vivo for the Design of AAV Vectors with Tailored Properties and Tropism

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Adeno-associated virus (AAV) capsid modification enables the generation of recombinant vectors with tailored properties and tropism. Most approaches to date depend on random screening, enrichment, and serendipity. The approach explored here, called BRAVE (Barcoded Rational AAV Vector Evolution), enables efficient selection of engineered capsid structures on a large scale using only a single screening round in vivo. The approach stands in contrast to previous methods that require multiple generations of enrichment. With the BRAVE approach, each virus particle displays a peptide, derived from a protein, of known function on the AAV capsid surface, and a unique molecular barcode in the packaged genome. The sequencing of RNA-expressed barcodes from a single generation in vivo screen allows the mapping of putative binding sequences from hundreds of proteins simultaneously. Using the BRAVE approach and hidden Markov model-based clustering, we present 25 synthetic capsid variants with refined properties, such as retro- grade axonal transport in specific subtypes of neurons, as shown for both rodent and human dopaminergic neurons.

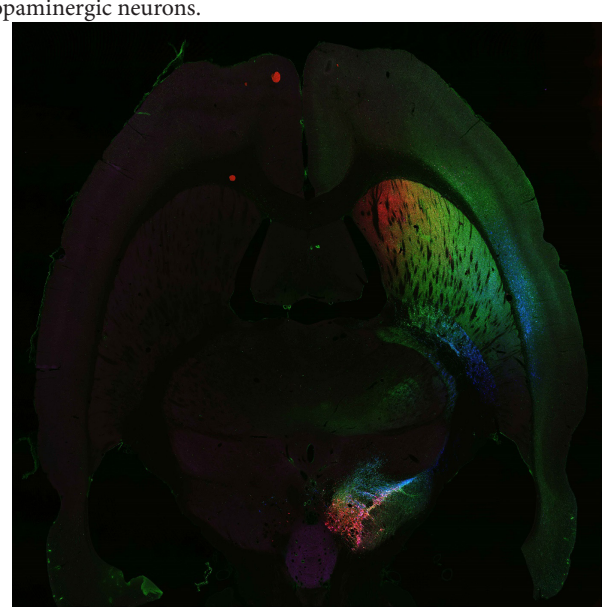

Figure 1. Mapping of nigral afferent topography to the striatum using three Cre-inducible genes (mCherry, red; alpha-synuclein, green; tagBFP2, blue) packaged in separate MNM008 vectors and injected into the striatum along the rostrocaudal axis. Here illustrated in a horizontal section including both striatum (Str) and substantia nigra pars compacta (SN) imaged using a laser-scanning confocal microscope.

146. Engineered Capsids for Efficient Gene Delivery to the Retina and Cornea

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Adeno-associated viral (AAV) vectors represent an ideal vehicle for human gene transfer. One advantage to the AAV vector system is the availability of multiple naturally occurring serotypes that provide selective tropisms for various target cells. Strategies to enhance the properties of the natural AAV isolates have been developed and can be divided into two approaches, rational design or directed evolution. The rational design approach utilizes knowledge of AAV capsids to make targeted changes to the capsid to alter transduction efficiency or specificity, while the directed evolution approach does not require *a priori* knowledge of capsid structure and includes random mutagenesis, capsid shuffling, or random peptide insertion. Here we describe the generation of novel variants using a rational design approach and knowledge of AAV receptor binding, surface charge and AAV capsid protein post translational modifications. Previously, we described an AAV2HBKO variant devoid of canonical heparin sulphate binding residues. In this study, the effect of adding surface arginines was further explored with another capsid, AAV5. AAV5 - arginine variants were generated at yields that were 2-3 fold less than yields achieved with parental AAV5, but variants retained the same capsid protein ratio as the parental capsid. The performance of AAV5 was compared to the AAV5-arginine variants following subretinal delivery of 1 x 10^9 vgs of each vector harboring the identical CBA-eGFP expression cassette. Analysis of native eGFP fluorescence in transduced retinae, revealed that the variants transduced the ONL and RPE cells to the same level as parental AAV5-eGFP. In contrast, intravitreal delivery of the AAV5 arginine variants to the mouse retina, at a vector dose of 1 x 10^9 vgs per eye, followed by EGFP fluorescence analysis of the transduced retinae, confirmed that each of the AAV5 arginine variants had acquired a novel tropism for corneal endothelium cells. In contrast parental AAV5 showed no transduction activity with intravitreal delivery. To understand the general function of post translational modifications (PTMs), on the AAV capsid, we examined the role of N-terminal acetylation on AAV5 transduction in the retina. A reduction in acetylation on VP1, as verified by LC/MS, resulted in a concomitant reduction in mouse retinal transduction. This reduced transduction was evident when the N-terminal acetylation of VP1 was decreased in isolation, or in combination with reduced N-terminal acetylation in the AAV5 VP3 protein. Notably, when VP1 N terminal acetylation was preserved and VP3 N-terminal acetylation reduced, there was a significant improvement in retinal transduction compared to parental

AAV5; the improved transduction was evident in both the mouse retina and NHP retinal explants. These data underscore the importance of VP1 N terminal acetylation in AAV5 transduction and suggests that this PTM maybe a requirement for AAV endosomal release, a process that requires a pH conformational change in the VP1 N terminus, prior to trafficking to the nucleus. The AAV5 variants demonstrate improved transduction properties in both the mouse retina and cornea, while a non-human primate, (NHP), tissue explant model was established to allow the rapid assessment of translational fidelity between species for the novel capsids. In conclusion, the AAV5 variants described here have novel attributes that will add to the efficacy and specificity of their potential use in gene therapy for a range of human ocular diseases.

147. Selection of Adeno-Associated Virus Vectors Targeting the Central Nervous System Usingan *In Vitro* Model of Human Blood-Brain Barrier

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Recombinant adeno-associated virus (AAV) vectors offer great promises to treat various genetic and acquired central nervous system (CNS) disorders. Currently, the most successful CNS gene therapy is ZolgensmaR, an FDA approved drug where rAAV9 delivers the SMN1 gene into motor neurons for the treatment of patients with lethal spinal muscular atrophy. However, extremely high doses of the drug are required, leading to varying degrees of adverse effects, including death. A major limitation in the field is that none of the AAV vectors efficiently penetrate the human blood-brain barrier (BBB) to the same degree achieved in certain mouse strains. Our goal has been to create and isolate AAVs with enhanced ability to penetrate the human BBB and transduce specific cell types in the CNS. The cell type specificity after crossing the BBB is important for targeting different CNS diseases. To achieve this goal, we have been developing two key techniques. The first component involves establishing an *in vitro* transwell BBB system with human endothelial cells as well as human iPS neurons and astrocytes from 3D organoid cultures. We have tested various naturally isolated AAV vectors in this model and validated that the AAVs known to cross the BBB more efficiently, such as AAV9 and AAV-rh.10, perform better than other AAV vectors. The second component involves AAV vector evolution in the established humanized BBB transwell system. We have created a complex AAV library with ~1 million variants, each containing a unique DNA barcode, using multispecies interbreeding. Following three different genetic evolutionary selection schemes, the viral library is screened multiple passages through the humanized BBB transwell system. We used a combination of high-throughput single molecule capsid DNA (2.2kb) sequencing (PacBio) as well as barcode sequencing (Illumina), to identify the capsids that were enriched in the various screens we performed. Multiple enriched AAV variants were made up of complex capsid chimeric sequences that not only have enhanced ability to pass through the BBB but also efficiently transduce neurons and/or astrocytes. We have validated six viral vectors from the screen for astrocytes and all six are able to cross the endothelial cell layer and transduce astrocytes 37.2 to 91.4-times better than AAV9. We also found four viral vectors from the screen for neurons that are 3.6 to 19.5-times better than AAV9 at crossing both the endothelial and astrocyte cell layers. These four vectors are also 1.6 to 4.0-times better than AAV9 at directly transducing human iPS-derived neurons. The best capsids will be pooled and tested for BBB penetration and CNS transduction in non-human primates prior to selecting a few candidates for consideration in human clinical trials.

148. Using Machine Learning to Predict Adeno-Associated Virus Tropism In Vivo Using Cells as the Input Layer

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The variable and unpredictable tropisms of AAVs pose a significant challenge in the delivery of gene therapies to specific cells and tissues, thus requiring the discovery and engineering of novel AAVs to target the more recalcitrant cells as well as to enhance the specificity of AAVs. This difficulty also highlights the need to develop better predictive models. Presently, the tropisms of novel capsids are characterized by performing biodistribution studies in animal models. However, this takes time and often has to be repeated for every new application of the viral capsid. Here, we present a simple machine learning paradigm, Bioscaling, that can predict how well different AAV capsids will perform in different *in vivo* murine tissues by observing how each virus performs in a panel of diverse cell lines *in vitro*. To build our model, we first examined the previously reported capabilities of different AAVs to transduce different cells *in vitro* as well as *in vivo*. We noticed that each AAV has a very distinct profile, both *in vitro* and *in vivo*. If we can identify an AAV by its *in vitro* transduction performance in different cells, and if we can repeat this process using *in vivo* data in various tissues, then through the identity of the AAVs, we can link the transduction capabilities of AAVs in cells *in vitro* to various tissues *in vivo*. As a proof of concept, we built a classifying model to identify each AAV by their *in vitro* or *in vivo* transduction "fingerprints", and we were able to achieve predictive accuracy greater than 95% on average for all nine serotypes (AAV1, AAV2, AAV3B, AAV4, AAV5, AAV6, AAV7, AAV8, and AAV9). This shows that we can indeed identify the AAVs by their transduction patterns. We then performed high-throughput transduction screening using AAVs with barcoded-GFP transgenes on this panel of cells to get the *in vitro* "fingerprint" of each AAV. This pool of AAVs was also intravenously injected into C56BL/6J mice. Our preliminary results show that our model can predict transduction efficiency for heart, liver, kidney, and muscle with correlation values of 0.74, 0.72, 0.93, and 0.82, respectively. With sufficient data, this machine learning approach may allow us to predict how different new AAVs will perform *in vivo*.

149. Simultaneous Control of Endogenous and User Defined Genetic Pathways Using New DHFR Pharmacological Chaperones

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Small molecule-regulated destabilizing domains (DDs) confer inherent instability and degradation to proteins fused to them yet allow for positive regulation of fusion protein abundance in the presence of a small molecule stabilizer. Thus, DDs serve as a unique approach for the conditional regulation of protein abundance both in experimental models and gene therapy contexts *in vitro* and *in vivo*. One such DD is a mutated form of E. coli dihydrofolate reductase (ecDHFR) which can be stabilized with the antibiotic, trimethoprim (TMP). While TMP is a potent pharmacological chaperone of the ecDHFR DD, we hypothesized that additional stabilizers exist, some of which may confer additional benefits (i.e., non-antibiotic, or dual function) when compared to TMP. Using a combinatorial screening approach, we validated seventeen new 2,4-diaminopyrimidine/triazine ecDHFR DD stabilizers (AC_{50} ~0.11 μM to > 10 μM), fifteen of which were non-antibiotics. The 2,4-diamino structure as well as the presence of a separate aryl group were both required for stabilization of the ecDHFR DD, which appears to occur through combinatorial interactions amongst Ile5, Met16, Phe31 and Ile94 in the dihydrofolate binding pocket. Representative compounds from each screening approach were confirmed to conditionally regulate the abundance of an adeno-associated virus (AAV)-introduced ecDHFR DD-firefly luciferase (ecDHFR.FLuc) *in vivo* in the eye and/or the liver of mice. Next, stabilizers were leveraged to perform dual functions; the human DHFR inhibitors aminopterin and methotrexate (which also stabilize ecDHFR DD), significantly sensitized HeLa cells to death at nanomolar concentrations when combined with regulation of a dominant negative heat shock factor fused to ecDHFR. GW2580, a previously characterized cFMS tyrosine kinase inhibitor, stabilized ecDHFR.FLuc in the eye at levels equivalent to TMP while concurrently repressing microglia abundance in the ganglion cell and plexiform layers. Thus, these newly identified pharmacological chaperones allow for simultaneous control of specified endogenous and user-defined genetic pathways, the combination of which may provide synergistic effects in complex biologic scenarios.

150. Small Alphaherpesvirus Latency-Associated Promoters Drive Efficient and Long-Term Transgene Expression in The Central Nervous System after systemic AAV administration

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Recombinant adeno-associated viral vectors (rAAV) are used as gene therapy vectors to treat central nervous system (CNS) diseases. Despite their safety and broad tropism, important issues need to be corrected such as the limited payload capacity and the lack of small gene promoters providing long-term, pan-neuronal transgene expression in the CNS. This seems critically important for AAV gene therapies administered to newborns that should last 80+ years. Commonly used gene promoters are relatively large and can be repressed a few months after CNS transduction, risking the long-term performance of single-dose gene therapy applications. We used a whole-CNS screening approach based on systemic delivery of AAV-PHP.eB, iDisco+ tissue-clearing and light-sheet microscopy, to identify three small latency-associated promoters (LAP) from the herpesvirus pseudorabies virus (PRV). These promoters are LAP1 (404bp), LAP2 (498bp) and LAP1_2 (880bp). They drive chronic transcription of the virus encoded latency-associated transcript (LAT) during productive and latent phases of PRV infection. We observed stable, pan-neuronal transgene transcription and translation from AAV-LAP in the CNS for six months post AAV transduction. In several CNS areas, the number of cells expressing the transgene was higher for LAP2 than the large conventional EF1α promoter (1264bp). Our data suggests that the LAP are suitable candidates for viral vector-based CNS gene therapies requiring chronic transgene expression after one-time viral-vector administration.

151. AAV-S: A Novel AAV Vector Selected in Brain Transduces the Cochlea with High Efficiency

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Recent years have seen the development of new AAV vectors, designed to target specific organs and cell types with high efficacy. Many of these utilize 7-mer peptide insertions that modify the transduction properties of a particular AAV serotype. However, these vectors can often be repurposed to transduce other tissues, including those that are difficult to treat, such as the inner ear. Transducing certain cell types in the inner ear - such as hair cells - remains a challenge, with many conventional AAV vectors failing to consistently target one class of hair cells, outer hair cells (OHCs). We previously reported the development of new vectors using a Cre-based expression library system called iTransduce. We used this to generate vectors capable of crossing the blood brain barrier and

transducing the brain. Of the two vectors tested, one (AAV-F) showed great promise and high CNS expression, whereas the other (AAV-S) did not. While systemically injected AAV-S did not cross the blood-brain barrier effectively, it transduced a variety of tissues including heart, liver, and muscle. After direct injection into the brain, AAV-S displayed high local transduction efficiency in neurons, even at relatively low doses. To assay its transduction properties in the inner ear, we produced AAV-S encoding a single-stranded expression cassette driving EGFP under the CBA promoter, and injected it in neonatal (P1) mice via the round window. We found that AAV-S transduces hair cells of the cochlea extremely well. Both inner and outer hair cells were transduced with efficiencies of up to 100% and 99% (Fig. 1a, b) at the dose tested ($2x10^{10}$ VG). We also observed significant transduction of the spiral limbus and spiral ganglion (Fig. 1c, d). We are now examining the transduction efficiency of AAV-S in other organs of the inner ear, in adult mice, and in deafness disease models. Overall, we show here that AAV-S is a promising preclinical candidate for genetic therapies of the inner ear.

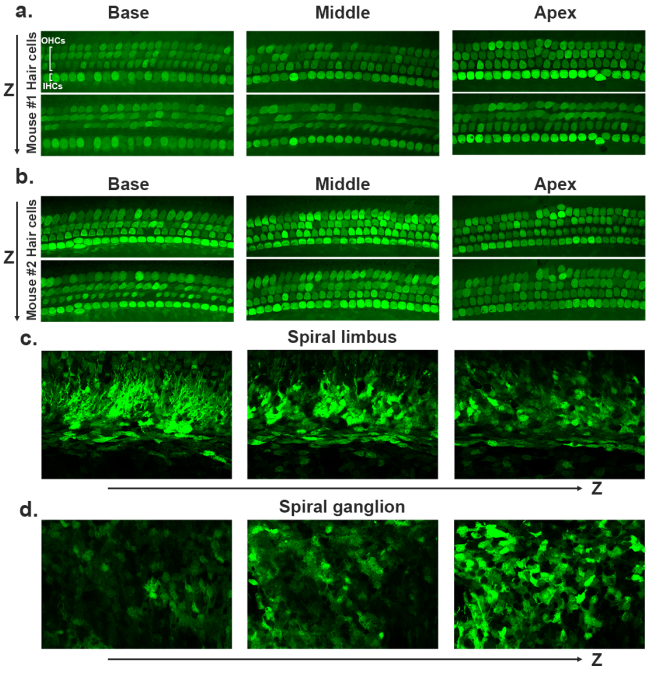

Figure 1: GFP fluorescence following AAV-S-CBA-GFP administration to the inner ear. (a,b): Representative images of cochlear sensory epithelium transduced with AAV-S (63x magnification). (c): Transduction in the spiral limbus. (d): Transduction in spiral ganglion region. Z and arrow indicates different layers of Z-stack. OHC: outer hair cells. IHC: inner hair cells.

152. Abstract Withdrawn

153. Utilization of Single-Particle Tracking to Identify and Characterize Cellular Glycan Receptors of Novel Adeno-Associated Virus Variants

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Adeno-associated viruses (AAVs) have gained a lot of attention in recent years, because they can serve as safe and reliable delivery vehicles for human gene therapy. Among the many fascinating aspects of AAVs is the existence of their many natural variants, some with the capacity to uniquely target various organs/tissues in mouse, non-human primates, and humans in a capsid-dependent manner. To develop more efficacious and safer AAV-based gene therapeutics, it is crucial to understand the receptorology of different AAVs. It has been reported that AAVs utilize different glycan receptors such as N-linked sialic acid, O-linked sialic acid, galactosides, or heparin sulfate proteoglycans (HSPG), for cell surface recognition. However, a majority of these studies were conducted *in vitro*, using cultured cells that express a mixture of sialic acids. Unfortunately, the exact sialic acids that are crucial for virus recognition cannot been assessed with these model systems. Recently, we discovered a novel AAV2 variant named AAVv66, which is ~13-fold more neurotropic than AAV2; yet, its capsid sequence is only 13 amino acids different from that of prototypical AAV2. To further understand the AAVv66 capsid, particularly if it binds to glycan receptors, we hypothesized that single-particle tracking (SPT) may provide both qualitative and quantitative information on AAV-receptor interaction and binding at single-virion resolution. The SPT assay uses an artificial and flat lipid membrane called a supported lipid bilayer (SLB), which is coated onto a glass substrate to mimic cell membranes. The composition of the SLB is fully controllable, so we can fix one glycan type (or more) at a time for each experiment. Combining SLB with total internal reflection fluorescence (TIRF) microscopy allows us to focus on a ~100 nm-thick interface above the glass substrate to visualize, with high signal-to-noise ratios, a fluorescently-labeled AAV particle binding to the target membrane. In this report, we will present our findings in developing, validating, and optimizing SPT to distinguish and characterize major glycan molecules that govern AAV tropism selection. Our study sheds light on fundamental AAV virology, which may provide crucial information in designing effective delivery strategies and patient treatment in the future.*Co-corresponding authors

154. AAV2-Based Capsid Variant, P2-V1 Avoids Neutralization by Anti-AAV Neutralizing Antibodies in Human Vitreous Samples

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PURPOSE: Intravitreal injection (IVI) of AAV is a promising approach for treating the retina as it can be performed in clinic rather than a surgical suite, thereby increasing accessibility of gene therapies to larger patient populations. However, the limited clinical data, and non-human primate (NHP) studies utilizing currently available capsids indicate that the barriers to IVI AAV may restrict gene expression to levels that are potentially 'sub-therapeutic' in a substantial proportion of patients. We previously screened an AAV2-based capsid library in IVI mice and macaques for AAV variants capable of overcoming these barriers. Subretinal injection (SRI) of AAV5-GFP was done to create GFP sortable photoreceptor cells prior to delivery of AAV library. The most enriched variant, "P2-V1", transduced IVI mouse retinas with equal efficiency to our benchmark, AAV2(quadY-F+T-V), but had reduced potency relative to other identified variants. A comparison of P2-V1 and AAV2 amino acid sequence revealed 3 substitutions (out of sixteen total) previously shown, individually, to confer resistance to AAV neutralizing antibodies (NAb). The purpose of our study was to ask whether enrichment of P2-V1 was driven by an additional selective pressure, more specifically, the ability to avoid neutralization by pre-existing NAbs against AAV. **APPROACH**: Self-complementary smCBA-mCherry vectors were packaged in AAV2, AAV7m8, or P2-V1 via triple transfection. Vitreous samples collected from patients undergoing vitrectomy were screened for the existence of anti-AAV2 NAbs. ARPE19 were infected with AAV2-mCherry in the presence of vitreous at a 1:4 dilution. Vitreous samples (n=15) were classified as 'inhibitory' if they resulted in a >95% reduction in transduction by AAV2. Inhibitory samples were then used to evaluate the relative performance of AAV2 and P2-V1 vectors across a range of dilutions. Later, AAV7m8 vector, a capsid currently under consideration for IVI delivered gene therapy, was added to experiments. mCherry expression was quantified via flow cytometry and used to determine the NAb50 titer (reciprocal dilution at which 50% inhibition of transduction occurs) for each vector in each vitreous sample. **RESULTS:** In all of the inhibiting vitreous samples tested, the NAb50 for P2V1 was lower than or equal to that for AAV2 and AAV7m8. Head to head relative to AAV2, P2V1 had a lower NAb50 in 10/15 samples (67%), in some cases by as much as 64 fold. In samples where both P2V1 and AAV7m8 were tested, P2V1 outperformed AAV7m8 (i.e. had a lower NAb50) in 3/8 samples. Interestingly, in samples where AAV7m8 outperformed AAV2, P2V1 also outperformed AAV2. Screening of additional patient vitreous samples is underway. **CONCLUSIONS**: P2-V1 is a promising AAV2-based variant identified via directed evolution in IVI macaques. This novel capsid displays improved transduction of retina following IVI as well as the ability to avoid neutralization by anti-AAV antibodies. P2-V1 outperformed parent capsid AAV2 as well as AAV7m8 in neutralization assays performed using human vitreous sample harboring anti-AAV NAbs. These results demonstrate the potential for P2-V1 as a gene therapy vector in patients with pre-existing immunity to AAV. Future experiments will explore the immune avoidance capabilities of P2-V1 *in vivo* in the presence of anti-AAV NAbs. We are also investigating whether P2-V1 can be improved by rational design aimed at increasing intrinsic transduction efficiency as well as NAb avoidance.

155. Method for High-Throughput, In Situ Characterization of AAV Variant Pools in Intact Tissue Using Ultrasensitive Sequential FISH

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Extensive efforts have been made to engineer adeno-associated viruses (AAVs) with desirable characteristics, such as enhanced transduction efficiency and tissue- or cell-type specific tropisms. In-vivo selection, followed by next-generation sequencing (NGS)-based screening, has enabled us to uncover novel viral capsid variants, such as the AAV-PHP series (Deverman et al., Nat Biotech, 2016; Chan et al., Nat Neurosci, 2017; Kumar et al., Nat Methods, 2020). Despite successful library-based selections, the characterization of viral tropisms is slow and labor-intensive and is thus limited to only a handful of variants. To overcome this bottleneck and allow for high-throughput screening, we introduce an imaging-based approach that detects viral transcripts in intact tissue by using ultrasensitive, sequential fluorescence in situ hybridization (FISH). We first developed a new FISH method to enable detection of relatively low abundance viral transcripts compared to endogenous genes in tissue. Compared to two signal amplification methods, rolling-circle amplification (RCA) and hybridization chain reaction (HCR), our method resulted in a 2.7- or 6.7-fold higher signal-to-background ratio, respectively, with the same number of probes. The high sensitivity of our method also allowed us to detect RNA transcripts with 1 probe and distinguish capsid variants packaging an identical viral genome with a short mutated region (7 amino acids, equivalent to 21 base pairs) transduced in HEK293T cells. We also developed an efficient two-step probe stripping method to enable multiple rounds of labeling (up to 8), which increases the number of targets that can be characterized in the same tissue beyond the spectral limit (e.g., 4 colors x 8 rounds = 32 variants). The high sensitivity and ability for sequential labeling allowed us to examine the cell-type tropism of capsid variants and/or gene regulatory elements in intact tissue. For this purpose, we generated AAV pools, comprising a combination of novel AAV-PHP.B-like capsids and cell-type specific promoters, that package the same coding sequence with a unique barcode in the 3'UTR. The pool was injected into one animal at a low dose (~1e10 for each), and after 3-4 weeks of injection, the transcripts of each variant were detected with a custom probe set targeting the unique barcodes. As a proof-of-concept, we were able to characterize the cell-type tropism of 6 variants in one tissue within 4 hours. Further refinement of barcode designs (e.g., temporal barcoding or in situ sequencing) and single-molecule imaging will allow us to either reduce the screening time or increase the number of variants that can be characterized to hundreds. These approaches enable high-throughput characterization of virally delivered transgenes in intact tissue, thus complementing the active field of viral vector engineering with scalable tropism identification

or validation. Moreover, visualizing the distribution of many variants while preserving spatial context will offer insights into AAV biology, which can include entry mechanisms as well as cell- and tissue-type associated expression.

156. SMRT Sequencing Allows High-Throughput Analysis of a Whole Capsid Shuffled AAV Capsid Library Following CNS Selection in Mice and NHPs

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The outcomes from AAV capsid engineering efforts since its discovery have been both encouraging and promising, but it remains a major challenge to engineer AAVs that are seamlessly compatible across mouse and non-human primate (NHP) species. The use of AAV variant libraries coupled with various selection strategies has become an increasingly useful strategy to generate novel AAV variants with enhanced properties. However, analysis of capsids recover post-selection has been hampered by the inability to accurately sequence the entire capsid gene in a high-throughput manner. As a result, many library approaches are shifting to small changes in the capsid such as peptide insertions or barcodes, which can be sequenced quickly and easily. This has restricted the diversity of capsids in libraries, shifting the field away from whole capsid shuffled libraries. We suggest that this could restrict libraries to select for relatively incremental changes in vector tropism. To overcome this and enable the use of whole-capsid changes to be evaluated, we employed single molecule real-time (SMRT) sequencing analysis for a high throughput evaluation of recovered whole AAV capsid genes. SMRT sequencing has the advantage of sequencing the entire capsid gene in a single read with high accuracy, enabling sequencing the entire shuffled capsid rather than only an insert region or barcode. We hypothesized that paralleled AAV whole capsid shuffling and directed evolution in both mice and NHPs have the propensity to yield AAVs that are compatible across mice and NHPs and that will be serologically distinct from natural AAVs. As first steps to test this hypothesis, we have developed a new approach which consists of AAV whole capsid shuffling and directed evolution in mice and NHPs conducted in parallel. The AAV library was subjective to mainly target tissues of the central nervous system (CNS) in these animals following intrathecal (IT) injection. SMRT sequencing was able to identify over 15 thousand distinct whole AAV capsid sequences present in the library. After the first round of IT injection of this library in mice, SMRT sequencing analysis of the AAV genomes that were recovered post injection showed that 65 new capsids were recovered from mouse brain and 31 new capsids were recovered from mouse spinal cord. IT injection of this library in NHPs revealed that hundreds of unique AAVs from the library were able to target the brain while over a thousand unique AAV variants targeted the spinal cord. In depth analysis of the sequencing data revealed that several of the AAVs that targeted the mouse brain were also able to target regions of NHP brain. Currently, we are screening the viruses that are present at the highest frequency in mouse and NHP brains by packaging them with GFP followed by IT injection in mice. The analysis of selected library clones thus far demonstrates the utility of SMRT sequencing for high-content screening of whole capsid shuffled libraries, which was previously limited to analysis by capsid gene subcloning and Sanger sequencing. This approach should expand the options for library-based AAV capsid development beyond barcoded analysis and/or peptide insertion libraries.

157. AAV Capsid Assembly Using *Escherichia Coli*

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Virus-like particles (VLPs) have been used for numerous therapeutic applications, especially in vaccination and drug delivery. While the recombinant adeno-associated virus (rAAV) has emerged as a leading candidate in gene therapy with approved products, rAAV production in HEK-293 or insect cells still remains a challenge. We established and analyzed AAV VLP production using *E. coli* cells. Such VLPs could be used as a bio-nanoparticles and also serve as a starting material for DNA encapsidation for production of rAAV type moieties. To do this, first the widely used serotype-AAV2 was chosen. The codon usage of the AAV2 VP3, the main structural capsid protein, coding gene was optimized, cloned into a pET vector, expressed effectively in inclusion bodies in *E. coli* and purified by ion-exchange chromatography. AAV2 VLPs were obtained by a chemically defined refolding reaction. Specifically, the purified protein in a denaturing buffer with 5 M GuHCl was dialyzed against an assembly buffer containing 2 M L-arginine for 60 h. In addition, we also tested for an assembly-activating-protein (AAP)-independent AAV serotype. VP3 capsid protein of AAV serotype 5, the most genetically divergent AAV serotype among known 13 human and non-human primate serotypes, was successfully expressed in soluble form and able to develop AAV5 VLPs inside *E. coli*. The correct formation of AAV2 and AAV5 VLPs was confirmed by ELISA with anti-intact-AAV antibodies and imaging by atomic force microscopy. The biological transduction functionality was confirmed with an internalization assay into human HeLa cells. Furthermore, to increase VLP production and assess the effect of AAP on capsid assembly, we expressed and purified AAP2 and added the protein to the *in-vitro* assembly reaction of AAV2 VP3. For AAV5 VLPs, due to the formation of capsid within *E. coli*, we co-transformed AAP5 and AAV5 VP3 genes into *E. coli* cells using two pET vectors harboring different antibiotic resistance genes. The results showed that AAP2 directly aided AAV2 VLPs formation and that AAP5 supported AAV5 capsid assembly inside *E. coli*. To our knowledge, this is the first report of AAV VLP production within a chemically defined reaction or inside *E. coli* cells. Even though the VLP production needs to be optimized in the future, our finding opens an opportunity to explore AAV VLPs in biomedical applications and paves the way to investigate rAAV production using *E. coli*.

158. Investigation of the Hepatocyte Population Amenable to Gene Targeting by rAAV-Mediated Homologous Recombination in Mice

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The liver is one of the most attractive target organs for gene therapy using recombinant adeno-associated viral (rAAV) vectors. Nevertheless, AAV-mediated liver gene transfer has several challenges. For example, in the case of neonatal or pediatric patients, loss of episomal rAAV vectors results from liver growth resulting in a decrease in the expression of the therapeutic gene. In order to expand the clinical application of rAAV, our group previously reported a strategy using a rAAV vector for homologous recombination (AAV-HR) without the use of nucleases(Barzel, et al, Nature, 2015) in which we can specifically target a therapeutic coding sequence onto the end of a endogenous coding gene before the translation termination signal. Thus a chimeric mRNA is produced that makes both the endogenous and therapeutic protein .In current and prior studies, we found the efficiency of AAV-HR in mouse liver is <1% of hepatocytes, but it is unclear whether these rare HR events are stochastic or if there is a sub-population of hepatocytes amenable to AAV-HR. Thus, the purposes of this study is to determine if a limited number of hepatocytes can undergo targeted HR, as well as identify the determinants of such a population. To address this question, we made two different AAV-HR vectors that target two loci - albumin (AAV-HR-alb-GFP) and Apoc1 (AAV-HR-apoc1-tdTomato), both of which are highly and specifically expressed in hepatocytes. We have co-injected mice with both vectors and then now analyzing fluorolecsent imaging data from liver sections. If the HR process is stochastic, we expect to find at most 1%x 1%= 0.01% percent red/green or yellow hepatocytes. If the numer of green/red (yellow) hepatocytes is much greater than 0.01%s it suggests there is a finite population of hepatocytes amenable to AAV-HR. The preliminary results sugges there may be a defined population of amenable hepatoyctes. In addition, we are also investigating the character of AAV-HR positive hepatocytes, including the status of DNA synthesis and cellular proliferation. The insights from our ongoing experiments will give us a more complehensive understanding of AAV-HR and help us develop better ways to improve its efficiency for clinical applications.

159. Applying Machine Learning to Predict Viral Assembly in AAV2 Capsid Libraries

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Machine learning (ML) has aided in novel discoveries across numerous fields, including medicine and virology. Viral gene therapy is lagging behind these fields when it comes to applications of ML. Specifically, big data gathered through next-generation sequencing (NGS) of complex capsid libraries is an especially prominent source of lost potential in data analysis and prediction. Furthermore, adeno-associated virus (AAV) based capsid libraries are becoming increasingly popular as a tool to select candidates for gene therapy vectors with increased transduction efficiency, higher capacity for antibody evasion, and other desirable properties. These higher complexity AAV capsid libraries have previously been created and selected *in vivo*; however, applying neural network algorithms to analyze and predict the viability of capsid assembly *in silico* may augment smarter and more robust libraries for selection. In this study, NGS data of AAV capsid libraries gathered before and after viral assembly are used to train machine learning algorithms in predicting whether newly designed variants will form a virus-like particle. We found that a 78% accuracy of predicting capsid assembly is possible. Furthermore, this model was used to test hypothetical mutation patterns in library construction to suggest the importance of three specific residues in the AAV2 capsid. In order to biologically validate these findings, two mini-libraries were constructed using a machine-guided approach. One mini-library contains mutations to residues predicted to be critical in capsid assembly using this neural network. The second mini-library contains mutations to residues predicted to be non-critical in capsid assembly. We found that the critical residue mini-library witnessed a significant drop in complexity as compared to the non-critical residue mini-library, further validating the model's ability to predict capsid assembly. Finally, we propose potential applications for this ML algorithm, such as assisting the rational construction of future libraries and establishing a foundation for *in silico* selection of variants.

160. A Computational and Experimental Platform for Detecting Full Transcriptome Cell Type Tropism of Lowly Expressed Barcoded and Pooled AAV Variants via Single-Cell RNA Sequencing

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Despite being one of the primary gene therapy delivery vehicles, adeno-associated viruses (AAVs) are limited in their specificity towards certain cell types implicated in disease. Recombinant AAVs (rAAVs) are addressing these limitations through both capsid engineering and gene regulatory approaches that alter viral tropism or viral expression patterns. Current rAAV targeting, selection, screening, and characterization methods are typically based on single- or few-molecule read-outs, such as promoter and enhancer-driven constructs, mouse lines expressing Cre recombinase under a cell type-specific promoter, or cell type-specific antibodies for imaging. Such methods harbor challenges for parallelizing rAAV characterization, or extending characterization and engineering to complex or previously unknown cell types. The recent advent of single-cell RNA sequencing (scRNA-seq) has revealed a rich diversity of cell types and states, many of which are not associated with canonical cell type markers, and can even be defined by multi-gene programs. To aid in the engineering of rAAVs aimed at such complex cell states and aid in the discovery of novel tropisms, we have developed a scRNA-seq AAV screening method, whereby we inspect full transcriptomes of cells transduced with pools of AAV vectors in a single animal. To generate pools of variants that can be differentiated in sequencing, we package variants with either unique transgenes, or the same transgene with unique barcodes incorporated

in the polyA region. We then co-inject mice with these pools of variants, wait for expression, and harvest tissue slices for downstream cell dissociation and single-cell sequencing using the Chromium 10X Single Cell Kit. In order to accommodate the low expression rates of virally delivered cargo and the loss of the region of mRNA upstream of the polyA capture site that identifies the capsid variant, we amplify the viral transcripts from the full cDNA library with primers near the differentiating region of the cargo. To characterize variants, we developed a customized computational pipeline that addresses the unique challenges of these datasets: (1) to discern the variant that delivered each transgene read, we demultiplex the amplified viral transgene reads based on their differentiating sequences; (2) to reduce the effects of PCR amplification noise, we convert variant transgene reads into probabilistic estimates of the number of transcripts per cell; and (3) to calculate cell type biases, we automatically identify a cell type hierarchy and compare the distribution of viral transcripts by cell type to a null model of empty droplets. Thus far, our platform has corroborated several expected virus tropism findings from imaging (e.g. for brain vasculature or neuronal preference). To apply this barcoding strategy to even larger pools without individually cloning and producing each variant, and link arbitrary mutations in the capsid genome to the barcode, we have further developed a plasmid that contains both the expressed transgene and the capsid gene, but inverted in orientation, with their 3' ends adjacent. With these barcoding strategies and computational methods, we enable fast identification and characterization of rAAV variant pools with precise disease-relevant tropisms, with the ultimate goal of aiding the gene therapy field in developing precision delivery vehicles.

161. Determination of Full vs Empty Ratio of AAV Vectors with Qubit Fluorometric Quantification Method

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Adeno-associated virus (AAV) vectors purified by column chromatography method generally consist of a mixture of empty and full virus particles, whereas AAV vectors purified by density gradient ultracentrifugation method generally consist of full virus particles. Since the empty virus particles in an AAV product are considered contaminants, it's critical to accurately determine the quantity of empty capsids in any AAV product so that the experimental results could be better interpreted. Currently there are several methods available in the gene therapy field for us to determine the ratio of empty vs full virus particles. One uses transmission electron microscopy (TEM) method to determine the empty vs full ratio by observing the staining of virus particles. However, this method suffers from false full or empty virus images due to the variation of staining process. A second uses ELISA method to determine the total virus particles and QPCR method the total virus genomes so that the ratio of empty vs full virus particles can be calculated. Due to the fact that QPCR titer can vary significantly and therefore the empty vs full ratio is generally not very reliable. A third method uses analytical ultracentrifugation method in which the empty and full virus particles are separated at different density and the ratio can be calculated based on the total protein content in the empty and the full fractions. Recently it has been reported that a size-exclusion chromatography (SEC) method using multi-angle light scattering (MALS) is able to determine the AAV empty vs full particle ratio. However, all these methods not only require expensive equipment, reagents, and long process time, but also have significant variations. We developed a simple, universal and economical method using the Qubit Fluorometric Quantification kit to quantify the contamination of the double-stranded DNA (dsDNA) outside of the virus particles, the single-stranded DNA (ssDNA) genome inside the virus particles, and the virus particles by using the dyes that specifically stain the dsDNA, ssDNA, and proteins. Based on the results, we were able to measure the total residual host cell DNA contamination, the total virus particles and the empty vs full particle ratio with comparable precision to the SEC-MALS method. This novel method should provide an additional tool for further characterization of AAV vectors.

162. Systematic Assessment of AAV Serotype Efficiency Reveals Regional Heterogeneity in Cellular Tropism and Infectivity in the Adult CNS

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Direct reprogramming of cell identity from a glial to neuronal phenotype has been demonstrated using viral vectors to deliver reprogramming transcription factors to glial cells. Efficiency and vector tropism have a major impact on the feasibility to reprogram a sufficient number of new neurons for meaningful functional integration. Previously, we have successfully targeted and reprogrammed NG2 glia to neurons in vitro and in vivo using retroviral vectors to target the actively proliferating NG2 cell population. rAAV vectors are an alternative delivery platform frequently used to achieve efficient and widespread in vivo gene delivery. However, no systematic assessment of AAV serotype efficiency in targeting NG2 glia has been reported. We evaluated tropism and infectivity of multiple AAV serotypes using a CMV-eGFP construct in the following AAV serotypes: 1, 2, 4, 5, 6, 6.2, 8, 9, rh10, DJ, PHP.s, PHP.B, PHP.eB. All vectors used were obtained from the University of Iowa Vector Core Facility and adjusted to a standard titer and volume for intracerebral delivery of each AAV serotypes into rat cortex, white matter, and striatum. We subsequently examined brains by confocal microscopy to evaluate number and phenotype of infected cells. Intracerebral delivery of AAV serotypes revealed substantial differences between the volume of tissue infected, with the largest volumes seen with PHP.eB>rh10>PHP.B>5>8. Comparing the number of GFP+ cell in a standardized cortical region, serotypes 1, 9, PHP.B, and PHP.s infected an equivalent number of cells to AAV2, while the number of GFP+ cells with serotypes 4, 5, and PhP.eB was substantially less. AAV8 produced the highest number of cortical GFP+ cells, 3.5x more than AAV2, with 8>10>DJ=6=6.2. Cellular tropism also varied widely between serotypes. In the cortex, half of GFP+ cells were NeuN+ neurons and the number of neurons infected was similar for DJ and PHP.eB. All other serotypes infected substantially fewer neurons. For GFAP+ astrocytes, serotypes 1

and DJ were similar to AAV2, while 4, 8, and PHP.eB infected substantially fewer astrocytes. The most astrocytes were infected by PHP.s (3x that of AAV2) with PHP.s>PHP.B>5=6=6.2=9=10. For Olig2+ oligodendrocytes, serotypes 1, 5, and 9 were similar to AAV2, while 4, 6.2, PHP.B, PHP.eB, and PHP.s infected substantially fewer oligodendrocytes. AAV8 was highly efficient at targeting oligodendrocytes (6.5x more than AAV2) with 8>10>DJ>6. The results found in the cortex differed from injections into white matter or striatum, revealing regional heterogeneity in both number and phenotype of infected cells. For example, striatal AAV8 delivery infected only half as many cells as in the cortex and the number of oligodendrocytes was a third of that seen in cortex. Striatal AAVrh10 delivery infected an equivalent number of cells as in the cortex, but the number neurons infected was 4.5x greater than in the cortex. These data reveal substantial regional heterogeneity and tropism between AAV serotypes, suggesting this variability should be considered when comparing and planning studies. Although many serotypes infected cultured NG2 glia, virtually no serotypes targeted NG2 glia in vivo in any region with only a few NG2+ cells observed with DJ and PHP.s, suggesting that infectivity in vitro is not always a valid predictor of in vivo performance. These data also suggest that targeting reprogramming constructs to NG2 glia will require designing new and specific AAV serotypes or utilizing alternatives to AAV. Supported by NIH NS100514

163. Optimization of Single AAV CRISPRi

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The power of CRISPRi for clinical applications has been inhibited by delivery challenges due to the small optimal packaging limitation of AAV and the relatively large size of Cas9, KRAB, and the need for two promoters and the guide RNA. We addressed this through optimization of *S. Aureus* dCas9 nuclear targeting and KRAB fusion, and the selection of effective mini promoters to result in a single AAV vector that contains all CRISPRi components, including the guide RNA, and remains within 4.7kb. We used Pcsk9 as our initial target for proof of principle. Our single pAAV.Pcsk9CRISPRi construct decreased Pcsk9 mRNA levels 3-fold and secreted protein levels 3.5-fold *in vitro*. We performed a tail vein injection of 1x10e11 rAAV.Pcsk9CRISPRi or a negative control, rAAV.gstop into mice and detected a 35% decrease in serum Pcsk9 protein only with the rAAV.Pcsk9CRISPRi. This single AAV approach provides a clinically-translatable method for decreasing gene transcription of specific genes by CRISPRi in any tissue or targetable cell of interest.

164. AAV Biology Imaging Platform: Uncovering the Cellular Mechanisms Behind the AAV5-Based Vector Delivery System

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Introduction: The efficiency of gene therapy delivery is partially dependent on the events that control adeno-associated virus (AAV) from attachment in the membrane to vector DNA release and transgene expression. This suit of events can be regarded as multi-step intracellular barriers and need to be fully understood. Therefore, we developed an Imaging Platform which enables the study at the cell and subcellular level of all the steps taken during the transduction process. The AAV Biology Imaging Platform supported the visualization by immunocytochemistry (ICC) and/or fluorescent *in situ* hybridization (FISH) of the different cellular organelles, the AAV capsid and the AAV vector DNA and their possible interactions. Combining ICC and FISH to tracing down the events involved in the AAV capsid binding, processing and vector DNA release into the target cells will provide us with crucial data not only for fully understanding but also optimizing our AAV-based delivery system. **Methods:** In order to investigate the different steps during AAV transduction, we developed an imaging platform to analyze and to quantify the interactions between AAVs and host cells using 2D and 3D images. Using HEK 293T cells, ICC protocol standardizations were performed for each antibody used targeting the different cell organelles. To trace down AAV capsid into the cell host, pre-labeled AAV5 capsids containing vector DNA/hFIX were used for transduction. Cells were inoculated with labeled capsids ($5x10^5$ viral particles/cell) as a pulse lasting for 40 minutes at 4°C. After that, cells were washed with PBS and then maintained in complete DMEM at 37°C in the incubator. For AAV5 visualization, samples were collected at 4 different time-points (from 0 to 2 hours post-infection; p.i.), while for vector DNA/hFIX detection, cells were collected in 12 different time-points (from 0 to 6 hours p.i.). When incubation time was completed, cells were washed in PBS and fixed in 4% PFA prior ICC and/or FISH. The latter was performed using probes specific for AAV-hFIX vector DNA/RNA following the manufacturer's protocol. After staining, images were acquired by a Leica TCS SP8 X DLS confocal microscope or by a ZEISS Scan Digital Slide Scanner or by a ZEISS Observer 7 microscope. For 3D images, stacks of 20-50 focal planes were captured at ~0.31 µm z-intervals through the depth of the cell. Images were processed for visualization and quantification using LAS X-LS (Leica Microsystems) or HALO (Indica labs) software. **Results:** By means of our AAV Imaging Platform we were able to visualize by ICC, individually or in combination, the plasma membrane, early endosomes, lysosomes, Golgi apparatus, microtubules and nuclear envelope at the cell level. The processing of labeled AAV5-hFIX capsids was visualized in transduced cells overtime. Additionally, the interaction between labeled AAV5-hFIX capsids and cell host organelles (i.e. plasma membrane, cell nucleus) was also successfully observed, demonstrating that the platform is suitable for co-localization type of analysis. Furthermore, vector DNA/hFIX positive cells were detected by FISH, and in combination with ICC, 3D image analyses showed that a higher AAV vector DNA/hFIX probe signal was detected inside the nucleus from 2 to 3,5 hours p.i., suggesting a time frame for AAV vector DNA release into the cell nuclei. In summary, we developed an Imaging Platform to visualize and to analyze at the cell and subcellular level the relevant steps of our AAV-based vector delivery system using 2D and 3D images. The flexibility in the platform (i.e. by adding new markers, different probes) allows us to expand its capabilities and deepen our understanding in the intracellular behavior of delivery viral vectors.

165. Analysis of AAV6.2FF Biodistribution and Transduction Efficiency in Mice When Administered by Five Different Routes of Injection

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Adeno-associated virus (AAV) vectors are rapidly progressing as gene delivery agents as evidenced by the regulatory approval of at least three AAV gene therapies. The tissue tropism of wildtype AAV serotypes has been carefully elucidated; however, as the field evolves towards using novel or rationally engineered recombinant AAV vectors designed to evade pre-existing immunity and/or increase transduction efficiency, it is important to determine the biodistribution and tissue targeting characteristics of these novel vectors. Here we investigated the biodistribution of AAV6.2FF in a comprehensive manner by comparing its tissue tropism and transducing efficiency when delivered by five different routes of administration. C57BL/6 mice were injected intranasally, retro-orbitally, intraperitoneally, intramuscularly or intravenously with an AAV6.2FF vector encoding a heat stable human placental alkaline phosphatase (AAV6.2FF-AP) reporter gene. At 6 weeks post-vector administration, mice were euthanized, and all major organs were harvested, stained for AP expression and imaged. Tissues were then divided such that half was cryopreserved in OCT and the other half was flash frozen at -80°C. Cryosectioned tissues were counterstained with nuclear fast red in order to determine cell tropism of the vector. Flash frozen tissues will be homogenized and alkaline phosphatase reporter gene expression quantified using an enzymatic assay. Gross analysis revealed markedly different biodistribution of AAV6.2FF depending on the route of administration (Figure 1). Intranasal administration of AAV6.2FF-AP led to robust transduction of the nasal cavity, trachea and lung parenchyma and a lack of transduction in any of the other major organs, including the brain. Retro-orbital and intraperitoneal administration of AAV6.2FF-AP revealed a much broader biodistribution. While retro-orbital administration of AAV6.2FF-AP targeted primarily the heart and liver with high rates of transduction, the lungs were also transduced, but at a lower efficiency than with intranasal administration. Intraperitoneal injection of AAV6.2FF-AP displayed a similar pattern of tissue transduction to that of the retro-orbital administration, with the distinct difference being the high transduction efficiency of the pancreas. These preliminary results demonstrate that AAV6.2FF tissue transduction is highly dependent on the route of administration and sheds light on which route of administration to use when targeting specific organs.

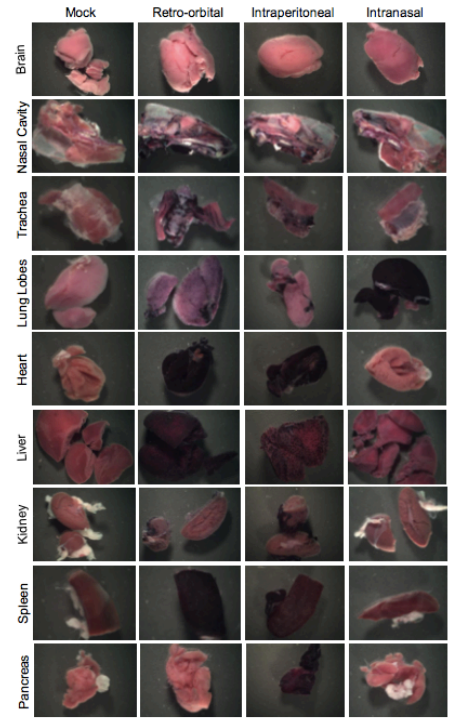

Figure 1. Representative gross images of alkaline phosphatase stained tissues from mice injected with AAV6.2FF-AP by different routes of administration. C57BL/6 mice were administered $1x10^{11}$ vg of AAV6.2FF-AP retro-orbitally, intraperitoneally or intranasally and tissues harvested 6 weeks later. All major organs were harvested and stained to detect alkaline phosphatase reporter gene expression.

166. Investigation of Potential Pseudo wtAAV Species Providing Replication Competence

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Background: The industry gold standard assay for analysis of replication competent AAV consists of amplification of AAV in the presence of a helper virus, followed by qPCR for either rep/inverted terminal repeats (ITR) junction or for rep and/or cap only sequences. However, a qPCR based read out targeting one single gene sequence is also able to detect other replicating variants than pseudo wtAAV carrying a rep-cap genome flanked by ITRs at both genome ends, therefore not necessarily reflecting replication competence of a pseudo wtAAV. In the end, any genome flanked by ITR sequences will be amplified and produce progeny virus in cells complemented with helper virus activity as well as Rep and Cap protein coding sequences. In order to investigate this further, we performed an in-depth study to characterize the primary potential replicating forms generated during rAAV manufacturing and what factors may influence their abundance. **Method**: We established a method to isolate highly concentrated DNA from packaged AAV particles after 2 rounds of infection in presence of Adenovirus 5. By qPCR and (nested-)PCR in combination with DNA sequencing we characterized the content (ITR, rep, cap), quantity, sequence orientation and functionality of packaged sequences and traced the results back to the molecular

design of the plasmids used for AAV manufacturing as well as to possible recombination events resulting in the formation of replicating rep and cap genomes. In this approach we compared a conventional plasmid packaging system carrying rep and cap sequences on the same plasmid with our unique FREELINE split-packaging system in which rep and cap genes are separated on two different plasmids. **Results**: Here, we provide data of an in-depth analysis of main potential replicating AAV species generated during rAAV vector packaging and show differences between vector preparations derived from the two alternative plasmid systems. We observe that emerging rcAAV species rely on the sequence arrangement of plasmids as well as the plasmid ratio used. We identified species which derive from homologous as well as from non-homologous recombination events and analyzed the packaged sequences for potential functionality of rep and cap. In this context, we confirmed the superiority of our split-packaging system for reducing the risk of formation of pseudo wtAAV particles containing functional rep and cap genes. **Discussion**: Our results show that rcAAV levels determined by conventional (regulatory-accepted) assays strongly depend on the sensitivity of the assay and the chosen read-out, showing examples for over- and underestimation of rcAAV titres. Furthermore, our results indicate that the quality of vector preparations manufactured with different platforms using different plasmid systems with respect to rcAAV payload cannot be compared when relying on an industry standard rcAAV assay. The analytical tools established here provide insight into molecular events resulting in the composition and formation of replicating forms other than rAAV vector sequences such as rcAAV which is a new, highly valuable quality indicator for vector batches and the plasmid packaging system used.

167. Data-Driven Identification of Capsid Residues That Can Define AAV Tropisms

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Adeno-associated viral (AAV) vectors can transduce many different types of cells and tissues, but they each have a preferential target that leads to very distinct transduction tropisms in different cell lines and the differences in each AAV's ability to transduce any particular cell type can be very drastic. Taking HEK293T cells as an example, AAV1, 2, 3B, and 6 have a very high transduction efficiency in this cell line, while AAV4, 5, 7, 8, and 9 have very poor transduction capabilities. There are many factors that have been discovered to be important variables in determining the transduction efficiencies AAVs in cells, like receptors such as AAVR or co-receptors such as galactose. Some AAV vectors are strongly reliant on such factors, and previous works were able to make use of such information to increase the transduction efficiencies of AAVs in specific cells by overexpressing the dependent factors in cells or by transplanting receptor binding footprints from one AAV to another (Pillay et al. 2016; Shen et al. 2012). However, these previous works required the knowledge of specific receptors or receptor binding motif in order to build a specific cell line or virus capsid capable of transduction. To further expand our capability to identify important factors and features on AAV capsids responsible for controlling transduction efficiencies, we present here a data-driven method to help elucidate the important features on AAV capsids responsible for transducing HEK293T cells and generate novel mutants with enhanced transduction capability. We performed multiple sequence alignment and then encoded each residue using the corresponding biophysical and biochemical properties. Using these inputs, we then trained a machine learning algorithm to identify key residues that might be responsible for transducing HEK293T cells efficiently. We were able to identify 8 amino acids that may each be capable of increasing the transduction efficiency of AAV9 - a serotype with poor transduction efficiency in HEK293T cells. Our preliminary data demonstrate that three out of the eight identified residues increase AAV9's transduction efficiency in HEK293T cells. Continued efforts can enable the generation of new AAV capsid mutants with desired tropisms.

168. AAV-Mediated Expression of Monoclonal Antibodies Protects Against Respiratory Syncytial Virus Infection in a Murine Model

Amira D. Rghei[1], Xuiaoyan Zhan[2], Matthew M. Guilleman[1], Laura P. van Lieshout[1], Khalil Karimi[1], Byram W. Bridle[1], James E. Crowe[2], Sarah K. Wootton[1]

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Vectored monoclonal antibody (mAb) expression mediated by adeno-associated virus (AAV) gene delivery has been demonstrated to generate protective and sustained concentrations of therapeutic mAbs in animal models for a variety of infectious diseases. Our rationally engineered AAV6 triple-mutant capsid, AAV6.2FF, facilitates rapid and sustained mAb expression following intramuscular administration. Given the impressive transduction efficiency of AAV6.2FF in the lungs and its ability to promote long-term mAb expression in the serum, we aimed to expand our AAV6.2FF-mAb platform for protection against respiratory infections of public health importance. Respiratory syncytial virus (RSV) infection causes acute lower respiratory tract infections and is an important cause of death in young children (<5 years of age) in low resource settings. Additionally, RSV infection is now recognized as a significant issue in the elderly and those who are immunocompromised. To date, there are no licenced vaccines to combat RSV. Prophylaxis of high-risk infants with palivizumab, a humanized murine mAb that binds to antigenic site II on the pre- and post-fusion conformation of the RSV fusion protein (F), is moderately effective in prevention of hospitalizations. More potent antibodies now are available and could be more effective. We recently identified a potent human mAb, designated RSV90, that binds to a highly conserved epitope in antigenic site V in the pre-fusion form of RSV F. Here we evaluated AAV6.2FF-mediated expression of RSV90 or palivizumab in mice and assessed the protective efficacy of AAV-vectored mAb expression in an RSV challenge model. Full-length RSV90 and palivizumab antibody genes were cloned downstream of the CASI promoter into an AAV vector containing a WPRE sequence. Intramuscular (IM) injection of mice with $1x10^{11}$ vector genomes (vg) of AAV6.2FF-RSV90 or AAV6.2FF-palivizumab resulted in rapid, robust, and sustained antibody expression in the serum beyond 10 weeks post-administration. Antibodies were also detected at numerous mucosal surfaces, including the lung, vagina, small intestine and peritoneal cavity for both vectors. Interestingly, intranasal (IN) administration of the two AAV vectors resulted in mAb expression exclusively in the

lungs. C57BL/6 mice injected with $1x10^{11}$ vg of AAV6.2FF-RSV90 or AAV6.2FF-palivizumab IM were protected completely from intranasal challenge with $1x10^{6}$ PFU RSV strain A2. Future directions include evaluating the protective efficacy of the two AAV-vectored mAbs in a cotton rat model, a more permissive model for RSV replication than mice. In conclusion, AAV-mediated antibody gene delivery is a promising method for extending the therapeutic effects of recombinant mAbs, which potentially could be used as a passive vaccination strategy for immunocompromised individuals or for use against pathogens for which effective vaccines are not available.

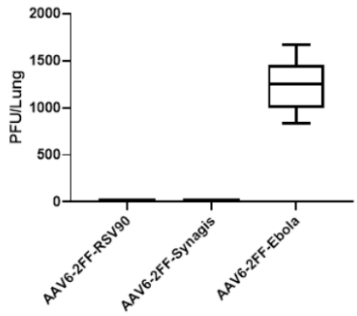

Figure 2. Mice expressing AAV6.2FF-palivizumab and AAV6.2FF-RSV90 completely protect mice from RSV challenge. Groups of C57BL/6J (n=8) were injected with AAV6.2FF-palivizumab (Synagis), AAV6.2FF-RSV90 or AAV6.2FF expressing an Ebola virus-specific antibody as a control. Four week-post AAV-mAb administration, mice were challenged with $1x10^{6}$ PFU of RSV A2 and sacrificed five days later. Lungs were homogenized RSV infectious units quantified. Both AAV6.2FF-palivizumab and AAV6.2FF-RSV90 showed no detectable infectious RSV in the lungs.

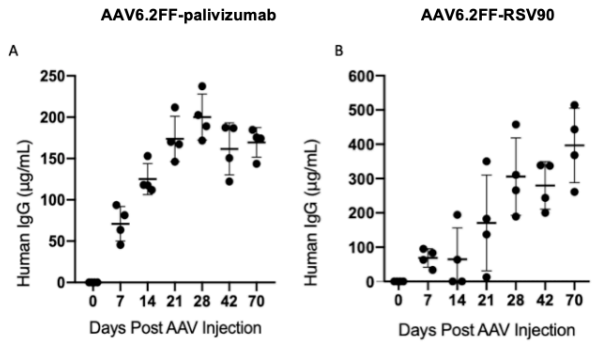

Figure 1. Serum antibody expression levels after intramuscular administration of $1x10^{11}$ vg of AAV6.2FF-palivizumab or AAV6.2FF-RSV90. Balb/c mice (n=4) were IM administered $1x10^{11}$ vg AAV6.2FF-palivizumab (A) or AAV6.2FF-RSV9 (B) and their serum human IgG levels monitored for 10 weeks.

169. Abstract Withdrawn

170. Abstract Withdrawn

AAV Vectors - Preclinical and Proof-of-Concept Studies

171. Customized Xenograft Model Development: Liver Repopulation of FRG KO Mice with Primary Murine, NHP, or Human Hepatocytes

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Background: Liver-humanized mice have proven to be of great use for in vivo studies in many application areas including infectious diseases, NAFLD/NASH, gene editing/therapy, metabolism, regenerative medicine, and pharmacology/toxicology. The FRG KO mice can be repopulated with hepatocytes from different species. This allows the replacement with "lower" species by, instead of using primates, for example, using a mouse transplanted with primate hepatocytes to achieve the same results. **Methods:** FRG KO mice were transplanted with hepatocytes from various sources. These include different species: mouse, rat, pig, non-human primate, or human. The level of repopulation correlated to the level of species-specific albumin concentration in the blood. Alternatively, the repopulation can be measured by IHC with a FAH specific staining. **Results:** Using a standardized protocol, we were able to generate FRG KO mice that were highly repopulated with a xenograft liver. On average, it takes approximately 12 weeks to repopulate the animal's liver with healthy hepatocytes. The percentage of repopulation was at least 70% and was consistently above 90%. Once repopulated, the xenograft remains stable for the remainder of the life of the animal. The liver-xenograft mice show a metabolism profile of the original donor species. This allows the testing of novel compounds in a small animal model. Moreover, these different xenograft models can be used to test the efficacy and tropism of viral or non-viral delivery of gene therapy in vivo. The test article dose needed is much smaller than what would be needed when testing in the original NHP model or in humans. **Conclusion:** FRG KO mice can be repopulated with hepatocytes from different species. These liver-xenograft mice can be used either for in vivo studies, or their hepatocytes can be harvested and used for in vitro applications.

172. Characterization and Correction of an *In Vitro* Model of Wilson Disease by Recombinant Adeno-Associated Virus (rAAV) Delivered *ATP7B* Transgene

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Wilson disease (WD) is a rare genetic disorder affecting roughly 1:50,000 people worldwide, caused by mutations in the *ATP7B* gene. In healthy hepatocytes, the copper-transporting ATPase 2 protein (ATP7B) produced localizes to the Trans-Golgi Network (TGN) and shuttles excess cellular copper in two major pathways: 1) direct

excretion of copper into the bile mediated by lysosomes or 2) the secretion of copper into the blood stream through a downstream copper transporter Ceruloplasmin (CP). Patients with WD present with a deficiency or complete loss of function of the ATP7B protein leading to toxic levels of copper primarily within the liver and the CNS. Excess copper levels drive the accumulation of several dysfunctional metabolic and neurological symptoms as the disease progresses. Current therapies aim to limit circulating copper leaking from the liver using chelators or remove the entire organ through an invasive transplant. Due to the toxicity and risks of these therapeutic strategies, treatment options that specifically address the underlying cause of the disease are needed. In order to target *ATP7B*, the genetic cause of WD, our approach involves using rAAV vectors generated from a HeLa producer cell line (PCL) carrying a modified human *ATP7B* transgene. Of the many rAAVs generated, containing various capsids, transgene sequences, and promoters, we identified the most therapeutically optimal vector. These vectors can transduce HepG2 cells and ideally correct the phenotypic deficiencies commonly seen in WD. To establish a cell-based model of WD, we developed an ATP7B KO HepG2 cell line, validated the specificity of the gene deletion, and confirmed the loss of ATP7B protein compared to the parental HepG2 cell line. Additionally, we applied several molecular and biochemical assays to assess copper sensitivity, ATP7B localization and trafficking through the TGN as well as in ATP7B KO cells treated with vehicle or rAAV-ATP7B. Overall, our ATP7B KO cell line functions well as an in vitro model system of WD and provides an efficient means of evaluating rAAV efficacy and potency prior to *in vivo* studies.

173. Abstract Withdrawn

174. Anatomical Characterization of AAV Chimeric Capsids in the Central Nervous System of Macaque Monkeys

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Introduction: Natural AAV capsids containing two primary receptors (i.e. AAV6 with the HS and Integrin motifs) can be leveraged to generate and characterize novel capsids, via rational design, that feature multiple receptor binding loci. For example, the galactose-binding footprint from rAAV9 and heparin sulfate-binding locus from rAAV2 have been engineered into chimeric capsids for gene delivery. Preliminary characterization has demonstrated that chimeric rAAV2g9 has the binding activity of both rAAV9 and rAAV2, thus providing gain-of-function over parental capsids. Further, mutant rAAV2g9 show enhanced features over the parental rAAV2g9. In rodents, rAAV2g9 features enhanced transduction capacity and minimal leakage from the central nervous system to mitigate off-target effects, compared with both parental capsids. A second capsid of interest, rAAV2-retro, was directly evolved in the striatum of rodents and selected for its ability to provide robust post-transduction retrograde transport. Our goal is to characterize and compare the properties of AAV2g9, rAAV2g9 mutants, and rAAV2-retro in the non-human primate brain. **Methods**: AAV2g9, double mutant rAAV2g9, and rAAV2-retro were injected into the prefrontal cortex, visual cortex, or superior colliculi of cynomolgus macaques (*Macaca fascicularis*). After a 2-month survival duration, we used neuroanatomical and histological approaches to assess (1) proximal neuronal transduction at the injection site and (2) distal transduction in regions with known anatomical connectivity to the injection site to assess for retrograde or anterograde transport. **Results/Discussion**: Despite its namesake, a major finding is that rAAV2-retro provides conditional retrograde transduction in macaque neuronal populations, in contrast to what has been found in rodents. Further, in a number of injections, we have discovered anterograde labeling of the exogenous gene in axonal terminals, in addition to local transduction of neurons around the injection sites. This finding was confirmed in multiple injections across the primate neuroaxis in multiple animals. Conversely, our working hypotheses are that (1) AAV2g9 provides greater transduction at the injection site compared to recombinant parental AAV serotypes in the non-human primate model, and (2) based on previously published rodent data, rAAV2g9 double mutants may spreads further from the injection site and provide better retrograde transport to neurons with projections to the injection site when compared to AAV2g9. Primate data with respect to these and other AAV capsid mutants will be discussed.

175. Recombinant AAVs Target Custom Frataxin Therapeutic Cassettes to Tissues of Pathophysiologic Relevance in Friedreich's Ataxia

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Friedreich's Ataxia (FRDA) is a genetic disease affecting multiple organ systems, in which an intronic triplet repeat expansion (GAA) suppresses expression of frataxin (FXN). This mitochondrial protein is involved in iron-sulfur clustering, and non-regenerating tissues become especially vulnerable to oxidative stress and cell damage. In FRDA patients, neuronal damage in the CNS and PNS impairs motor coordination, manifesting as progressive gait and limb ataxia. In addition, oxidative stress in the heart leads to hypertrophic cardiomyopathy in early adulthood, which is often lethal. Naturally occurring AAV serotypes are widely used in research settings and clinical trials to deliver genetic cargo throughout the body when administered systemically. However, broad transduction of current systemic vectors (e.g. AAV9) can have deleterious off-target effects[1], underscoring the need for vectors that limit efficient transduction to specific disease targets. In this study, we aimed to develop precision AAV-mediated FXN gene replacement therapy to replace physiologic levels of FXN in the deep cerebellar nuclei, dorsal root ganglia, and the heart in two mouse models of FRDA, while minimizing transduction of organ systems and cell types that are not major contributors to the disease process, including the liver. To this aim, we used a dual-engineering approach to optimize both the delivery system and therapeutic cargo for targeted replacement of physiologic levels of FXN. Three constructs of the FXN gene containing different

putative regulatory elements were cloned and packaged into AAVs for CNS and PNS targeting. In early experiments, a cocktail of PHP.eB and PHP.S was used for broad CNS and PNS targeting of these constructs after intravenous delivery in a knock-in-knock-out (KIKO) mouse model of FRDA, which recapitulates the genetic etiology of the disease, but exhibits a mild motor phenotype. With this approach, we achieved broad FXN expression throughout the target regions. Notably, cassettes containing FXN regulatory elements yielded expression patterns and levels mimicking the healthy endogenous state. To evaluate whether restoring expression patterns is sufficient to rescue sensorimotor deficits of FRDA, we transitioned to an inducible shRNA knockdown mouse model of FRDA (FXNiKD) which exhibits a more severe motor and coordination deficit than the KIKO model, as well as a cardiac phenotype. For CNS targeting in these experiments, we instead utilized the next generation vector AAV.CAP-B10, selected for its equally high efficiency of crossing the rodent BBB as PHP.eB but with unique bias toward neurons, and superior detargeting of the liver, cerebellar Purkinje cells, astrocytes and oligodendrocytes, which are clinical off-targets in FRDA. Using this new combination of CAP-B10 and PHP.S, FXN was systemically delivered to a small pilot cohort of symptomatic FXNiKD mice and wild-type controls, and sensorimotor phenotype was evaluated. In addition, due to the degenerative nature of FRDA, we are investigating whether targeted exogenous FXN therapy is able to prevent motor and coordination deficit development in FXNiKD mice when prophylactically applied.1.Hinderer, C., et al., Severe Toxicity in Nonhuman Primates and Piglets Following High-Dose Intravenous Administration of an Adeno-Associated Virus Vector Expressing Human SMN. Human Gene Therapy, 2018. 29(3): p. 285-298.

176. AAV.PLP Stops Disease and Prevents Epitope Spreading in a Model of Relapsing-Remitting Multiple Sclerosis

Addelynn Sagadevan[1], Isabelle Cote[1], Geoffrey D. Keeler[1], Cristina D. Gaddie[1], Brad E. Hoffman[1,2]

[1]Pediatrics, University of Florida College of Medicine, Gainesville, FL,[2]Neurosciences, University of Florida, Gainesville, FL

In the autoimmune disease Multiple Sclerosis (MS) there is a failure of thymically derived regulatory T cells (Tregs) to maintain tolerance. Unfortunately, antigen-specific therapies have remained elusive due to the genetic diversity of multiple unknown encephalitogenic epitopes present in patients. To address this, we have developed relevant AAV-based gene-immunotherapies that can restore tolerance in order prevent or reverse clinical symptoms and neuroinflammation in preexisting disease by inducing antigen specific Tregs in the experimental autoimmune encephalomyelitis (EAE) model.

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Given that 85% of MS patients are diagnosed with relapsing-remitting (RR) MS, demonstrating the therapeutic efficacy of our novel gene-immunotherapy in models that closely resemble clinical disease is an essential step toward a cure. Proteolipid protein (PLP)-induced EAE presents with an initial relapsing-remitting disease (RR-EAE), followed by a secondary progressive stage in SJL mice and is similar to that of human MS disease.

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Using a hepatocyte directed AAV vector expressing mouse PLP (AAV.PLP), we have effectively induced/restored antigen-specific tolerance to multiple epitopes of PLP and abrogated disease. To initially test if the gene-immunotherapy vector could prevent disease, AAV.PLP or Null vector was given 14 days prior to EAE induction. EAE was then induce in SJL (H-2^s) mice by immunization using $PLP_{139-151}$ in adjuvant. Beginning 10 days later, all control mice quickly developed severe RR-EAE. Remarkably, the AAV.PLP treated mice were protected and never developed any signs of neurological disease (Fig1A). For therapeutic reversal of pre-existing disease, immunotherapy treatment was administered after the first appearance of symptoms (early reversal) or during the first remission (late reversal) (Fig1B). Again, following the initial response, AAV.PLP completely blocked or ameliorated clinical disease, reduced cellular infiltration, and suppressed demyelination in $PLP_{139-151}$ immunized mice, regardless of treatment timing. To demonstrate that AAV.PLP is not restricted to a specific epitope, the vector was tested using a secondary immunogenic PLP epitope ($PLP_{178-191}$) that results from the epitope spreading process demonstrated in SJL mice that were induced with $PLP_{139-151}$. Remarkably, in both prevention and reversal experiments, disease was significantly reduced or resolved in AAV.PLP treated mice (Fig1C). In contrast, 100% of the control mice relapsed after the initial remission phase. Lastly, to show that AAV.PLP can adjust to genetic diversity (MHC-unrestricted), EAE was induced in a cohort of C57BL (H-2^b) mice using $PLP_{178-191}$. As before, the AAV.PLP gene-immunotherapy completely prevented disease (Fig1D).

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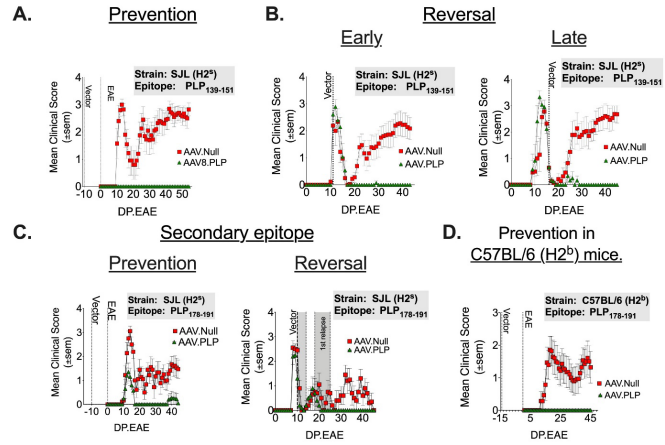
For the first time we provide definitive evidence that AAV directed gene-immunotherapy not only efficiently prevents the development of MS-like disease, but can also abrogate active pre-existing disease and relapses in a murine model of RRMS. The data further supports that treatment simultaneously provides protection against epitope spreading. Clinical translation of this novel gene therapy could result in prevention of the early inflammatory responses, thus stopping the transition into secondary progressive MS and significantly improving the quality of life for MS patients.



177. Intraparenchymal Spinal Cord Delivery of AAV VY-SOD102 Reduces Disease Burden in the G93A Mouse Model of ALS-SOD1

Jeffrey M. Brown, Heather Yonutas, Carol Huang, Michael P. Hefferan, Jenna Tocci, Ruo Jie Wang, Jeffrey S. Thompson, Emily M. Christensen, Qingmin Chen, Dinah W. Y. Sah, Holger Patzke

Voyager Therapeutics, Cambridge, MA

Mutations in superoxide dismutase 1 (SOD1) result in progressive motor neuron loss through gain-of-function toxic properties and are responsible for up to 20% of familial ALS, or 2-4% of all ALS patients in the U.S. Studies using transgenic mice expressing SOD1 mutations have demonstrated reduced neuropathology, improved motor behavior and extension of survival with partial lowering of SOD1. RNA interference (RNAi) is a naturally occurring process that mediates gene silencing. Expressing RNAi using artificial pri-miRNAs is the preferred approach for an AAV gene therapy targeting SOD1 for inhibition by RNAi. Previously reported studies using intraparenchymal spinal cord dosing demonstrated significant SOD1 knockdown by RT-qPCR on laser captured motor neurons and by branched DNA assay on tissue punches from the ventral horn along most of the length of the pig spinal cord. Notably, the identified lead AAV vector substantially suppressed SOD1 in motor neurons in cervical levels of the spinal cord, critical for respiratory function. The current studies extend our previous findings by demonstrating efficacy of our lead construct in the G93A mouse model of ALS. VY-SOD102 demonstrated a dose-responsive increase in survival of G93A mice when dosed during disease onset (P50-55). This increased survival was associated with a delay in overall disease onset, reduction or full prevention of hindlimb paralysis and improved grip strength and rotarod performance. Evaluation of in vivo pharmacology showed a dose-dependent reduction in hSOD1 mRNA in the spinal cord which correlated with overall tissue vector genome levels. Evaluating vector genome levels required in large animals for spinal cord SOD1 reduction show potent knockdown at vector genome tissue levels consistent with published data following intra-cisterna magna dosing. Collectively, our findings support the use of AAV gene therapy targeting SOD1 with RNAi as a potential approach for the treatment of SOD1-ALS

178. Transgene Expression and Tolerability After a Secondary Subretinal Injection of an AAV Vector to the Ipsilateral Eye in Non-Human Primates

Judith Newmark[1], Adrian Timmers[1], Ryan Boyd[2], Thomas Vihtelic[2], Mark Shearman[1]

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Purpose: Secondary subretinal injection of an AAV vector to the contralateral eye is an accepted practice in preclinical and clinical studies. Limited information is available, however, on the expression and safety outcomes of performing a secondary subretinal injection of AAV vector to the same eye, and so with this specific aim, we conducted a study in non-human primates.**Methods:** Cynomolgus macaques were divided into low dose (n=4) and high dose groups (n=4). Both eyes were given a single subretinal injection of AAV vector containing a photoreceptor specific promoter and hGFP reporter on Day 1. During Week 8 the left eye was given a second subretinal injection of the vector at high dose, at a different location from the first injection. At Week 16 the animals were sacrificed, and the eyes were processed for immunohistochemistry of hGFP and histopathological assessment by a veterinary pathologist. Ophthalmic exams were performed throughout the study to assess tolerability and inflammation, and fundus autofluorescence confocal scanning laser ophthalmoscopy (cSLO) was performed to evaluate hGFP expression. Neutralizing antibodies, fluorescein angiography, and clinical pathology were also assessed.**Results:** Ocular examinations showed a minimal level of inflammation after the first injection. Similarly, and importantly, no apparent increase in inflammation was recorded following the second subretinal injection. Quantitative analysis of fundus autofluorescence cSLO images demonstrated increasing hGFP expression after the first injection that continued through Week 16, with higher expression for the high dose group. The hGFP fluorescence levels in the right and left eye were similar at the same time point. Quantitative analysis of hGFP fluorescence following the second injection showed a steadily increasing hGFP fluorescence in the second location, similar to the increase in the first location following dosing. hGFP expression in the second location did not inhibit the fluorescence of hGFP in the first location and was similar to hGFP fluorescence in the contralateral eye at equivalent time points post-dose.**Conclusions:** Secondary subretinal injection of an AAV vector to the ipsilateral eye was well tolerated in non-human primates and did not inhibit transgene expression in the first injection site. The option to perform additional subretinal injections provides patients with a better chance for effective therapy if an initial injection was ineffective or failed, additional retinal regions require treatment, or if transgene expression or efficacy diminishes over time.

179. AAV9-Mediated Delivery of an NFAT-Neutralizing Decoy Oligonucleotide Ameliorates Heart Failure in Mice

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Introduction: Heart failure represents the leading cause of death in the Western world. However, recent treatment options focus solely on reducing the severity of symptoms. Previous studies have underlined the substantial role of nuclear factor of activated T cells (NFAT) in the regulation of cardiac growth, fetal gene expression and extracellular matrix deposition. Collectively, these processes contribute to pathological myocardial hypertrophy and ultimately lead to heart failure. **Purpose**: We aimed at simultaneously neutralizing four members of the NFAT family of transcription factors as a therapeutic strategy for myocardial hypertrophy transiting to heart failure through AAV-mediated cardiac expression of shRNA-based

decoy oligodeoxynucleotides (ONs) targeting NFATc1-c4. **Methods**: NFAT consensus binding sequence (NFAT ON, 40 bp) and mutated control oligonucleotide were cloned into an AAV plasmid under the control of H1 promotor. *In vitro* experiments were performed in neonatal rat ventricular cardiomyocytes (NRVCMs) using AAV6 vectors. Endothelin-1 (ET-1) was used as a pro-hypertrophic stimulus, and mRNA levels of fetal genes were measured by real-time PCR. Cell size was assessed by α-actinin immunocytochemistry. For *in vivo* studies, AAV9 vectors expressing NFAT ON were injected 2 days after induction of cardiac hypertrophy by transverse aortic constriction (TAC). Expression of ONs following transduction was demonstrated by fluorescent *in situ* hybridization (FISH). Cardiac function (ejection fraction, EF; fractional shortening, FS) and left ventricular mass were measured by echocardiography. Myocardial fibrosis was assessed in frozen sections by Masson's Trichrome staining. **Results:** Transduction with AAV6 vectors expressing NFAT ONs markedly decreased ET-1 induced NRVCM hypertrophy, evidenced by a marked reduction in fetal gene programme and decrease of cardiomyocyte size as compared to controls. Tail-vein injection of the designed AAV9 into adult mice led to efficient expression of the nucleic acid compound as evidenced by FISH. Importantly, *in vivo* experiments showed that although cardiac function severely deteriorated 6 weeks after TAC in control mice (EF: 58±6,4% in sham operated mice, 42±4% in TAC control mice), AAV9-mediated delivery of ONs after TAC resulted in preservation of cardiac function to basal levels (EF: 59±3,58%). Moreover, we could show that our treatment largely ameliorated cardiac hypertrophy, demonstrated by a reduction in HW/TL (26±2% decrease) and LV mass (41±2,3% decrease) as compared to controls. Additionally, reducing NFAT transcriptional activity led to marked reduction in extracellular matrix development in the myocardium. **Conclusion:** Our study proves that continuous generation of ONs targeting NFAT transcriptional activity in cardiomyocytes potently inhibits cardiac hypertrophy caused by hemodynamic stress and improves left ventricular function. Antagonizing increased NFAT activity thus seems to be a promising approach for translation into treatment of heart failure.

180. AAV Gene-Immunotherapy is Not Inhibited by Immunosupression in a Model for Multiple Sclerosis

Addelynn Sagadevan[1], Isabelle Cote[1], Geoffrey D. Keeler[1], Cristina D. Gaddie[1], Brad E. Hoffman[1,2]

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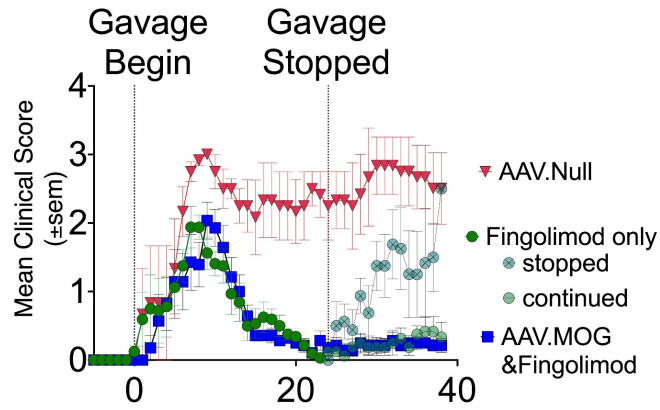
While there is no cure for MS, current Disease Modifying Therapies (DMTs) focus on generalized immune suppression to slow disease progression. To increase the specificity, our lab has developed a novel gene immunotherapy that can selectively modulate the adverse immune response against specific myelin proteins. We have demonstrated our novel approach can not only prevent, but can also reverse Experimental Autoimmune Encephalomyelitis (EAE), the animal model of MS, using an Adeno-associated virus (AAV) vector expressing a specific neuro-protein. When considering the development of a clinical trial, patients are likely to be receiving a DMT or will start a treatment protocol as the established standard of care. The prevailing goal of AAV gene-immunotherapy is to restore immune tolerance so that long-term continuous use of a DMT is unnecessary. As such, it is vital to ensure that the medical treatment currently being undertaken by the patient does not adversely affect the ability of our gene-immunotherapy to re-establish and maintain tolerance. Thus, it is necessary to assess the ability of our AAV vector expressing myelin oligodendrocyte glycoprotein (AAV.MOG) to induce immunological tolerance while a patient is currently being treated with the drug Fingolimod, an established immunosuppressant used in the treatment of MS patients. Using 8-week-old Female C57BL/6 mice, EAE induced with MOG35-55. At first sign of clinical disease, mice were injected with AAV.MOG vector and began daily administration of fingolimod via oral gavage until day 24. Control mice were treated with fingolimod only, without the administration of vector. At Day 24, fingolimod treatment was discontinued, whereas half of the control group receiving treatment continued receiving fingolimod and the remaining half stopped receiving treatment. By day 20 post treatment, all treated mice had recovered. However, after treatment was stopped mice that were only receiving fingolimod relapsed and developed severe EAE (Fig. 1). Whereas mice treated with AAV.MOG and fingolimod remained nearly disease free. Our data of concomitant administration of vector and fingolimod demonstrates a synergistic effect that results in a significant long-term reversal of disease even upon withdrawal of DMT treatment. In sum, simultaneously treatment using AAV.MOG and the DMT, fingolimod, shows no apparent inhibitory effect on the function of the vector. In fact, it appears to work synergistically to reverse disease progression and increase the effectiveness of gene-immunotherapy. In conclusion, our AAV gene-immunotherapy has significant clinical relevance as it restores a persistent and continuous immune tolerance such that long-term continuous DMT may be unnecessary for MS patients.



181. AAV Gene Therapy to Provide Continuous C1-INH Expression to Treat Hereditary Angioedema

Jill Woloszynek, Atul Sathe, Katherine Webster, Stacy Sherman, Katie Black, Lin Xie, Natalie Fredette, Joseph Chen, Nicole Galicia, Brian Kaplowitz, Sherry Bullens, Heather Wenzel, Christa Cortesio, Vishal Agrawal, Alexander Giaramita, Stuart Bunting, Hassib Akeefe, Kahsay Gebretsadik, Gabor Veres, Peter Colosi

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Hereditary angioedema (HAE) is a rare autosomal dominant disorder that presents as localized, acute episodes of severe, painful swelling that can occur anywhere in the body at any time. The frequency of angioedemia episodes, which persist for 1-5 days and may not have an identifiable trigger, is variable among patients but can occur up to several times a week. Without treatment, a swelling event in the airway can lead to asphyxiation and death. The recurrent and unpredictable nature of these attacks significantly affects the patients' quality of life. HAE types 1 and 2 are due to mutations in SERPING1 that reduce C1 esterase inhibitor (C1-INH) activity. C1-INH is a highly expressed serum protein (16-32mg/dL) that plays roles in the coagulation, complement, fibrinolytic, and the contact activation pathways. Insufficient C1-INH activity causes dysregulation of the contact activation pathway leading to un-regulated bradykinin formation and B2 receptor signaling resulting in excessive vascular permeability and vasodilation. Current therapies are successful in reducing severity and attack frequency but patients' can still experience breakthrough attacks. We are developing a promising new therapeutic approach for HAE types 1 and 2 using an adeno-associated virus (AAV) SERPING1 gene therapy vector to augment functional C1-INH levels via continuous production of C1-INH from the liver with a single treatment. BMN331 is an AAV gene therapeutic utilizing the AAV5 capsid, with a liver specific promoter/enhancer, driving the expression of a SERPING1 cDNA. Intravenous administration of BMN331 in a dose response study in Rag2-/- mice produced stable levels of functional human C1-INH ranging from undetectable to 30 times the normal range, without ALT elevations, alterations in liver histo-architecture, or pathological increases in IBA1-positive Kupffer cells. A long-term durability study in mice is underway. Treatment of cynomolgus macaques with BMN331 produced significant circulating levels of C1-INH and was well-tolerated in an ongoing study.

182. Insulin Replacement Gene Therapy Using a Novel Glucose- and Drug-Inducible Adeno-Associated Virus

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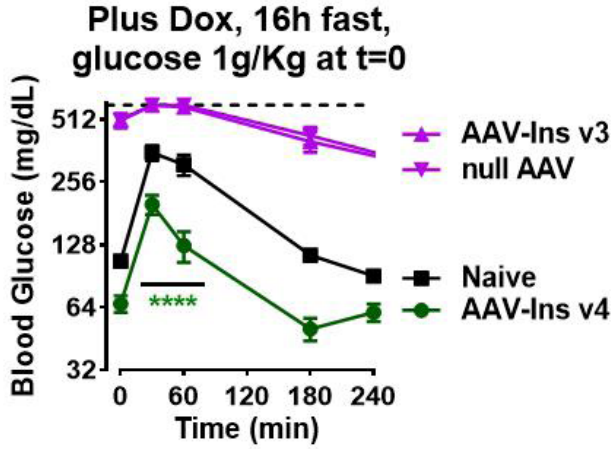
Objective: To develop AAV-based gene therapies that can be activated by the FDA-approved drug doxycycline (Dox). We present proof-of-concept studies using diabetic mice that show effective delivery and pharmacological regulation of an insulin gene replacement therapy. **Methods:** Glucose-inducible promoters have been developed by incorporating the DNA sequences that bind the carbohydrate response element binding protein, ChREBP. We developed a novel promoter with multiple repeats of ChREBP binding motif 1 and motif 2 (Jeong et al., 2011). For Dox regulation we engineered a single AAV to contain both components of the 3G Dox-on system (Clontech): 1) the reverse tetracycline transactivator (rtTA), whose expression is controlled by the glucose-inducible promoter; and 2) the tet operator, where the rtTA binds in the presence of Dox to induce the expression of recombinant insulin. Recombinant AAV8 pseudovirions were injected into the tail vein of diabetic mice (streptozotocin model). Blood glucose levels were monitored during ad libitum feeding and after an intra-peritoneal glucose tolerance test (GTT). Insulin production was monitored using serum samples and an ELISA directed against the human C-peptide produced by the rAAV. Expression of recombinant insulin mRNA was also validated using RT-qPCR. AAV delivery was confirmed by ddPCR on liver DNA.**Results:** Design of the AAV targeting plasmid was guided by in vitro studies measuring the expression of the fluorescent reporter, Fusion Red. The final designs showed modest regulation by glucose (2-fold), but tight regulation by doxycycline (Dox) with a large dynamic range (8,000-fold). All in vivo experiments were blinded and included 4 groups of mice (C57Bl/6, n=5 each): 1) naive (not diabetic, no AAV); 2) negative control (diabetic, null AAV); while 3) and 4) tested variations of the rAAV-Insulin gene therapy (versions 3 vs 4). Preliminary in vivo studies on diabetic mice measured glucose tolerance before doxycycline administration. These GTTs established that groups 2-4 were diabetic, showing an inability to lower blood glucose after glucose challenge. Strikingly, administration of Dox normalized glucose handling in groups 3 & 4 to similar levels as observed in the naive group. In a second experiment, we started Dox soon after rAAV injection, and then measured ad libitum blood glucose. The group with rAAV-Insulin version 4 began to show a response after 3.5 weeks, with blood glucose falling from 600 down to levels observed in naive mice, 200 mg/dl. Mice in this group also showed better glucose handling in the GTT test than naive mice. This result shows lower Dox doses can be used to normalize glucose handling, allowing for titration of the gene therapy response. **Significance:** The human insulin replacement market has been estimated at $42 billion dollars. Most commonly used strategies rely on the patient monitoring their blood glucose from a finger prick and daily subcutaneous injections of recombinant insulin. The present research suggests that a one-and-done pharmacologically inducible insulin gene replacement strategy can be developed.



183. AAV-Mediated Anti-Eosinophil Gene Therapy for Eosinophilic Esophagitis

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Eosinophils are specialized granulocytic effector cells that store and release highly active mediators and participate in the immune defense

against pathogens. However, eosinophils are also implicated in the pathogenesis of a variety of chronic allergic disorders, including eosinophilic esophagitis, characterized by persistent blood eosinophilia, infiltration of eosinophils into the esophagus, and release of eosinophil mediators that damage the tissue, resulting in upper gastrointestinal morbidity, food impaction and dysphagia. Treatment with elimination diets and/or corticosteroid therapy can slow disease progression, but are complicated by adverse effects and limited compliance. We hypothesized that a single intravenous administration of an adeno-associated virus (AAV) coding for an anti-eosinophil monoclonal antibody that induces eosinophil apoptosis (anti-Siglec-F) would reduce on a persistent basis the number of blood and tissue eosinophils in a murine model of eosinophilic esophagitis. To assess this concept, we established a novel mouse model of eosinophilic esophagitis that mimics the human disease by sensitization [intraperitoneal administration] and challenge [intranasal administration] with peanut extract with saline as a control. After challenge, these mice exhibit an eosinophilic esophagitis phenotype demonstrated by elevated levels of blood eosinophils, elevated IgE levels, infiltration of eosinophils in the esophagus and food impaction. The mice were treated with a single administration (10^{11} genome copies) intravenously with AAVrh.10mAnti-Eos, a serotype rh.10 AAV vector coding for an anti-Siglec-F monoclonal antibody. Vector administration resulted in persistent, high levels of anti-Siglec-F antibody expression. Administration of AAVrh.10mAnti-Eos to the mouse model of eosinophilic esophagitis, reduced peripheral ($p<0.007$) and esophageal ($p<0.003$) eosinophil numbers and alleviated food impaction 7 wk after AAVrh.10mAnti-mEos administration when compared with untreated mice. These results suggest that a single treatment with AAVrh.10m.anti-Eos has the potential to provide substantial therapeutic benefit to patients with eosinophilic esophagitis, primarily alleviating the burden of eosinophilic esophagitis.

184. Developing a rAAV-Based Gene Replacement Therapy for GM3 Synthase Deficiency

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GM3 synthase (ST3GAL5) deficiency is a rare monogenic neurological disorder common within Old Order Amish communities due to a population-specific founder variant (*ST3GAL5* c.862C>T) that segregates with a carrier frequency of ~4%. ST3GAL5 mediates synthesis of GM3, the common precursor for all a- and b-series gangliosides. Biallelic *ST3GAL5*c.862C>T mutations abolish ganglioside biosynthesis and result in systemic and cerebral ganglioside deficiency. *ST3GAL5* c.862C>T homozygotes appear healthy at birth, but develop progressive microcephaly, neurodevelopmental stagnation, intractable epilepsy, irritability, insomnia, deafness, blindness, and dyskinesia within a few months of life. No treatment is currently available. Here, we hypothesize that gene replacement therapy targeted to the central nervous system (CNS) could restore cerebral ST3GAL5 expression and ganglioside biosynthesis and thereby rescue the severe neurodevelopmental phenotype. Using fibroblast cell lines and iPSC-derived cortical neurons from patients with ST3GAL5 deficiency, we used lentivirus-mediated gene replacement to transfer human *ST3GAL5* cDNAs encoding all isoforms of the ST3GAL5 protein. We first examined the expression efficiency by Western blot. As expected, all constructs produced the ST3GAL5. Moreover, we showed that h*ST3GAL5* gene replacement restored GM3 production in patient-derived fibroblasts. More importantly, gene replacement in iPSC-derived cortical neurons reconstituted GM3 as well as its major downstream a- and b-series brain gangliosides (e.g., GD1a, GD1b, and GT1b). These *in vitro* data indicate the strong therapeutic potential of human *ST3GAL5* cDNA gene replacement. We next packaged h*ST3GAL5* constructs into AAV9 capsid that is known to target neurons and astrocytes following systemic delivery. To examine the safety and efficacy of transgene expression, we first delivered AAV vectors intravenously to wild type C57BL/6 mice. We observed that at high doses ($2x10^{14}$vg/kg for neonates, $1x10^{14}$vg/kg for young adults), AAV9-h*ST3GAL5* vectors compromise survival. Although lower vector doses ($2.7x10^{13}$vg/kg) were tolerable in wild type mice, such a dose regimen may or may not be therapeutically efficacious when targeting the CNS via systemic delivery. In addition, after recording elevations of hepatic alanine aminotransferase following AAV9-h*ST3GAL5* injection, we attempted to de-target h*ST3GAL5* transgene expression from liver through endogenous miR-122, but little improvement was observed. We are currently trying to understand the pathomechanism and optimize the vector design to mitigate vector associated toxicity. In parallel, we injected neonatal wild type mice via direct CNS injection. Enhanced rAAV genome biodistribution and h*ST3GAL5* over-expression was measured in the brain tissue by Droplet Digital PCR (ddPCR) and Western blot, respectively, and we observed no short-term vector-associated toxicity. Studies are underway to further develop rAAV-based h*ST3GAL5* replacement vectors and test their safety and efficacy in *St3gal5*-/- and *St3gal5-B4galnt1* double knockout mice. [a]Co-corresponding authors

185. AAVHSC-Mediated Editing of the Factor VIII Gene for the Correction of the Inversion 22 Mutation in Hemophilia A

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Hemophilia A is an X-linked recessive genetic disease caused by a deficiency of the clotting protein Factor VIII. Approximately half of all hemophilia A patients carry the I22I mutation in which exons 1-22 of the Factor VIII gene (F8) are inverted relative to exons 23-26. This I22I inversion results in the expression of a defective truncated Factor VIII protein. We have isolated a group of Clade F adeno-associated viruses from normal CD34+ hematopoietic stem cells (AAVHSCs). We and others have previously shown that AAVHSC mediate precise and efficient on-target genome editing without the need for prior

exogenous nuclease-mediated cleavage of genomic DNA. Here we test the hypothesis that utilizing AAVHSC vectors to target the insertion of the exons 23-26 cDNA downstream of exon 22 would restore the full length FVIII protein and confer clotting ability. In the abstract by Bugga et al, we showed that AAVHSC editing vectors successfully inserted the promoterless GFP open reading frame (ORF) into the F8 locus in cell lines and primary human liver sinusoidal endothelial cells (LSEC) *in vitro*. Sequence analyses revealed that the AAVHSC vectors were precisely inserted directly downstream of F8 exon 22, indicating that the edited cells expressed GFP under the control of the chromosomal F8 promoter. No evidence of indel mutations or insertion of AAV ITR sequences was observed in the edited locus. We also showed that *in vivo* intravenous injection of AAVHSC editing vectors resulted in the targeted insertion of the promoterless luciferase ORF into the mouse F8 gene, with stable long-term liver-specific expression of luciferase. Based upon these studies we have designed an F8 correction vector that encodes a codon optimized cDNA of exons 23 to 26 of the F8 gene, followed by a FLAG tag, a T2A sequence and the promoterless mCherry ORF. This was followed by a transcription termination and polyadenylation signals. The homology arms were designed to insert this correction cassette immediately at the end of exon 22 such that exon 23 to 26 will be fused to exon 22 to generate a full-length Factor VIII protein with a C terminal FLAG tag. Transduction of primary human LSECs with this FVIII correction vector resulted in mCherry expression, suggesting successful targeted insertion of the correction sequence in the F8 gene. Studies are underway to test the production of the full-length Factor VIII clotting protein and correction of the I22I truncation.

186. Improved AAV9 Transduction of the Central Nervous System and Retina After Co-Treatment with Neuraminidase

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Production costs and potential safety concerns related to high viral doses are major challenges in the gene therapy field. Thus, efforts to reduce the amount of viral vector needed for efficient targeting of the organ/cell type(s) of interest are of high value. In this study, we evaluated the potential of combining AAV9 delivery with neuraminidase (NA) for cerebrospinal fluid and retinal applications. Neuraminidase is an enzyme that cleaves sialic acid (SIA) residues from cell surface glycans. Via this mechanism, the AAV9 galactose receptors may transiently become more exposed and easier to access for the vector. Importantly, multiple groups previously demonstrated that co-injection of AAV9 and neuraminidase increases in vivo gene transfer to heart, lung, and muscle. We evaluated whether the same principle could be applied to increase AAV9 transduction in the central nervous system (CNS) following cerebrospinal fluid delivery of AAV9 and in the retina following intra-ocular injections. After intracerebroventricular injections of AAV9.GFP and neuraminidase into the CSF, we saw a substantial increase in overall GFP transduction in spinal cord and sagittal brain sections. In spinal cord, counts of GFP+ neurons colocalized with ChAT suggest ~15% increased transduction of motor neurons while non-neuronal cell targeting was also increased. For intra-ocular delivery, we injected AAV9.GFP and neuraminidase into the vitreous fluid of the eyeball in 2-month-old mice. Again, we found a substantial increase in the number of retinal cells transduced. Colocalization of GFP+ cells with Sox2 and Otx2 counterstains showed increased targeting of Müller glia and bipolar cells in the inner retinal cell layer, which is particularly exciting since previous experiments with AAV9 alone showed low targeting of these cells. In summary, our data suggests that the addition of neuraminidase during viral delivery to the CNS and retina using CSF and intravitreal injections, respectively, might be an attractive option to enhance transduction of target cell types. This would allow for lower viral loads to reach maximum efficacy, thus decreasing therapy production costs and the potential for viral-mediated immune responses.

187. Miniaturizing Usher Syndrome Type 2A Gene for Gene Therapy via Cell Proliferation

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Usher Syndrome (USH), an inherited disorder, is the leading cause of deaf-blindness with prevalence estimated to be approximately 1 in 6000. According to the onset age of vision loss, severity of hearing impairment, the presence or absence of vestibular dysfunction, it can be classified into three type, USH1, USH2, and USH3. Multiple genes associated Usher syndrome have been identified. This study focused on the gene causes type 2A Usher syndrome, USH2A, which encodes Usherin with an open reading frame of 15.6kb. Currently the most expedited path to clinical application for inherited photoreceptor degeneration is subretinal AAV-mediated gene augmentation. Since AAV has a size limit for packaging transgene cargo <~5kb, the goal of this study is to engineer constructs with reduced size (minigenes) and yet retaining Usherin protein function sufficient to have therapeutic effects. Full length Usherin consists of large motif repeats (35 Fibronectin type III motifs (FN3), 10 Laminin EGF motifs (EGF Lam), and 2 Laminin G domains). Although several key positions have been shown to affect fibronectin and collagen binding, the role of most motifs are yet to be examined and potentially could be functional redundant. We aim to achieve miniaturization by reducing the number of the motif repeats. Due to the large number of motif repeats and lack of information on their individual significance, we work on parallel strategies—rational design of exploratory Ush2a minigenes, meanwhile building combinatorial library to establish high-throughput screening to identify which minigenes allow for superior rescue of disease phenotype. So far we have designed seven minigenes, interrogating the significance of EGF_Lam (5~10), Laminin G(1~2) and FN3 repeats (2~35). To test these minigenes, we use Oc-k1 cell line which originated from the organ of Corti of a P14 Immortomouse and expresses inner ear cells' protein marker OCP2. We have created Ush2a knockout Oc-k1 cell line. Both the KO cell line and WT Oc-k1 was used. The in vitro model bases on the observation that 1) wild type Oc-k1 cells have significant faster proliferation rate than the USH2A null Oc-k1 cells, and 2) after transfection of full length human USH2A gene, proliferation

of null Oc-k1 cells increased dramatically. Three out of these seven minigenes achieved 50% or more rescue effect of the full length USH2A (normalized to the cell count 96 hours after transfection of full length human USH2A), three displayed moderate increase (~30%) on cell proliferation and one had no effect compared to control group. Based on the result from these seven exploratory Ush2a minigenes, we are currently building a combinatorial library targeting LamG2 + FN (#5~13). Establishment of the in-vitro model can allow us to batch screen the combinatorial library of USH2A minigene in a fast, (semi-)quantitative, and cost-efficient fashion. Our future direction also includes further validation of the therapeutic effect in USH2A-/- mouse model.

188. Lack of Toxicity in Non-Human Primates Receiving Clinically Relevant Doses of an AAV9.U7snRNA Vector Designed to Induce *DMD* Exon 2 Skipping

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Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by progressive *muscle* degeneration and weakness affecting approximately 1 in 5,000 male births worldwide. Duplications of exon 2 account for 10% of all duplication mutations in the *DMD* gene. We have previously demonstrated prolonged therapeutic efficacy and an absence of off-target splicing effects in AAV9.U7snRNA mediated skipping of exon 2 in a murine Dmd model. To evaluate the potential toxicity of AAV9.U7snRNA, male juvenile cynomolgus macaque (NHPs, aged 29-32 months) received a single intravenous infusion of an AAV9 vector containing four copies of a non-coding U7snRNA with additional sequences targeting the DMD exon 2 splice acceptor and donor sites (scAAV9.U7.ACCA vector). Three animals received the vector at 3e13 vg/kg (the minimal efficacious dose (MED) for a planned clinical trial), three received 8e13 vg/kg, and three received vehicle control in a study performed in accordance with good laboratory practice (GLP). In each group, n=2 animals were euthanized at 3 months and n=1 animal was euthanized at 6 months post-injection. We present here the results of the analysis at 3 months post-administration, with 6 months analysis underway. Administration of vector at both doses resulted in no significant clinical changes, including animal body weights, qualitative food consumption, ophthalmology, neurological examinations, cardiac biomarkers, urinalysis parameters, bone marrow cytology, gross necropsy, or changes in absolute or relative organ weights. There were no remarkable echocardiography findings in any injected animal. Treated NHPs demonstrated the minimally increased monocytes and large unstained cells at both doses with a return to control/baseline levels within a month of injection. The treated NHPs had transient increases in triglycerides, alanine aminotransferase, and aspartate aminotransferase at various levels. Minimal single cell, hepatocyte necrosis was observed at ≥ 3e13 vg/kg; mild, diffuse hepatocellular vacuolization was observed in one 8e13 vg/kg dose group animal. The RNA-seq data showed that total *DMD* mRNA levels decreased in a dose-dependent manner in all muscle tissues (heart, quadriceps, diaphragm), reaching up to 38% reduction in expression in the hearts of animals treated with 8E13 vg/kg of scAAV9.U7.ACCA. DMD expression was relatively stable in the liver and testis tissues at both doses and vehicle control. Exon 2 skipping was most pronounced in the hearts of animals treated with a high dose, displaying approximately 67% skipping. The biodistribution completed by qPCR assay confirmed the presence of AAV9.U7.ACCA vector in all tested tissue and organs demonstrating the dose-dependent exclusion of exon 2 in skeletal muscles, diaphragm, and heart. These results were consistent with our previous finding in the Dup2 and C57Bl6 mice, and showed that systemic delivery of AAV9.U7.ACCA to NHPs was safe and well-tolerated at clinically relevant doses. These findings support initiating the first in human study of this vector for treatment of Dup2 DMD patients.

189. A Quantitative Systems Pharmacology Approach for Translational Modeling of AAV8-Based Gene Therapy Products in Rodents, Macaques and Man

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A major obstacle in the development of Adeno-associated Virus (AAV) based gene therapy treatments is an incomplete understanding of in vitro-in vivo correlation and nonclinical-clinical extrapolation which necessitates highly empirical approaches to clinical dose selection. To address this gap, we present a Quantitative Systems Pharmacology (QSP) framework that includes a minimal physiologically based pharmacokinetic model linking AAV dose and systemic exposure levels to tissue biodistribution of vector genomes, intracellular trafficking and transcription/translation of the target gene. We have parameterized this model for AAV8 using appropriate *in vitro* derived biomeasures describing capsid-receptor interactions, internalization, transduction and validated it using a database of previously published pre-clinical and clinical data on dose-dependent AAV8-based liver-targeted gene therapy treatments. We used this framework to investigate key rate-limiting processes and inter-species differences in AAV directed gene expression. Our analysis shows that transgene expression kinetics are rate-limited by steps of intracellular vector trafficking, nuclear import and DNA processing rather than cell entry. Our model also predicted a linear dependence of vector genome/liver cell transduction as a function of AAV8 dose which was consistent with reported data for various single-stranded AAV8 constructs that exhibit a fixed efficiency of 1-3% for liver-delivery of vector genomes in both mice and non-human primates. In contrast, data for self-complementary AAV8 constructs showed much higher liver-delivery efficiencies and significant species differences with mice exhibiting

efficiencies up to ~70% which could be accounted for in the model with appropriate modification of DNA processing rates. Model-based analysis of per-genome protein expression including corrections for species dependent turnover/clearance where appropriate indicated transcription/translation rates varied between species with a decreasing trend with increasing body weight that could partly be explained by mass-based metabolic scaling. Altogether we show how accounting for these processes and species-scaling factors in our QSP framework can partially reconcile observed differences between pre-clinical and clinical gene expression thereby enabling better estimates of clinically efficacious doses for AAV8 constructs. Finally, we discuss potential extensions of the model to AAV9 and other non-liver targeted treatments and prediction of tissue transduction efficiency.

190. AAV-Mediated Antibody Delivery for Hereditary Angioedema

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Hereditary angioedema (HAE) is a rare life-threatening disorder characterized by recurring attacks of subcutaneous or mucosal edema affecting the face, larynx, extremities, gastrointestinal tract, and genitalia. Attacks may be frequent (weekly) and disabling with a substantial impact on the quality of life. HAE is usually caused by a haploinsufficiency in the SERPING1 gene encoding the C1 esterase inhibitor (C1INH), a serpin that normally functions to inhibit factor XIIa and plasma kallikrein. Failure to inhibit these targets results in poorly controlled plasma kallikrein activity and overproduction of bradykinin, resulting in pain and swelling. Severe disease is often treated prophylactically with intravenous (IV) infusions of C1INH or subcutaneous administration of an antibody specific for plasma kallikrein. Our approach is to deliver an adeno-associated viral (AAV) vector capable of expressing anti-kallikrein antibody from the liver, resulting in sustained circulating levels of therapeutic antibody. The longer half-life and higher specific activity of an anti-kallikrein antibody over C1-INH provides a rationale for our AAV-mediated antibody gene therapy approach for HAE. We conducted vector optimization studies, evaluating multiple antibody expression cassette designs, different AAV capsids and routes of administration to select a lead vector to advance for clinical development. The optimized expression cassette containing a liver-specific promoter and a codon optimized and CpG depleted transgene with a modified furin-2A processing signal resulted in robust serum antibody concentration when delivered intravenously using an AAV8 vector. We have achieved very high (>1mg/ml) and sustained levels of functional anti-kallikrein antibody in the serum of C57BL/6 mice following IV vector administration at a dose of 1e13gc/kg. Serum or plasma from vector injected mice effectively inhibited human kallikrein activity *in vitro* and inhibited endogenous mouse kallikrein in an *ex vivo* assay, clearly demonstrating the functionality of the vector expressed anti-kallikrein antibody. We are currently assessing the efficacy of our lead vector in a mouse model of carrageenan induced paw swelling and planning the evaluation of its expression kinetics and pharmacodynamics in non-human primates.

191. AAV Gene Delivery in Lung Organoids Derived from Human Embryonic Stem Cells

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We wished to use recombinant adeno-associated virus (rAAV) for transduction of human lung parenchyma, in particular Alveolar Type II (ATII) cells, to treat genetic surfactant deficiencies. For efficient transduction by rAAV, careful selection of serotypes with the appropriate tropism is required, but such serotypes have not yet been identified. To screen for rAAV serotypes with the required tropism we chose a human 3D lung model generated from human embryonic stem cells. These lung bud organoids (LBOs), generated as described by Chen and colleagues [Nat Cell Biol, (2017) 19: 542], exhibit a strong bias towards generation of lung parenchyma, especially surfactant-producing ATII cells. After sequential differentiation of human embryonic stem cells to anterior foregut endoderm and subsequent maturation and branching induction of nascent LBOs, matured LBOs (at days 59 and 79) were microinjected with multiple rAAV vectors. Approximately 3.5E8 - 1E9 genome copies (GC) of rAAV vectors carrying a CMV-EGFP transgene cassette were injected in a volume of approximately 0.5 µl. Delivery of vectors to the lumen of the organoids in this way allowed modelling of transduction from the air-facing, apical surface of the lung. By monitoring native EGFP fluorescence in the LBOs, we identified rAAV2, rAAV6 and rAAV6 variants as serotypes with strong tropisms for human lung parenchyma (n=3-4, rAAV6.2 in two independent experiments). Serotypes 1 and 8 resulted in low levels of transduction (n=3), whereas serotypes 5 and 9 were indistinguishable from naive (n=3, two independent experiments). Staining of LBOs for the ATII cell markers surfactant proteins B and C was positive and confirmed distal lung identity, suggesting suitability of the vectors for transduction of ATII cells. Staining for known primary rAAV entry receptors such as alpha-2,3- and α-2,6-linked sialic acid was similar to mature human lung. Immunohistochemistry also confirmed the presence of the universal AAV receptor (AAVR), providing potential to further explore vector entry mechanisms. As a comparator to LBOs, we also screened rAAV serotypes in a second model of the human lung - precision cut lung slices (PCLS) - generated from surgical lung resections. Freshly excised human distal lung tissue was sectioned (500 µm), cultured with antibiotics and antifungals and treated *ex vivo* with 3E10 GC of rAAV vector expressing CMV-EGFP. As for the LBO model, transduction of human PCLS was confirmed for rAAV2 (n=3-6, two donors), rAAV6 (n=4, one donor), and rAAV6 variants (n=3-6, two donors). There was minimal transduction with rAAV serotypes 1, 8 and 9 (n=4, one donor) and serotype 5 was negative (n=4, two donors). Results from both models were concordant, indicating that rAAV vectors based on serotypes 2

and 6 may be suitable for transduction of human lung parenchyma. In summary, our results establish LBOs as a new model for pulmonary gene transfer which, together with human lung slices, help identify rAAV serotypes suitable as candidates for the treatment of genetic diseases originating in ATII cells, such as surfactant deficiencies. Bibliography: Chen, Y.W., et al., *A three-dimensional model of human lung development and disease from pluripotent stem cells*. Nat Cell Biol, 2017. **19**(5): p. 542-549.

192. Abstract Withdrawn

193. Phenotypic Benefit of Intrastriatal Administration of the AAV Gene Therapy VY-HTT01 in the YAC128 Mouse Model of Huntington's Disease

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Huntington's disease (HD) is a fatal, monogenic neurodegenerative disease characterized by progressive motor, cognitive and neuropsychiatric impairment, and caused by toxic gain-of-function expansion of trinucleotide repeat in the huntingtin gene (HTT). Partial suppression of HTT in the brain has been demonstrated to be both safe and effective in animal models of HD, providing proof-of-concept for an HTT lowering therapeutic strategy. VY-HTT01 is an AAV gene therapy encoding a primary miRNA selectively targeting human HTT mRNA for knockdown. In the present study, we investigated the efficacy of VY-HTT01 treatment in the YAC128 mouse model of HD. YAC128 mice received bilateral intrastriatal injections of different doses of VY-HTT01 or vehicle at 2 months of age, and human HTT lowering, motor function and neuropathology were evaluated. Our data show that VY-HTT01 treatment resulted in significant and dose-dependent human HTT mRNA and human mutant HTT protein lowering in the striatum of YAC128 mice. Concomitant with significant human HTT reduction, VY-HTT01-treated YAC128 mice showed significant improvement in motor function at all three doses tested compared to vehicle-treated YAC128 mice. In addition, no overt neuronal toxicity or gliosis was observed in VY-HTT01-treated YAC128 mice at 24 weeks post-dosing. Thus, these results demonstrate that VY-HTT01 rescues behavioral dysfunction in a mouse model of HD concomitant with human HTT reduction and is well-tolerated in this model. This study supports further clinical development of VY-HTT01 as a therapeutic gene therapy for treating patients with HD.

194. AAV9 Gene Therapy Rescue of an eEF1A2 Knockout Mouse Model

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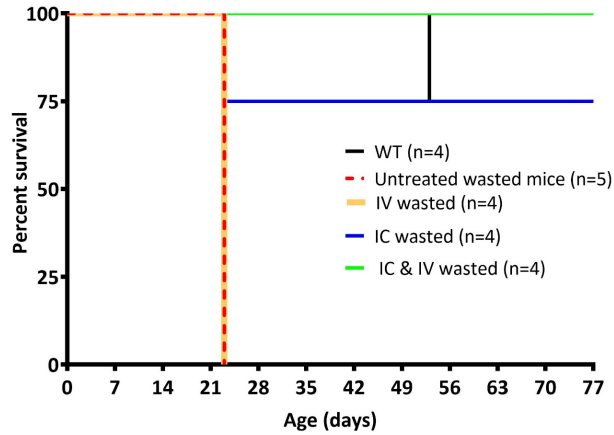
Eukaryotic translation elongation factor 1 alpha 2 (eEF1A2) is essential for the delivery of aminoacyl transfer RNA to the ribosome for protein synthesis. Mutations in the *EEF1A2* gene have been associated with severe intellectual disability, autism and epilepsy. There are currently no effective treatments. An eEF1A2 knock out mouse model (wasted mice) has been well-characterised. The wasted mice exhibit gait disturbances and tremor after weaning, followed by paralysis and motor neuron degeneration by 23 days of age. Using this mouse model, we wanted to test the hypothesis that the function of the protein could be restored with gene therapy. We therefore designed an adeno-associated virus serotype 9 (AAV9) to drive expression of the eEF1A2 cDNA and green fluorescent protein (GFP). We interrogated its bio-distribution after single intravenous or intracerebroventricular injections to new born wild-type mice. We found widespread transgene expression in the CNS after both routes of administration from a single injection of a GFP marker gene. Following this we treated cohorts of neonatal mice in a randomised, blinded trial with AAV9-eEF1A2. Mice with combined intracerebroventricular and intravenous administration (n=4), or intracerebroventricular (n=3) only were completely rescued by AAV9-eEF1A2 (Figure 1). Behavioral studies such as rota-rod and inverted grid, further validated the efficacy of the treatment. Both treated groups showed no significant difference compared to wild-type controls and survived until the experiment was terminated when the entire cohort had reached 72 days of development. We present for the first time a successful gene therapy approach for the eEF1A2 wasted mice, which raises the potential for a new treatment approach for children affected by mutations in this gene.



195. Use of the Adeno-Associated Viral Anc80 (AAVAnc80) Vector for the Development of Precision Genetic Medicines to Address Hearing Loss

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In vivo cochlear gene therapy has effectively restored or improved cochlear function in mouse models of genetic deafness, even when the implicated gene has a transcript exceeding the packaging capacity of AAV vectors (Akil et al., 2019; Al-Moyed et al., 2019). Recent nonclinical experiments in non-human primates (NHPs) have shown that: (1) a single administration of AAVAnc80 vectors efficiently transfers genes encoding enhanced green fluorescent protein (eGFP) into the cochlear sensory epithelium in macaques (Andres-Mateos et al., 2019) and baboons (Gao et al., 2020); (2) the transduction efficiency of AAVAnc80 vectors, as assessed by eGFP expression, is dose-dependent (Francis et al., 2019); and (3) administration of dual-AAVAnc80 vector encoding eGFP, in which the transgene is divided into two fragments and packaged into separate AAVAnc80 vectors, results in efficient transduction of cells within the sensory epithelium in NHPs (Darcy et al., 2020) and, as shown here, in mice. Following gene transfer of both vectors into cochlear cells, reconstitution, recombination, and/or transplicing results in the production of a full-length cDNA and expression of the target protein. In developing a potential gene therapy to address *OTOF*-mediated hearing loss, an autosomal recessive form of sensorineural hearing loss caused by mutations in the otoferlin gene, we generated a dual AAVAnc80 vector to deliver the nearly 6 kB cDNA encoding human otoferlin (AAVAnc80-hOTOF) to the appropriate target cells, the inner hair cells (IHCs) of the cochlea. We characterized age-related changes to cochlear function in an otoferlin knock-out (*Otof* $^{-/-}$) mouse model from 3 weeks to ~10 months of age, relative to wild-type (WT) controls, using the auditory brainstem response (ABR) and distortion-product otoacoustic emissions (DPOAEs). In *Otof* $^{-/-}$mice, ABR peaks were not observed. DPOAE thresholds increased with age in both WT and *Otof* $^{-/-}$ mice, and threshold elevations were more severe in *Otof* $^{-/-}$ mice. In addition, we will present evidence that a single administration of AAVAnc80-hOTOF to *Otof* $^{-/-}$ mice can achieve otoferlin expression in IHCs as well as long-term improvements in cochlear function.

196. MBP-Specific Gene-Immunotherapy Prevents and Reverses Severe Disease in a Mouse Model of Multiple Sclerosis

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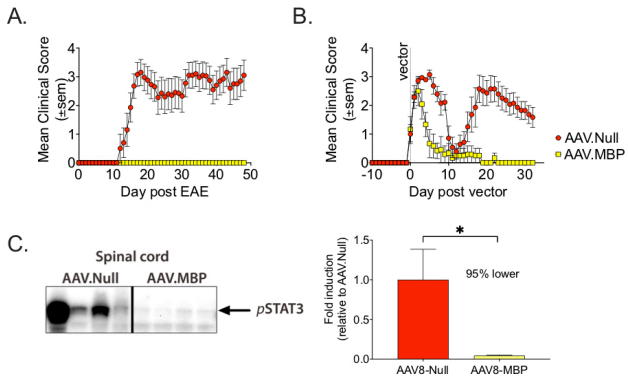
Multiple sclerosis (MS) is an autoimmune of the central nervous system that results from a loss of tolerance to specific neuroproteins. It is the most prevalent non-traumatic neurological disorder incapacitating adults. Currently, approved therapies are based upon generalized immune suppression which become less effective with disease progression and are associated with significant adverse effects. We have recently developed antigen-specific gene-immunotherapies capable of suppressing and reversing severe experimental autoimmune encephalomyelitis (EAE) induced with multiple neuroproteins, regardless of the epitope or genetic haplotype of the mouse. Adding to our platform, we have engineered a new viral vector (AAV.MBP) designed to provide immune tolerance to myelin basic protein (MBP), a well-characterized autoantigen highly associated with MS and linked to epitope spread in the CNS of patients. Here we demonstrate the preventative and therapeutic potential of this novel gene therapy using cohorts of (SJL x PL) F1 mice immunized with the immunogenic MBP_{Ac1-9} epitope located within the N-terminal portion of the MBP protein. To demonstrate prevention of disease, AAV.MBP or AAV.Null vector (10^{11} vg/mouse; i.v.) was given to two groups of mice (n=10/group) 2weeks prior to being immunized with the MBP epitope emulsified in CFA; in order to prophylactically induce tolerance to MBP. Beginning on day 10 post EAE induction, the control mice developed severe EAE with bilateral hindlimb paralysis that persisted for >50 days. In stark contrast, none of the mice that received AAV.MBP showed any signs of EAE throughout the entire course of the study (MCS peak of 3.2 +/- 0.5 vs 0; P <0.001) (Fig1A). At endpoint, AAV.MBP mice had 95% less phosphorylated STAT-3 in the spinal cord compared to Control (P<0.05) and it has been shown that development of pathogenic Th17 cells *in vivo* is dependent on STAT3 signaling (Fig1C). Next, we demonstrate that the gene-immunotherapy can also reverse active-preexisting disease. This time EAE was induced first using the immunogenic MBP_{Ac1-9} epitope emulsified in CFA. At disease onset, AAV.MBP (or AAV.Null) vector was given. AAV.MBP mice reached MCS peak to that of Control (2.5 +/- 0.5 vs 2.9 +/- 0.1; N.S.) (Fig 1B). Notably, AAV.MBP mice started recovering as early as 3 days post vector delivery whereas clinical disease continued to worsen until day 6 post injection in AAV.Null treated mice. Furthermore, by day 9 post vector delivery, AAV.MBP mice regained significant motor function (lower MCS) compared to the control mice (Ps<0.05). At day 12 post vector injection, mice from both groups almost completely recovered; however, disease in mice that received AAV.Null quickly relapsed and remained significantly disabled until the end of the experiment (Ps<0.05). Whereas, after the initial response, the AAV.MBP treated mice remained stable and free of clinical disease symptoms. These results clearly demonstrate ability of our AAV gene-immunotherapy to suppress autoimmune response against MBP, a known MS related antigen. These results, combined with those presented in other submitted abstracts, could potentially be the foundation for a cure to MS.



197. Optimization of AAVHSC-Based Genome Editing Vectors for Targeted Insertion in the Factor VIII gene

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The intron 22 inversion (I22I) mutation of the coagulation Factor 8 (F8) gene accounts for about half of Hemophilia A, an X-linked recessive bleeding disorder. This inversion is caused by an intra-chromosomal recombination event leading to an inversion of exons 1 to 22 relative to exons 23 to 26. We hypothesized that the targeted insertion of the cDNA of exons 23-26 of the FVIII gene after intron 22 would restore the full length F8 protein. Here we describe the optimization of AAVHSC-based genome editing vectors to edit the FVIII gene with the ultimate goal of correcting the I22 inversion. We and others have previously shown that AAVHSC mediate efficient and precise homologous recombination-based genome editing in the absence of prior nuclease-mediated double stranded DNA breaks. Here we tested parameters to optimize the design of F8 I22I editing vectors for the insertion of a promoterless GFP after exon 22 of the F8 gene. We tested editing vectors designed to insert the GFP open reading frame (ORF) either immediately at the end of exon 22 on within intron 22 of the human F8 gene, such that GFP expression would be dependent upon the F8 promoter and in certain cases, following appropriate splicing. In addition, we tested different homology arm (HA) configurations and symmetries for editing these two locations within the F8 gene. We also compared different AAVHSC serotypes. Editing efficiency was measured at the cellular level by flow cytometry for GFP expression in both cell lines as well as primary human liver sinusoidal endothelial cells (LSEC). The frequency of editing was found to range from 1% to 20% in K562 cells, depending upon the HA configuration and genomic location. In primary human LSEC, GFP expression ranged from 4% to >40%. Editing was confirmed at the molecular level by targeted integration (TI) assays employing amplification of junction sequences using GFP-specific and chromosome-specific primers. Sequence analysis revealed precise and seamless insertion of the GFP cassette into the F8 gene and the absence of insertion/deletion mutations. Finally we tested editing of the F8 gene in vivo using AAVHSC vectors designed to insert the promoterless luciferase ORF into the murine F8 gene. Editing vectors of different HA configurations and packaged in different AAVHSC serotypes were compared. Using this strategy, luciferase expression would be under the control of the endogenous F8 promoter and would only occur following chromosomal insertion in the correct orientation. Intravenous injection of all murine F8-Luciferase editing vectors tested, led to in vivo luciferase expression. Luciferase expression was detected 5-7 days post-injection, plateaued by day 21- 30 post-injection and was stable until the end of the experiment, >120 days post-injection. No toxicity was observed following the injection of the AAVHSC editing vectors. Our results indicate that AAVHSC editing vectors successfully edit the human and murine F8 genes in primary human cells in vitro and in mice in vivo in a stable fashion and form the basis for the design of correction vectors to edit the I22I inversion FVIII mutation.

Gene Targeting and Gene Correction

198. Efficient Non-Viral Ablation of *COL7A1* Exon 73 Splice Acceptor for the Treatment of Dystrophic Epidermolysis Bullosa (DEB)

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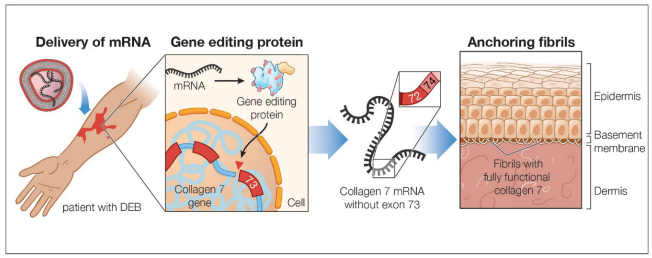
Dystrophic Epidermolysis Bullosa (DEB) is a devastating rare disease associated with chronic wounding of the skin. Patients suffer from a wide range of complications including: scarring, blistering, infection, and squamous cell carcinoma. DEB is phenotypically characterized by a lack of functional anchoring fibrils at the dermal-epidermal junction due to mutations in the Collagen type VII Alpha I (*COL7A1*) gene. Viral gene therapies are being explored for the treatment of DEB. However, viral vectors can elicit an immune response, are susceptible to silencing over time, and can carry a risk of insertional mutagenesis. *COL7A1* is a large gene, encompassing 118 exons, several of which are dispensable to protein function. The use of antisense oligonucleotides (ASOs) to cause transient skipping of exon 73 and 80 restored functional anchoring fibrils in patient-derived fibroblasts and keratinocytes. However, ASO-based approaches suffer from limited tissue penetration and require chronic dosing. Gene-editing approaches using TALENs and CRISPR-Cas9 are also being explored for the treatment of DEB. However, current gene-editing approaches suffer from low efficiency, necessitating the use of selection markers that prevent *in vivo* application. Here we show efficient ablation of the *COL7A1* splice acceptor site in primary human keratinocytes and fibroblasts using gene-editing endonucleases delivered via a novel, minimally immunogenic mRNA. We targeted multiple sequences near the exon 73 splice acceptor using TALENs and a novel chromatin-context-sensitive gene-editing endonuclease (NoveSlice). We observed high-efficiency cutting with both endonucleases in a cell-free cleavage assay. We then transfected primary keratinocytes and fibroblasts and characterized editing events using both biased and unbiased methods. The T7 endonuclease I assay confirmed high on-target editing, while RT-PCR of the exon 71-75 region showed durable exclusion of exon 73 from collagen 7 mRNA. Genome-wide off-target screening was conducted to assess potential unintended edit sites. We demonstrate efficient, non-viral ablation of the *COL7A1* exon 73 splice acceptor

using gene-editing endonucleases delivered via a novel minimally immunogenic mRNA, and we show durable exon exclusion in primary human cells. This approach overcomes the low-efficiency of previous gene-editing approaches, and is being explored for clinical use.



199. CRISPR/Cas9 Double Edited CD34⁺ Hematopoietic Stem Cells from Normal Donors and Patients with Thalassemia, Effectively Re-Activate Endogenous γ-Globin

Aphrodite Georgakopoulou[1,2], Kiriaki Paschoudi[1,3], Nikoletta Psatha[4], Chang Li[4], Achilles Anagnostopoulos[1], André Lieber[4], Evangelia Yannaki[1]

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Recently, gene therapy of β-thalassemia, involving *ex vivo* lentivirus vector-mediated β-globin gene addition in hematopoietic stem cells (HSCs) has provided therapeutic clinical responses. In parallel, alternative approaches using gene editing platforms are being intensely explored as novel, curative and potentially safer opportunities for patients with hemoglobinopathies. Given that the hereditary persistence of fetal hemoglobin (HPFH) greatly ameliorates the clinical phenotype of β-thalassemia, we used CRISPR/Cas9-mediated gene editing to simultaneously disrupt both the binding sites of the gamma globin suppressor BCL11A within HBG1 and HBG2 promoters and the erythroid BCL11A enhancer, thus reactivating the expression of γ-globin. For the delivery of CRISPR/Cas9, a non-integrating helper-dependent adenoviral HDAd5/35++ vector (HDAd-CRISPR-Dual) was used, displaying high affinity to the hCD46 receptor which is uniformly expressed on human hematopoietic stem and progenitor cells. This CRISPR/Cas9 system was tested in CD34⁺ cells from healthy donors and thalassemia-major patients, previously enrolled in mobilization trials. Following 48-hour transduction, the cells were cultured in methylcellulose and erythroid differentiation medium and the efficacy of genome editing was evaluated by T7E1 assay for cleavage rates and flow cytometry for γ-globin expression. Gene disruption by T7 assay was 12.1±2.1% for the BCL11A enhancer and 15.6±1.7% for the HBG1/HBG2 site in normal CD34⁺ cells and 14.6±1.1% and 16.7±2.6% in thalassemic CD34⁺ cells, respectively, translating into significantly increased γ-globin expression post erythroid differentiation, despite the high background γ-expression in the untransduced samples, due to activated fetal globin synthesis by culture conditions. Specifically, the HbF expression in BFU-Es derived from the edited samples, in terms of % HbF expressing cells and intensity of expression, was higher than their counterparts in the untransduced BFU-Es [*% HbF expressing cells* : Normal 57.7±8.3% *vs* 32.5±8.2%, respectively, p=0.03, Thalassemic: 76.6±1.85% *vs* 61.8±5.6%, respectively, p=ns / *Mean fluorescence intensity (MFI)* : Normal 259.3±81 *vs* 96.1±6, respectively, p=0.05, Thalassemic: 1338.7±86.3 *vs* 355.6±54.4, respectively, p=0.000001]. Importantly, the expression was erythroid-specific as the edited myeloid colonies (CFU-GM) derived from normal and thalassemic CD34+ cells expressed HbF at barely detectable levels. In erythroid culture, HbF expression was again significantly higher in the edited over non-edited differentiated erythroid cells [*% HbF expressing cells*: Normal 30.9±1.6% vs 12.3±4.8%, respectively, p=0.01, Thal 81.4±1.18% vs 44±5.02%, respectively, p=0.0003 / *MFI*: Normal 44.6±0.6 vs 25.1±1.0, respectively, p=0.001, Thal 398.7±40.2 vs 76.3±32.1, respectively, p=0.00003]. *In vivo* studies in xenografts are in progress. Overall, we present a novel and potentially effective approach of HSC gene editing by CRISPR/Cas9-mediated reactivation of γ-globin expression that can be used as an alternative gene therapy platform for the treatment of hemoglobinopathies.

201. Abstract Withdrawn

202. Investigation of GeoCas9 as an Alternative CRISPR/Cas9 System to Treat Retinal Disease

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Introduction: CRISPR/Cas9 is a promising tool to treat hereditary retinal dystrophies. CRISPR/Cas9 consists of two components: a single guide RNA (sgRNA) and a Cas9 protein. The sgRNA allows for sequence-specific binding while Cas9 creates a double-stranded break in the target DNA. CRISPR/Cas9 can be packaged within an adeno-associated viral (AAV) vector for delivery into the retina. Only certain Cas9 proteins are small enough to be packaged within an AAV, which has a coding capacity of ~4.7 kb. SaCas9, which is ~3.2 kb in size, is commonly used. However, one limitation with SaCas9 is its PAM sequence (5'-NNGRRT-3' or 5'-NNGRR-3'), which restricts CRISPR/Cas9 editing to certain regions of the DNA. Exploring the potential of other Cas9 proteins with different PAM sequences would allow for targeting of a wider range of DNA sequences. One such alternative is GeoCas9, which is ~3.2 kb in size and has a PAM sequence that is 5'-NNNCRAA-3'. An additional advantage of GeoCas9 is that it may be less likely to induce an immune response because, unlike SaCas9, it is derived from a bacterium that is not known to cause disease in humans. We explore the DNA cleavage ability of GeoCas9 in HEK293 cells expressing enhanced green fluorescent protein (*eGFP*). **Methods:** Two transgenes were created for dual-transfection: one containing GeoCas9 driven by a CMV promoter (from plasmid #87703, Addgene) and another containing sgRNA driven by a U6 promoter. Four sgRNA variants targeting *eGFP*, one targeting rhodopsin (*RHO*), and one targeting vascular endothelial growth factor A (*VEGFA*) in HEK293 cells were inserted into the sgRNA construct downstream of the U6 promoter. A third transgene was used, which contained SaCas9 driven by a CMV promoter and

a *VEGFA*-targeting sgRNA driven by a U6 promoter. The ability of the GeoCas9 to cleave DNA after dual-transfection in HEK293-*eGFP* cells was assessed using tracking of indels by decomposition (TIDE) analysis. DNA cleavage by SaCas9 was used as a comparison. sgRNA and GeoCas9 RNA expression was assessed using quantitative polymerase chain reaction (QPCR) and GeoCas9 protein expression was determined with western blot. **Results:** Disruption of *VEGFA* by SaCas9 and GeoCas9 was compared using sgRNAs that target adjacent regions of DNA. The targeting efficiency of SaCas9 (62.7±4.7%) was significantly higher than that of GeoCas9 (0.33±0.24%) ($p<0.0001$). There was no significant disruption of the *eGFP* locus when the *eGFP*-targeting sgRNA plasmids (G1, G2, G3, and G4) were dual-transfected with the GeoCas9 plasmid (Geo) [$F_{Geo+G1}(5,12) = 6.679$, $p = 0.0034$, $p>0.05$ after correcting for false discovery rate; $F_{Geo+G2}(5,12) = 0.6746$, $p = 0.6507$; $F_{Geo+G3}(5,17) = 3.082$, $p = 0.0368$, $p>0.05$ after multiple comparisons; $F_{Geo+G4}(5,12) = 2.819$, $p = 0.0656$]. There was also no significant difference in rhodopsin disruption between any of the experimental samples [$F_{Geo+RHO}(5,12) = 0.7098$, $p = 0.6276$]. sgRNA and GeoCas9 RNA expression after transfection with plasmids was determined using qPCR. There was significantly more sgRNA expression in the cells transfected with sgRNA-coding plasmids compared to the controls ($p<0.0001$). There was also significantly more GeoCas9 expression in the cells transfected with GeoCas9-coding plasmids compared to the controls ($p<0.0001$). Presence of the GeoCas9 protein was confirmed by western blot. **Conclusion:** We show that *VEGFA*-targeting SaCas9 plasmids successfully cleave DNA while plasmids encoding GeoCas9 and the associated sgRNA cannot disrupt the *VEGFA*, *RHO*, and *eGFP* genes in HEK293-*eGFP* cells, despite expression of both sgRNA and GeoCas9 RNA and protein. Our findings may be due to the effects of post-translational processing on GeoCas9 activity, reduced GeoCas9 activity at 37°C, an incompatible sgRNA scaffold (tracrRNA) sequence, or reduced PAM site affinity.

203. Validation of CRISPR/Cas9 Off-Target Discovery Profiles from *In Silico* Prediction, Cell-Based & Biochemical-Based Assays with Targeted Off-Target Sequencing

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For CRISPR/Cas9 to be an effective and safe therapeutic drug in the treatment of diseases that would benefit from the reduction of pathogenic proteins, it can be beneficial to induce a high rate of knockout through indel frameshifts at the on-target locus and minimize any off-target editing. We present an integrative genomics approach that combines *in silico*, biochemical and cell-based genome-wide off-target discovery of 11 CRISPR/Cas9 guides designed to eliminate the expression of a secreted protein. A compendium of loci in the human genome were curated *in silico* predicted off-target profiles, the biochemical off-target discovery assay SITE-Seq®, and the cell-based oligo capture assay GUIDE-Seq. Validation of these off-target discovery profiles was conducted through targeted off-target sequencing on the empirical loci discovered and the top 30 *in silico* predicted coordinates. Our targeted off-target sequencing revealed that *in silico*-based methods had the lowest contribution to validated discovery of off-target editing. The biochemical discovery assay SITE-Seq® was the most sensitive and identified validated indels that were missed in the cell-based assay GUIDE-Seq. Therefore, an integrative genomics approach that combines *in silico* CRISPR guide design followed by empirical biochemical off-target discovery and validation through targeted off-target sequencing is useful to identify potential human CRISPR/Cas9 therapeutic candidates.

204. High-Throughput Screens of PAM-Flexible Cas9 Variants for Gene Knock-Out and Transcriptional Modulation

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A key limitation of the commonly-used CRISPR enzyme *S. pyogenes* Cas9 is the strict requirement of an NGG protospacer-adjacent motif (PAM) at the target site, which reduces the number of accessible genomic loci. This constraint can be limiting for genome editing applications that require precise Cas9 positioning. Recently, two Cas9 variants with a relaxed PAM requirement (NG) have been developed (xCas9 and Cas9-NG) but their activity has been measured at only a small number of endogenous sites. Here we devised a high-throughput Cas9 pooled competition screen to compare the performance of both PAM-flexible Cas9 variants and wild-type Cas9 at thousands of genomic loci and across 3 modalities (gene knock-out, transcriptional activation and suppression). We show that PAM flexibility comes at a substantial cost of decreased DNA targeting and cutting. Of the PAM-flexible variants, we found that Cas9-NG outperforms xCas9 regardless of genome engineering modality or PAM. Finally, we combined xCas9 mutations with those of Cas9-NG, creating a stronger transcriptional modulator than existing PAM-flexible Cas9 variants.

205. Novel CRISPR-Associated Enzymes for Gene Editing

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The field of genome editing has accelerated since the discovery of CRISPR-associated nucleases. These enzymes are part of adaptive microbial immune systems and make double-stranded breaks in viral genomic DNA at locations specified by a co-expressed guide RNA. The ability to reprogram these enzymes with a user-specified guide has made them compelling tools for genome editing. The large natural diversity of microbial viruses has driven the evolution of equally varied CRISPR systems. Despite the diversity of these systems in nature, few have been developed sufficiently for therapeutic applications, even though the systems currently in use have significant limitations. Here, we identify novel families of CRISPR systems and develop them into genome-editing platforms. These families were identified based on a big-data analysis of more than 11,000 metagenomes that were

collected from a variety of complex environments. Representative enzymes were further analyzed to determine nuclease characteristics (e.g. PAM sequence, cut site, type of DNA break, and specificity) and principles for guide design that enable their use. With select systems, we demonstrated editing of therapeutically-relevant genes in human cell lines. These novel CRISPR-associated effectors share low sequence identity with each other and with commonly used gene-editing systems. The systems exhibit novel predicted guide RNA structures and overcome limitations of current enzymes by expanding targetable genomic loci. The small sizes of some of these nucleases have the potential to simplify and improve delivery by AAV, facilitating in vivo applications. We anticipate that this expanded toolkit of CRISPR-associated enzymes will enable the majority of genome editing needs.

206. Targeted *piggyBac* Transposition Using Guide RNA in Human Cells

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Novel gene replacement technologies have the potential to safely and efficiently reverse genetic defects underlying many diseases. Many current approaches act passively, first initiating a double-stranded break, then relying on host repair to uptake donor DNA. Alternatively, we delivered an actively integrating transposase to the target sequence to initiate gene insertion. We fused the *piggyBac* transposase to catalytically dead Cas9 (dCas9) and designed guide RNAs (gRNAs) to the CCR5 safe harbor sequence. We introduced mutations to the native DNA binding domain of *piggyBac* to reduce non-specific binding of the transposase and cause the fusion protein to favor binding by dCas9. This strategy enabled us, for the first time, to direct transposition to the genome using gRNA. We showed that increasing the number of gRNAs improved targeting efficiency. Interestingly, over half of the recovered insertions were found at a single TTAA hotspot. We also found that the fusion increased the error rate at the genome-transposon junction. We isolated clonal cell lines containing a single insertion at CCR5 and demonstrated long-term expression from this locus. This system can easily be designed to bind nearly any target sequence, facilitating applications by an expanded number of research groups. We observed high levels of off-target integration which will need to be addressed in future studies and have identified several strategies to improve specificity. Ultimately, we hope to develop an improved vector that exclusively integrates at the target sequence. This would enable targeted delivery of large cargos such as multiple gene cassettes or long endogenous sequences. A strictly site-specific, RNA-guided transposase would have important applications including transgenic animal generation, modification of cell lines for research and diagnostics, or delivery of therapeutic cargo to a designated location in the genome. The proof-of-concept that dCas9 can direct *piggyBac* genome insertions is a crucial step for this early stage technology.

207. *Apoa1*, a Safe Harbor Locus for Therapeutic Liver Genome Editing

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The liver is an organ of particular interest for gene therapy, both for correcting inherited metabolic disorders, as well as a biofactory for secretion of therapeutic proteins. The CRISPR/Cas9 genome editing system has greatly improved specificity and precision of gene targeting and can be efficiently delivered to the liver with adeno associated viral vectors (AAV). The goal of this project is to identify novel safe harbor sites in the liver that could drive high expression of therapeutic proteins. We performed ChIP-Seq for histone H3K27 acetylation and RNA Pol II binding and ATAC-Seq, and identified Apolipoprotein a1 (*Apoa1*) as one of the most highly expressed and accessible loci in mouse and human liver. To target the *Apoa1* locus, we injected adult or postnatal 4 (P4) *C57BL/6J* mice with a dual AAV8 system, consisting of the CRISPR/*Staphylococcus aureus* Cas9 machinery (AAV-CRISPR) and a donor cassette (AAV-Donor), which includes the final coding exon of *Apoa1* (Exon 4) fused to a promoterless marker gene (mKate2) by a 2A self-cleaving peptide coding sequence, flanked by homology arms to the *Apoa1* locus. By deep sequencing, we observed efficient on-target nuclease activity at the *Apoa1* locus (indels frequency: 51.5 ± 3.2%) and no off-target activity in any of the predicted sites. We detected two major integration events at the *Apoa1* locus: the expected homology directed repair (HDR)-mediated integration of the donor cassette and the non-homologous end joining (NHEJ)-insertion of the whole AAV genome. By droplet digital PCR, we observed 11.8 ± 2.5 and 1.1 ± 0.6 % of NHEJ- and HDR-insertion in adult injected-mice, which resulted in 5.7 ± 2.9 % of mKate2-positive hepatocytes. In mice injected at P4, the NHEJ- and HDR-insertion frequency was respectively 21.5 ± 8 and 9.5 ± 3.3 % and the percentage of mKate2-positive hepatocytes increased up to 16.4 ± 3.6 %. The genome editing of the *Apoa1* locus did not adversely affect the endogenous level of apoA1 as well as neighboring genes, and no liver toxicity or histopathological abnormalities were observed. To test whether the *Apoa1* locus could support the production of a secreted therapeutic protein, we designed an AAV-Donor carrying the coding sequence of human Factor IX (*FIX*) fused to the 2A sequence. We observed the efficient and sustained expression of FIX from the *Apoa1* locus over a period of six months post-injection (0.23 ± 0.06 μg/ml at 24 weeks). Finally, we sought to determine whether a targeted gene therapy at the *Apoa1* locus could correct an inherited metabolic liver disorder. We injected a mouse model of Hereditary tyrosinemia type I, fumarylacetoacetate hydrolase deficient ($Fah^{-/-}$) mice, with AAV-CRISPR plus an AAV-Donor carrying human *FAH*. The targeted expression of *FAH* by the *Apoa1* locus resulted in rescue of lethality, repopulation of the liver by FAH positive cells, recovery of normal histology and liver function. In addition, the targeted expression of human apolipoprotein E (*APOE*) by the *Apoa1* locus resulted in APOE restoration and amelioration of

plasma cholesterol levels in a mouse model of hypercholesterolemia (*Apoe*$^{-/-}$ mice). Overall, these results show *Apoa1* as novel safe harbor site for targeted gene therapy in the liver.

208. Durable and Efficacious Expression of Factor IX Driven by GeneRide in Liver Injury Models

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Recent progress in AAV gene therapy has shown great promise in numerous monogenetic diseases. However, pediatric patients may not be suitable for such approach due to loss of episomal DNA copies in growing tissue such as liver. Though promising clinical data have been documented in adult patients, the long-term effect of liver-targeted canonical AAV therapy remains to be demonstrated, especially in cases where adult liver starts to regenerate/repair in response to acute or chronic stimuli. GeneRide™ is a novel AAV-based, promoterless, nuclease-free, genome editing technology that leverages the natural process of homologous recombination to insert, site-specifically, a copy of therapeutic transgene into the genome (*Barzel A, et al. 2015 Nature*). By being stably integrated in the genome, GeneRide can overcome the limitation of canonical AAV in situations of rapid liver proliferation during regeneration, as evidenced by stable transgene expression following 70% partial hepatectomy. Using human factor IX (hFIX) as transgene, we have demonstrated that GeneRide can achieve durable and stable therapeutic levels of hFIX when dosed to both neonatal and adult mice. In contrast, canonical AAV with liver specific promoter driven the same transgene showed over 100-fold reduction in hFIX expression level when dosed to neonatal mice compared to that in adult mice. We further evaluated the durability of GeneRide in two clinically relevant liver injury models, namely, (1) non-alcoholic fatty liver disease (NAFLD, 28-week high fat diet feeding) and (2) Tylenol overdose-induced acute liver toxicity (Acetaminophen, 400mg/kg, ip). The selection of these liver injury models is based on the prevalence and mechanism. NAFLD is the most common cause of chronic liver disease affecting 1 billion population worldwide. Acetaminophen is a commonly used analgesic in US which can cause serious acute hepatotoxicity at doses over 4 g/day. Each year in US, more than 30000 patients are hospitalized due to acetaminophen overdose. The NAFLD mouse model showed progressive elevation of ALT and steatosis (H&E staining) compared to control diet. The chronic feeding also induced 6-fold increase of hepatocyte turnover as evidenced by increased Ki67+ hepatocytes (Ki67 immunohistochemistry). As expected, the acute Tylenol overdose model showed a much higher ALT elevation and thus a corresponding 50-fold increase of hepatocyte proliferation. Interestingly, in both liver injury models, GeneRide treatment achieved stable and durable hFIX levels despite of liver damage and increased hepatocyte proliferation. In conclusion, GeneRide can retain durable gene expression not only in neonatal animals with a rapidly growing liver, but also in adult animals with liver injury conditions that are of high prevalence in general population. Taken together, GeneRide has shown great potential in achieving long-term benefits for both pediatric and adult patients.

209. CALITAS: A CRISPR/Cas-Aware ALigner for In Silico Off-Target Search

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CRISPR/Cas-based medicines are being developed to treat serious diseases. Assessing the specificity of the DNA endonuclease is important to evaluating the safety of CRISPR-based medicines. In silico predictions are a common step for assembling lists of candidate off-target sites which are closely related to the guide RNA (gRNA) targeting sequence (in addition to experimental methods such as GUIDE-seq, BLISS, Digenome-Seq, or CIRCLE-Seq). We describe CALITAS, a CRISPR/CAS-aware aligner that predicts off-targets efficiently and more accurately than current in silico tools. CALITAS performs an exhaustive search across the genome using a modified version of the Needleman-Wunsch (NW) algorithm. Unlike other methods, the guide sequence without PAM is aligned first. The candidate guide alignments are then extended to include the PAM, allowing for possible gaps between guide and PAM, and for mismatches within the PAM. This extension process supports zero or more PAM sequences and selects the best matching PAM per off-target site. Additionally, the NW mismatch and gap alignment penalties have been tuned to support short alignments, i.e. typical gRNA length, against much larger genomic sequences. This ensures that all candidate sites can be detected for a given target site within a genome up to any given number of mismatches plus gaps. Key new features compared to other aligners and off-target finding algorithms are the following: Unlimited gRNA mismatches and gaps. Mismatches in the PAM are tolerated. Ability to use multiple PAM sequences or no PAM. Option to produce either the single best alignment per off-target site or all alignments meeting mismatch/gap limits. Ability to set base pair overlap cutoff for differentiating unique adjacent alignments. Ability to align against alternate alleles in the reference, via user-provided VCF files, for example from the 1000 Genomes Project. We used our algorithm to make predictions for 41 guides, that were selected to have optimal on-target PAMs for the nucleases SpCas9, SaCas9 and Cas12a. We also made predictions performing a PAM-less search. Our results show that among the CRISPR/Cas nucleases, the number of predicted off-target sites is the lowest for Cas12a and the highest for SpCas9. To assess the accuracy of our in silico predictions we compared our off-target predictions with experimentally detected Digenome-Seq off-targets obtained for a SpCas9 guide with many (~1000) off-targets. This shows that allowing multiple gaps for alignments is important for making accurate in silico off-target predictions. Furthermore, we also compared our predictions with off-targets and alignments found in the literature and show that CALITAS produces more optimal alignments with reduced overall mismatches plus gaps. Finally, we compared our predictions with the ones obtained using other available methods: Cas-OFFinder and CRISPRitz. Besides the improvement listed above we show that CALITAS genome wide searches finds more off-target sites.

CALITAS is a novel tool that allows precise and relevant prediction of candidate off-target sites and is a valuable method to help in the specificity assessment of CRISPR/Cas-based medicines. We believe that CALITAS is a best-in-class tool for CRISPR/CAS in silico alignment and off-target searches.

210. Highly Efficient Multi-Gene Knockout and Transgene Knock-in Using CRISPR-Cas12a in Induced Pluripotent Stem Cells for the Generation of Engineered Cell Immunotherapies

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To realize the full potential of cell-based immunotherapies for the treatment of solid tumors, it is likely necessary to highly modify cells to overcome challenges in the tumor microenvironment (TME), improve persistence, increase tumor homing, and resist immune rejection. Induced pluripotent stem cells (iPSCs) offer a renewable source of highly characterized cells that can be differentiated into an array of immune effector cells, including, but not limited to, iPSC-derived natural killer cells (iNKs). Edited iPSC clones can be screened and selected to contain only the desired edits (i.e., no off-target edits), thus ensuring a pure and 100% edited final population of iNKs with an economical manufacturing process. The generation of highly engineered iPSCs is challenging as these cells are less amenable to physical and genetic manipulation than other cell types. For example, prolonged existence as a single-cell suspension is associated with cell death, whereas overcrowding can lead to spontaneous differentiation. To overcome these challenges, we developed a method where iPSCs were first electroporated from a thawed aliquot that had been previously frozen rather than from a continuously growing culture of cells. This method minimized user variability, reduced contamination risk, and enabled rapid testing. To maximize editing efficiency, we used CRISPR-Cas12a, and optimized parameters such as guide identity, RNP concentration, electroporation buffer, electroporation setting, and cell number. The optimized method yielded >70% editing in the bulk iPSC population for all immune-effector gene targets with some targets reaching >99% efficiency by next generation sequencing. In addition to excellent efficiency, Cas12a is more specific than other widely used nucleases such as SpCas9, thereby reducing the potential for undesired off-target editing. Having achieved highly efficient and specific gene editing, we then optimized transgene knock-in efficiency using Cas12a by optimizing parameters such as homology arm length, SNPs to avoid re-cutting, electroporation conditions, and small molecule additives to transiently modulate DNA repair pathways. This work showed that plasmid DNA templates were good substrates for target integration as plasmids allow insertion of several different transgenes into a single target locus, and cargoes are not limited by size like AAV vectors (<5 kb cargo limit). The optimized method reproducibly achieved transgene knock-in of >5% using double-stranded DNA plasmids, as measured by ddPCR. Taken together, these findings show that Cas12a, with its robust editing capability and high intrinsic specificity, can be used to make a highly edited iPSC clone. The resulting highly characterized iPSC clones can be differentiated into various immune-effector cells to potentially overcome the challenges of treating solid tumors.

211. IDT Alt-R System - Enhancing Genome Editing and Homology-Directed Repair (HDR) with Improved CRISPR Reagents and Novel Design Tools

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Precise genome editing using the CRISPR-Cas systems has been a groundbreaking development for basic research and commercial endeavors, alike. However, poor editing efficiency, off-target cleavage, and low rates of precise homology-directed repair (HDR) present challenges to experimental success. Improvements to efficiency and specificity, in particular, can save valuable time and resources for scientists executing genome engineering experiments. Here, we present the complete Alt-R CRISPR genome editing workflow, showcasing the innovative solutions that have been developed to address these hurdles. The Alt-R CRISPR-Cas9 design tool applies empirically defined rules to identify potent and specific guide RNAs (gRNA). IDT has developed oligonucleotide synthesis methods to rapidly generate high-quality gRNAs which are customizable for many applications. These gRNAs are made available as both the native 2-part crRNA:tracrRNA complex and the fused single guide RNA (sgRNA) with chemical modifications to protect against nuclease degradation. Through protein evolution, we have developed novel Cas9 and Cas12a variants that have improved activity and specificity which allow for an expanded genome editing space. The highly specific Alt-R components are validated using rhAmpSeq™ - a multiplexed, amplicon enrichment method for next generation sequencing that affords simultaneous amplification of on- and off-target editing sites. Finally, specific edits made via HDR are enabled by a suite of reagents and the Alt-R CRISPR HDR Design Tool to increase the frequency of repair outcomes by this pathway. We will present this novel bioinformatics tool for ssDNA HDR template design. Further, we describe methods to improve rates of HDR by using modified donor templates as well as HDR-enhancing small molecules. Taken together, the Alt-R CRISPR system provides incremental enhancements to the gRNA, CRISPR nucleases, HDR donor template design, and genome editing analyses to collectively result in efficient and specific genome editing.

212. Optimization and Comparison of High-Throughput Methods for Assessing PAM Specificity *In Vitro* and in Cells

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CRISPR-Cas genome editors are a transformative technology for both biomedical research and potential clinical applications. CRISPR-Cas RNA-guided nucleases are unique because their activities are

largely specified by RNA:DNA interactions between a short guide RNA (gRNA) and an intended target site. The targeting range of CRISPR-Cas nucleases is restricted by the requirement for a specific protospacer adjacent motif (PAM) that licenses initial DNA binding. Interestingly, some CRISPR-Cas nucleases have been reported to have high activity *in vitro* but low activity in mammalian cells. To understand potential differences in activity between *in vitro*, bacterial, and human cell contexts we have applied and compared several high-throughput randomized PAM specificity assays. In brief, we adapted: 1) an *in vitro* PAM enrichment assay that selectively sequences cleaved target sequences[1], 2) a bacterial PAM depletion assay that sequences surviving colonies with uncut targets[2], and 3) a barcoded lentiviral PAM activity assay in human cells where indel activity can be measured without selection[3]. In our cell-based lentiviral PAM specificity assay, we found that Cas9 exhibited robust and specific activity towards NGG PAMs with much less activity for reported non-canonical PAM sequences. While Cas9 showed the highest activity towards targets with NGG PAMs in our bacterial PAM depletion assay, Cas9 also targeted regions with NAG and NGA PAMs. Finally, using high-throughput PAM depletion and PAM enrichment assays *in vitro*, we found that Cas9 favored NGG PAMs yet displayed editing activity towards sequences with NAG, NGA, and NTG motifs. Mammalian Cas9 PAM specificity assays have the highest apparent stringency in our initial analyses. In future studies, comparisons using these types of methods may help define the true targeting range of engineered variants such as Cas9-NG and xCas9.
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213. Extracellular Vesicles - A Tattletale for Rare Gene Editing Events

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Introduction - Since its discovery, gene editing has provided the ability to meticulously change genes with a profound effect on both therapeutics and molecular research. Even with new tools constantly being developed to increase efficiency and precision of the technique, the repair mechanisms post-gene editing are still error prone making it critical to detect and/or select a desired genecorrected cell clone. Since the contents of extracellular vesicles (EVs) reflect the cells that produced them, if a gene editing event occurs, the EV cargo should contain the gene corrected products, such as a protein or RNA species. The catch lays in the fact that EVs are by their nature very heterogeneous and only a small fraction of the population may harbor the gene edited products. Methods - We designed a CD63 construct with a genomic DNA target sequence for detection of a desired gene editing event. The gene editing target harbors a premature stop codon. Only when the desired gene editing event occurs to correct stop codon truncations by genomic missense or frameshift mutations, is a bioluminescent signal detected, as it then allows the CD63 tethered luciferase reporter to be translated. Results -This reporter detects gene corrections 2 days post-introduction of Cas9 and a sgRNA targeting the stop codon. Next-Generation Sequencing confirmed that the signal resulted from 1.12% gDNA changes. Our observations highlight the sensitivity of our system to detect even highly inefficient non-homologous end joining repair after a double-strand break within the target DNA. The CD63 construct also contains a membrane surface immune affinity tag to facilitate isolation of cells that encode the full length reporter, excluding the non-gene edited cells, without the need for single cell FACS. Moreover, the latter tag enables isolation of a pure EV population from these corrected cells to be isolated in a 1-2 hr procedure from the cell media. These EVs are detectable with luminescence if the reporter is fully expressed in the target cells. Our data shows that with this construct, EVs can be selected out of a heterogeneous pool of EVs that contain RNA solely expressed in the corrected cell. The latter observation allows the EVs derived from corrected cells to report on RNA derived from CRISPR/Cas9 events without the need for cell lysis and gene sequencing. Summary/Conclusion -A CD63 transgenic reporter protein contained in the membrane of cells and EVs may be used to detect and select out correctly gene edited EV-donor cells early on, reducing effort in avoiding cells with off targets effects.

214. Comparison of Cas9 Orthologues for Gene Suppression Activity

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The development of CRISPR-Cas systems provides a powerful tool for basic research as well as a potential avenue for therapy of genetic diseases. A number of different Cas nucleases have been used for genome and epigenome perturbations. Nevertheless, their delivery via adeno-associated vector (AAV) is limited due to the small packaging capacity of the delivery platform. Furthermore, most published studies have utilized different genetic targets, construct designs and regulatory elements making it difficult to draw conclusions about the comparative efficiencies and specificities of each nuclease. Here we report the development and optimization of an all-in-one AAV system harboring Campylobacter jejuni (CjCas9): one of the smallest Cas9 nucleases. The system has been validated on HEK293 cells carrying a copy of a GFP reporter. Furthermore, we built similar counterparts harboring two different Cas9 variants: SpCas9, and SaCas9. All constructs used exactly the same regulatory elements and are assembled into identical vectors for delivery. We assayed comparative efficiencies of each nuclease by plasmid transfection into HEK293-GFP cells. Genomic DNA was isolated 3 days post transfection and regions in close proximity to target gRNAs were PCR-amplified for analysis of induction of insertions and deletions (indels) by TIDE analysis. Additionally, protein was isolated from the HEK293-GFP cells and GFP fluorescence was quantified using fluorescence spectroscopy. Our experimental strategies allowed us to compare the efficiency of different components for genome engineering on the same target and indicate that SpCas9 may have the highest efficiency among the tested Cas9 variants, although the SaCas9 and CjCas9 orthologues were also able to generate a high level of targeted indels within eGFP. We aim to further assess the

efficiency and specificity of CjCas9 paired with repressor domains DNA-methyltransferase3A (DNMT3A) and Krüppel-associated box (KRAB) for targeted epigenetic editing via AAV delivery.

215. Identification and Characterization of New Cas9 Variants within the Class II CRISPR-Cas Systems

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CRISPR-Cas9 system has transformed the genome engineering field. Despite the excitement around, the system still lacks precision and efficiency for clinical applications. The most efficient DNA repair pathway used for targeted integration are Homology Directed Repair (HDR) pathway and the Non-Homologous End Joining (NHEJ) pathway. HDR pathway has intrinsic problems such as restriction to cell cycle whereas NHEJ pathway is mostly error-prone but is active throughout the cell-cycle. Here we have performed in depth bioinformatic analysis within the type-II CRISPR-Cas systems and looked for Cas9 orthologues that are more precise and more prone to induce targeted integration. We have identified a new ortholog (MHCas9) from the sequenced gut metagenome and characterized its properties for genome editing both *in vitro* and *in vivo*. We have found that this new variant generates staggered end breaks by 5′-overhangs while retaining SpCas9 like properties. We show that MHCas9 requires more specific binding interaction with the target site with less mismatch tolerance. Most interestingly, we have proven that this new variant has unique HDR and NHEJ mediated transgene integration property with increased efficiency compared to SpCas9 at various target sites. By using genetically encoded fluorescent tags, we have also shown that MHCas9 can be used for large-cassette integration via NHEJ pathway and flow cytometry analysis showed that it dominantly facilitates directional knock-in of the transgene. Together, we speculate that this new variant can provide new opportunities for precise genome editing and can be harnessed for a broad range of cell types with more predictable outcome.

216. Gene Editing with CRISPR/Cas9 to Correct DMD Exon Duplication Mutations in Patient-Derived Cells and Mice

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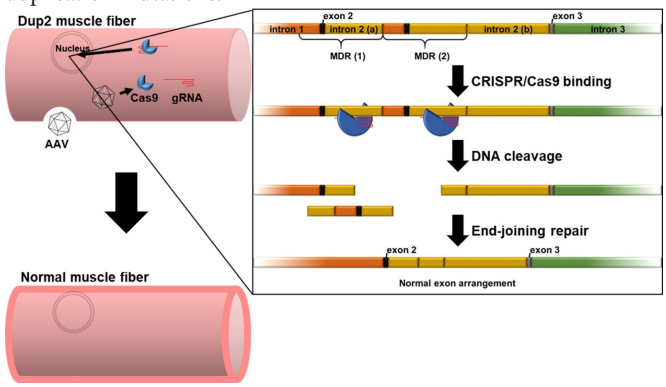
Dystrophinopathies are a spectrum of X-linked diseases including Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) caused by mutations in the DMD gene which codes for dystrophin, the vital, muscle-specific structural protein. Disease severity ranges from muscle weakness later in life for the mildest forms of BMD to complete loss of ambulation in adolescence and death from cardiac and respiratory complications in the teens and early twenties for the most severe forms of DMD. While dystrophinopathies can be caused by myriad mutations of the DMD gene, exon duplications are among the most common affecting approximately 11% of dystrophinopathy patients. Duplication of exon 2 (Dup2) is the most frequent and typically includes a minimal duplicated region (MDR) spanning the last ~1.5 kb of intron 1 through the first ~8.4 kb of intron 2. Somatic cell gene editing in patients' muscle tissues to excise duplicated exons is a potential therapeutic strategy for Dup2 patients. Here, we designed CRISPR guide RNAs (gRNAs) that target within the MDR of humans and mice and tested their gene editing activity in model human cells and in vivo. We identified highly-active gRNAs and demonstrated the feasibility of generating large genomic deletions that can correct exon duplications in the DMD gene when co-expressed with Cas9 via plasmid- or viral-based delivery. These results support further development of CRISPR-mediated gene editing for DMD duplication mutations.



217. Highly Precise Ex Vivo Correction of Sickle Cell Anemia Mutation in Patient Cells Using FnCas9

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Successful therapeutic gene correction is governed by the precision and efficacy of genome editing proteins. First generation CRISPR tools have relied on Cas9 from Streptococcus pyogenes (SpCas9) and its engineered variants for proof-of-concept correction of multiple monogenic mutations including sickle cell disease (SCD). However, persistent reports of off-targeting and large scale genomic changes have raised concerns about the safety and efficacy of such tools necessitating the exploration of better alternatives. We show that the orthogonal Cas protein from *Francisella novicida* (FnCas9) shows

remarkable ability to discriminate between off-targets even at the level of DNA binding making it highly suitable for both Homology directed repair (HDR) mediated gene correction as well as base and prime-editing. Our preliminary results of FnCas9 mediated editing in patient derived stem cells show successful correction of the SCD mutation and higher HDR rates than SpCas9 suggesting that the protein has fundamentally distinct properties of target recognition and DNA cleavage making it suitable for precise therapeutic applications. We have attempted to uncover its mode of DNA interrogation through biophysical measurements including bulk and single molecule studies and observed unique patterns of DNA:protein interactions that lie central to its targeting specificity and cleavage outcomes. At the genomic level, these translate to successful target discrimination from potentially similar sites, a pre-requisite for safe therapeutic editing. Taking cues from these studies, we have expanded the toolbox with base and prime editor versions of FnCas9 which reveal the scope of diverse applications of these proteins in targeted gene correction that can extend the platform to a larger number of diseases. We are currently moving towards preclinical validation of FnCas9 for SCD correction and developing effective strategies for its delivery for potential clinical applications in the coming years.

218. Development of Cytosine and Adenine Base Editors with an Expanded Targeting Range

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RNA-guided programmable cytosine and adenine base editors are a powerful class of genome editing tools for the introduction of localized base transitions without requiring the formation of a double-stranded DNA break. Base editors (BE) have an optimal window of activity relative to the protospacer adjacent motif (PAM) recognized by the Cas9 enzyme and these constructs are strand selective. Consequently, even for the widely used *Streptococcus pyogenes* Cas9 enzyme (SpyCas9), which requires an NGG PAM for target engagement, there are limitations in the number of genomic loci that can be effectively targeted. Broadening the genomic targeting range of base editing systems while maintaining robust and precise activity will extend the potential therapeutic genome editing applications amenable to these systems. Here we demonstrate that fusion of a programmable DNA-binding domain (pDBD) or another Cas9 orthologue to an SpyCas9 adenine or cytosine base editor can produce an RNA-programmable Cas9-pDBD or Cas9-Cas9 fusion base editor chimeras with dramatically improved base editing rates. These chimeric base editors also display an expanded targeting repertoire through the ability to utilize sub-optimal PAM sequences. These attributes should permit a larger fraction of disease-associated single base mutations to be efficiently corrected using these chimeric base editing platforms.

219. Modulating the Activity of CRISPR-Cas with Chemical Modifications in Single-Guide RNAs

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As gene editing and control systems move rapidly from research to therapeutic applications, adjusting the duration of their activity *in vitro* and *in vivo* becomes increasingly important. Leveraging our capability to robustly chemically synthesize single-guide RNAs and to incorporate modified nucleotides at any position, we have developed novel approaches for improving the activity and specificity of CRISPR systems using chemical modifications in guide RNAs. Previously, we developed a novel approach for enhancing efficiency of gene editing, especially in primary cells, by employing site-specific chemical modifications, e.g. 2'-O-methyl-3'phosphorothioate (MS) modification of the first and last three internucleotide linkages at both ends of guide RNAs (Hendel et al. Nature Biotechnology, 2015). We have performed further investigations of different types of chemical modifications at various positions in single-guide RNAs to identify configurations that further extend their lifetime in cells while maintaining strong activity of the CRISPR-Cas system for binding and cleavage of target DNA sequences. For example, single-guide RNAs with three MS modifications at both ends (3xMS-3xMS) or three MS modifications at the 5' end and two, three or four MP (2'-O-methyl-3'PACE) modifications at the 3' end (3xMS-2xMP, 3xMS-3xMP, and 3xMS-4xMP, respectively) were transfected into mammalian cells without Cas9 protein, and these exhibited increasingly longer half-lives across this series as measured directly by qRT-PCR of the guide RNAs, especially when larger numbers of MP modifications were added at the 3' end. Measurements of indel frequencies by deep sequencing of HepG2 cells co-transfected with these modified single-guide RNAs and Cas9 mRNA showed order of magnitude increases in editing activity by using guide RNAs with 3xMS-4xMP modifications compared to the 3xMS-3xMS modifications. We have also investigated the performance of guide RNAs including modifications at both ends such as 3xMS-3xMP with additional modifications placed throughout the guide RNA to further enhance stability of guide RNAs while maintaining good activity and specificity. These studies further demonstrate novel configurations of chemical modifications in guide RNAs that increase and/or modulate the activity of CRISPR-Cas systems, a capability that may be particularly useful when delivering guide RNAs into difficult to edit cells or when delivering guide RNAs *in vivo*.

220. A Novel System for Identifying Endogenous Promoters for Driving Expression of Selectable Guide RNAS In Vivo

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Precise recombinant adeno-associated virus (rAAV) gene editing is an attractive approach for gene therapies. While liver gene therapy

by rAAV is often limited by the small population of hepatocytes that have the desired gene edit, we have previously developed a method to link a desired genetic modification to a selectable gene disruption cassette in cis. Using this system, we can select and expand hepatocytes with proper gene targeting. We have previously targeted our construct to the highly expressed Albumin locus. However, it would be desirable to be able to target any disease gene locus in hepatocytes regardless of their level of expression. The promoterless GeneRide vector makes a targeted integration at a designated locus, and is then expressed from the endogenous promoter at that locus. To add a promoterless disruption cassette to the GeneRide vector, we utilized a self-cleaving guide RNA (scgRNA). The scgRNA consists of a gRNA flanked by two self-cleaving ribozymes. The ribozymes permit the proper processing of an active gRNA from a polymerase 2 transcript, keeping the promoterless GeneRide construct truly promoterless. Using a GeneRide vector expressing human Factor 9 (F9) and a scgRNA from the Albumin locus, with selection mice display supraphysiological levels of human F9. While this can be beneficial for some therapeutic genes, high levels of others can be detrimental due to toxicity. One way to modulate the expression of the transgene and achieve physiologically relevant levels is to target the rAAV scgRNA GeneRide construct to a lower expressing loci. However, the endogenous promoter must still be strong enough to produce sufficient expression of the scgRNA. Here we show that the scgRNA GeneRide vector can function from lower expressing loci using an Ai9-tdTomato reporter mouse which constitutively expresses SpCas9. The Ai9-tdTomato mouse contains a transgene consisting of a CAG promoter followed by a stop cassette and the fluorescent protein tdTomato. We have designed GeneRide constructs containing an scgRNA against the stop cassette to integrate into the transthyretin (Ttr), phenylalanine hydroxylase (Pah) and factor IX (F9) loci, which are respectively 10, 80 and 400-fold lower expressing than albumin. In this system, expression of tdTomato occurs when the stop cassette is inactivated by an indel mutation caused by the scgRNA. Frequency of integration of the GeneRide construct by homologous recombination can then be assessed by determining the percentage of tdTomato positive hepatocytes. In both neonate and adult mice analyzed after rAAV delivery, we observed homologous recombination resulting from all genes targeted at a frequency of 0.15% to 0.61% of hepatocytes, indicating that homologous recombination and expression of the scgRNA occurred. Furthermore, we demonstrate that homologous recombination occurs during cell division of hepatocytes. A time course in neonates showed that homologous recombination began to occur 4 days following injection. Overall, we show that targeting and expression of the scgRNA from lower expressing loci can be successfully achieved suggesting that sufficient amounts of scgRNA can be produced from weakly expressed polymerase 2 promoters. Based on these results we will aim to generate reporter mice for low expressing genes utilizing this system.

221. A Nanoparticle Mediated Genome Editing Technique for Single-Cell Murine Embryos

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Genome editing has the potential to treat numerous genetic disorders. Gene editing at the embryonic stage may have advantages due to the small number of cells, which are rapidly dividing. If heritable, genetic changes made in the early embryo will be passed onto daughter cells that will go on to form the rest of the organism, potentially allowing high rates of editing to be maintained throughout life. Embryonic gene editing is potentially useful in the creation of genetic mouse models and improving the output of livestock products. If found to be safe and ethical, it could also be used during in vitro fertilization (IVF) for the treatment or prevention of human disease. Here, we have used peptide nucleic acids (PNA) and a donor DNA oligonucleotide delivered with poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) to achieve gene editing in single-cell, fertilized embryos from an eGFP reporter mouse. In this mouse, successful editing results in eGFP expression. PNA/DNA NPs were added to media containing single-cell embryos. After treatment, the embryos developed normally in culture, with no apparent cytotoxicity -- treated embryos had similar rates of development to the 2-cell stage and blastocyst stage to untreated controls and exhibited normal zona pellucida hatching. Rates of gene editing increased with NP dose in the medium: The highest tested dose of 500 μg/mL NPs resulted in an average of 94% editing four days after treatment, at the blastocyst stage, as measured by ddPCR. Treated embryos also appear green under a fluorescence microscope. NP treated embryos were reimplanted into pseudopregnant dams at the two-cell stage. PNA/DNA NP treatment did not appear to be toxic, with a two-cell reimplantation rate of 43% (49 of 113 embryos) compared to an untreated reimplantation rate of 51% (21 of 41 embryos). Over 30 days after birth, treated pups also had normal growth compared to untreated mice and had normal morphology, behavior, and no gross developmental defects. Treatment with PNA/DNA NPs at the single-cell stage resulted in pups that expressed eGFP by fluorescence microscopy throughout their tissues, evaluated at e17.5 and p21. 89% of analyzed treated pups (17 of 19) had gene editing in at least one organ by ddPCR. 37% of treated pups (7 of 19) had editing in all analyzed organs by ddPCR. This work demonstrates the proof of concept that PNA/DNA NPs can be safely administered to developing murine embryos and can produce high levels of editing that are sustained after birth.

222. Toward Clinical Indel-Free Genome Editing by Shifting from DSB-Mediated CRISPR-Cas9 Gene Targeting to Breakthrough DSB-Free Prime Editing Driven by a Cas9 Nickase Fused to an Engineered Reverse Transcriptase

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Programmable CRISPR-Cas9 RNA-guided DNA endonuclease and derived nickase mutants and nuclease-null double mutant are at the origin of the current genome/epigenome editing revolution thanks to the easy and cost-effective customization of the ≈100 nt-long single guide RNA (sgRNA) with ≈20 nt complementary to the genomic target adjacent to a protospacer-adjacent motif (PAM). Hot-off-the press prime editing (PE) breakthrough drives direct polymerization

of edited DNA from an extended PE sgRNA (pegRNA) into the target genomic site. PE relies on a Cas9 nickase fused to an engineered reverse transcriptase (RT) and programmed by a pegRNA that comprises a 3'-end extension including a primer binding site (PBS) and an edit-encoding sequence that will be converted into a RT template upon PBS binding to the nicked PAM-containing DNA strand. By contrast, Cas9 (and zinc finger/TALE nucleases) relies on a double-strand break (DSB) in target genomic DNA in order to boost homologous recombination with donor DNA (gene targeting), thereby providing efficient targeted genome editing; mediated by the DSB homology-directed repair (HDR) pathway, gene targeting is however hampered by the deleterious genesis of mixtures of insertion/deletion mutations (indels) resulting from the overwhelming activity of the error-prone non-homologous end-joining (NHEJ) DSB-repair pathway. Unlike Cas9, PE has been shown, by its inventors, to mediate efficient targeted genomic insertions (up to 44 bp), deletions (up to 80 bp), all 12 possible base-to-base conversions and combinations in human cells without requiring DSBs or donor DNA templates. The current most sophisticated PE method (PE3b) includes an ancillary sgRNA with a spacer matching the edited target DNA strand but not the original sequence, in order to nick the non-edited strand and induce its replacement from the transient heteroduplex, thereby increasing editing efficiency. Under PE3b conditions, very high editing efficiencies have been described (> 50%) with low on-target indel numbers (< 1%), a ratio of editing to indels strikingly higher than with Cas9-initiated HDR. Such an on-target efficiency and specificity is a major achievement critically dependent on pegRNA optimization (PBS length and RT/edit template) and ancillary-sgRNA design. Regarding off-target genotoxicity, genome-wide evaluation has not yet been done; however, PE induced much less off-target indels than Cas9 at known Cas9 off-target sites. Both off-target and on-target DSBs are major genotoxic hazards that hamper Cas9-initiated HDR, the main drive of the current genome editing arm of our proposed universal stem cell gene therapy platform. We are thus shifting from hazardous DSB-mediated Cas9 gene targeting to breakthrough DSB-free donor DNA-free PE. So far, PE has been mediated by transfected/transduced transgenes encoding the fusion protein and peg/sgRNAs. Although split dual-AAV vectors have been successfully used for in vivo base editing at therapeutically relevant efficiencies in mouse organs, we believe that a transient expression strategy mediated by the delivery of ribonucleoprotein (RNP) complexes is a more promising way to raise PE safety and efficiency to a level compatible with ex vivo/in vivo clinical trials. Our hit-and-run approach mediated by premade PE3b RNP complexes is thus discussed in light of 1) the four arms of our proposed universal stem gene therapy platform (transient epigenome editing arm included) and their synergistic use, 2) published Cas9:sgRNA RNP delivery mediated by lentiviral capsid-based bionanoparticles, electroporation, cationic lipids and other vehicles, 3) lentiviral vector integration in resting/dividing cells, 4) base editing and 5) CRISPR-associated transposases.

223. CRISPR/Cas9-Mediated Gene Editing in Human Airway Cells

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Lung expression of the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) is highly regulated in various cell types. Because of this, CRISPR/Cas9-mediated gene editing, which enables the precise correction of *CFTR* mutation and retention of its endogenous expression pattern, has theoretical advantages for disease complementation over conventional *CFTR* gene replacement strategies. To test gene editing in human airway cells, we used lentivirus to create populations of CuFi8 cells (an immortalized CF human airway cell line that can differentiate into a multi-ciliated epithelium) that express the self-cleaving Cas9-p2a-EGFP^{Y66S}. EGFP^{Y66S} bears a point mutation leading to a non-fluorescent protein product, thus the efficiency of homology-directed repair (HDR) gene editing can be assessed by the restoration of green fluorescence. We used rAAV vectors to deliver the correction template, a 630bp truncated wildtype eGFP cDNA, without or with a U6 promoter-driving sgRNA that selectively targeted cleavage at the EGFP^{Y66S}mutant, but not wild type, sequence. Vectors were also incorporated an mCherry reporter for the transduction efficiency. In the proliferating CuFi8-(Cas9-p2a-EGFP^{Y66S}) monolayer cultures, infection of rAAV rAAV2/6.temp630 (no sgRNA) led to some restoration of fluorescence through HRD; however, infection of rAAV2/6.gRNAtemp630 (with EGFP^{Y66S} sgRNA) achieved 7.3-fold higher levels of correction. Fluorescence-activated cell sorting (FACS) revealed that the relative efficiency of HDR in rAAV2/6.gRNAtemp630 -transduced cells was 39.4%, while that in rAAV2/6.temp630 -transduced cells was only 5.2%. We also tested the efficiency of gene editing in polarized CuFi8-(Cas9-p2a-EGFP^{Y66S}) epithelium cultured at an air-liquid interface (ALI). Although the transduction efficiencies of rAAV2/HBoV1.gRNAtemp630 (apical) and rAAV2/6.gRNAtemp630 (basolateral) were high, based on mCherry expression, green fluorescence was not observed in the rAAV-transduced epithelia, which are largely non-dividing at ALI. Two weeks post-infection, genomic DNA was prepared from the ALI cultures, including non-infected controls. 763 bp PCR products across the Y66S codon (with a primer anchored outside the HR template) were cloned for Sanger sequencing. Whereas Cas9 mediated indels were not found in PCR products from non-infected cultures, non-homologous end joining (NHEJ)-induced indels at the Cas9 cleavage site were observed in the products from rAAV2/HBoV1.gRNAtemp630- and rAAV2/6.gRNAtemp630- infected cultures at the frequencies of 14.3% and 5.0%, respectively, indicating that although HDR is inactive in both the quiescent basal cells and well-differentiated epithelial cells of the ALI cultures, CRISPR/Cas9-mediated cleavage and NHJE are still efficient. These studies demonstrate that the CRISPR/Cas9 approach is able to generate desired double-strand DNA break at a chosen gene locus in both dividing airway basal cells and mitosis-quiescent cells of polarized human airway epithelium, suggesting the potential of gene-editing based therapeutic approach for CF lung disease.

224. Iterative Screen Identifies Amphiphilic Peptides That Confer Enhanced Delivery of CRISPR Associated Nucleases to Airway Epithelia

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Disease-causing mutations in the CFTR gene lead to the dysfunction of somatic airway epithelial cells, contributing to the onset and progression of cystic fibrosis (CF). The use of therapeutic proteins or genome-editing reagents to modify these disease-associated mutations in airway epithelia holds promise, but the lack of an appropriate delivery mechanism in vivo remains a challenge. We developed amphiphilic peptides with properties that facilitate the effective delivery of CRISPR ribonucleoproteins (RNPs) to well differentiated primary cultures of human airway epithelia in vitro and mouse large and small airway epithelia in vivo. Here, we optimized these amphiphilic peptides to deliver SpCas9 and AsCas12a RNPs in primary cultured human airway epithelial cells at an air-liquid interface. An iterative approach was used involving rational design and screening. Following the application of the peptides and RNPs to airway epithelia, the targeted genomic locus was PCR amplified and next generation sequencing (NGS) was used to quantify the frequency of double strand breaks (DSB) as a surrogate for delivery efficiency. This approach successfully identified peptides with improved delivery properties over starting materials. These candidate peptides were then used as platform designs for further iterative screening. Ongoing studies will evaluate in vivo efficacy using transgenic Rosa mT/mG mice (tdTomato mouse). In summary, this screening strategy facilitated identification of novel delivery peptides that improve the delivery of CRISPR RNPs to airway epithelia. These peptides provide new resources for therapeutic protein delivery to airway epithelial cells.

225. Characterizing and Reducing Spurious DNA Edits by CRISPR Base Editors

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Cytosine Base Editors (CBEs) enable precise cytosine-to-thymine genetic mutations via APOBEC-mediated deamination of CRISPR-targeted cytosines. However, due to the natural DNA-editing capacity of the APOBEC domains they harbor, CBEs possess the ability to create substantial pseudorandom cytosine editing events across the genomes and transcriptomes of affected cells in a gRNA-independent manner. These unpredictable off-target editing events, here termed spurious DNA editing, are an important parameter to understand while adapting CBEs to clinical use; however, since they occur rarely on a per-cytosine basis, detecting such spurious editing events usually involves laborious and time-intensive clonal expansion and whole-genome sequencing. To overcome these challenges and enable rapid assessment of CBE spurious DNA editing capacity, we developed an *in situ* approach termed Base Editing at Anchored R-Loop DNA (BE-ARD) to study this critical parameter. Using this assay, we can rapidly compare the rates of spurious editing events using various engineered CBEs, which reveals the surprising result that CBEs bearing the R33A and K34A mutations (previously described as the SECURE variants) possess markedly lower rates of spurious DNA editing than their parent enzymes.

226. Dual AAV-Based 'Knock-Out-and-Replace' of *RHO* as a Therapeutic Approach to Treat RHO-Associated Autosomal Dominant Retinitis Pigmentosa (RHO adRP)

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The *RHO* gene encodes for rhodopsin, a G-protein coupled receptor essential for normal vision in low-light conditions and photoreceptor survival. *RHO* gain-of-function, autosomal dominant (ad) mutations lead to photoreceptor death and ultimately vision loss. Among the 20,000 Rho-associated adRP patients worldwide, over 150 *RHO* mutations have been identified that span the length of the entire gene. Given the penetrance and location of these mutations, we developed a CRISPR-Cas-based therapeutic that simultaneously 'knocks out' endogenous RHO, and 'replaces' it with exogenous functional RHO. To 'knock out' RHO, we identified potent guide RNAs (gRNAs) in the 5' end of *RHO gene* with *S. aureus* CRISPR-Cas9 target sites that were predicted to disrupt Rho protein function. gRNAs with minimal off-target cutting were then identified using orthogonal methods that included Digenome-Seq and GUIDE-Seq. Because CRISPR-Cas9-induced double-stranded DNA breaks generate indel mutations via non-homologous end-joining repair, we assessed the biological impact of several predicted novel *RHO* alleles that could potentially be created by our gene editing approach. To this end, we developed an *in vitro* overexpression system and several orthogonal assays to measure novel *RHO* allele RNA and protein expression, as well as gene product localization and cytotoxicity. These studies showed that out-of-frame edits (-1 and -2 bp) exhibited ~10-fold reduction in protein levels and demonstrated 166% cell viability compared with the highly cytotoxic deleterious P23H ad *RHO* allele, on average. In contrast, in-frame edits (-3 bp) of single amino acid deletions exhibited a mean of 94% the protein abundance of wt *RHO* and 120% the cell viability of P23H. Based on these experiments, we have identified an editing strategy that not only edits and knocks down *RHO* expression at a high rate, but also minimizes off-target editing and the generation of novel deleterious *RHO* alleles. Because RHO is highly expressed and essential for rod photoreceptor survival, a 'replace' vector that provides sufficient exogenous RHO, upon endogenous RHO knockout, is critical. To this end, we developed an AAV overexpression vector by systematically optimizing RHO cDNA codon usage, as well as promoter, 5' UTR, intronic, and 3' UTR elements. In a head-to-head comparison with a previously published vector, our optimized transgene expressed 3-fold more exogenous RHO in HEK293T cells. As the published vector

demonstrated protection from retinal degeneration in an animal model, it is very likely our optimized 'replace' vector will achieve sufficient RHO expression and maintain photoreceptor viability. Rho adRP represents a significant unmet need. To address this disease, we identified an editing strategy that is potent, specific, and minimizes the generation potentially deleterious *RHO* alleles. We also identified a Rho expression system that is expected to deliver sufficient levels of the Rho protein. Experiments to further test this therapeutic approach in relevant biological systems such as human retinal explants and humanized mice are ongoing.

227. Molecular Characterization of Precise *In Vivo* Targeted Gene Editing in Human Cells using AAVHSC15, a New AAV Derived from Hematopoietic Stem Cells (AAVHSC)

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Targeted gene integration via precise homologous recombination (HR)-based gene editing has the potential to correct genetic diseases. AAV (adeno-associated virus) is being used to target the underlying cause of disease by nuclease-free gene integration at a disease-causing locus. Rapid advancements in the application of AAV-mediated, non-nuclease *in vivo* targeted integration as a novel therapeutic modality should require precise characterization of the efficiency and the nature of the changes being introduced to the genome at the molecular level. Here we describe a framework for assessing *in vivo* AAV-mediated non-nuclease targeted integration using two orthogonal methods to measure on-target integration frequencies: a method to detect *de novo* mutations, and long-read sequencing technologies to query inverted terminal repeats (ITR) integrations. Although these methods require optimization for each target genomic loci, these methods can be applied to fully characterize any targeted HR-based integration. Here we used AAVHSC15, a vector targeting integration into the human phenylalanine hydroxylase gene (*PAH*), the causal gene underlying the autosomal recessive disorder phenylketonuria (PKU). In a humanized liver model of PKU, we demonstrated that the hematopoietic stem-cell derived AAVHSC15 targeting vector achieved non-nuclease mediated gene integration at a frequency of 6% based on two independent assays measuring the integration into the PAH locus. Characterization with these molecular methods establishes *in vivo* non-nuclease mediated integration at levels higher than previously reported with AAV. In addition, no *de novo* mutations or ITR integrations at or above a lower limit of detection of 0.5%. Variant correction analysis aligns with previous findings demonstrating HR as the mechanism of AAV-mediated, targeted integration. These methods characterize targeted integration, or *in vivo* editing events, and measure editing precision at the molecular level, offering a more robust scientific approach for confirmation of editing and potential comparability across platforms.

228. Abstract Withdrawn

Oligonucleotide Therapeutics

229. MDR3/ABCB4 mRNA Therapy for Treating Progressive Familial Intrahepatic Cholestasis 3 (PFIC3)

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Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare lethal autosomal recessive liver disorder, characterized by early onset of persistent cholestasis that rapidly progresses to liver cirrhosis and failure during early childhood. PFIC3 is caused by loss-of-function mutations of the *ABCB4* gene (also called *MDR3*), which encodes a liver specific phosphatidylcholine transporter (ABCB4/MDR3) localized in the canalicular membrane of hepatocytes. Currently, therapeutic options for patients with PFIC3 are extremely limited and normalize liver function and clinical symptoms only in a small subgroup of affected patients. Liver transplantation remains the only curative therapeutic option, but its application has been limited by the shortage of donor liver and the lifetime burden of immunosuppressive drugs). Although protein replacement therapy (like enzyme replacement therapy or ERT) has proven to be successful for many inherited metabolic diseases, it has yet to show feasibility for therapeutic applications involving intracellular membrane-bound proteins like ABCB4/MDR3. Recently, gene replacement therapies based on viral vector delivery technologies have shown promising efficacy for genetic diseases caused by deficiency of a broad range of proteins, including membrane proteins. However, inherent risks remain in terms of genotoxicity and immunogenicity. For all these reasons, there is a dire need for the development of alternative therapeutic strategies and to overcome many of these concerns, we have produced and screened chemically- and genetically-modified mRNA variants encoding human ABCB4 (hABCB4 mRNA) encapsulated in lipid nanoparticles (LNPs). We examined their pharmacological effects in a cell-based and in an *in vivo* mouse model resembling human PFIC3 due to homozygous disruption of *ABCB4* gene (*Abcb4*-/- mice). Our data showed that administration of hepatocyte-targeted hABCB4 mRNA resulted in de novo expression of functional hABCB4 protein and restored phospholipid transport in cultured cells and in PFIC3 mouse livers. Importantly, repeat injections of the hABCB4 mRNA effectively rescued the severe disease phenotype in *Abcb4*-/- mice, with rapid and dramatic normalization of all clinically-relevant parameters such as inflammation, ductular reaction and fibrosis. mRNA therapy also promoted favorable hepatocyte-driven liver regeneration to restore normal homeostasis (including liver weight, body weight, liver enzymes, and portal vein blood pressure). In conclusion, our data provide strong preclinical proof-of-concept for systemic mRNA-LNP therapy as a potential treatment option for patients with PFIC3.

230. Regulation of tRNA Gene Transcription During *In Vivo* Short Hairpin-RNA-Mediated Immune Cell Activation

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The emerging evidences reveal that transfer RNA (tRNA) is not only a necessary component for protein synthesis, but is involved in regulation of various physiological and pathological processes. Activation of immune cell is also affected by the expression levels and post-transcriptional modifications of tRNAs, and the tRNA derivatives (including tRNA-derived stress-induced RNAs, tRNA-derived fragments, and tRNA-derived small RNAs). Currently, the regulatory mechanism of tRNA gene transcription during *in vivo* short hairpin RNA (shRNA)-mediated antitumor immune responses is not well-known. Our previous studies demonstrate that delivery of the shRNA plasmid targeting Indoleamine 2,3-dioxygenase (IDO) via intramuscular injection delayed tumor growth and induced Th1-bias immune activation and tumor specific cytotoxicity in multiple murine tumor models. Moreover, we observed that the level of tryptophan tRNA ($tRNA^{Trp}$) increased in spleen cells of IDO shRNA-treated mice. However, the physiological role of $tRNA^{Trp}$ was still unknown. The purpose of this study is to evaluate the profiles of tRNA gene transcription and determine its regulatory mechanism in splenic immune cells. The immune cells were collected from spleen of LLC1 lung tumor-bearing mice after treatment of 50 μg of IDO shRNA or control shRNA. The profile of tRNA gene expression was determined via RNA sequencing. The results showed that only some of tRNA gene expression was significantly changed in IDO shRNA-treated immune cells when compared to control shRNA. Because the expression of regulatory proteins for RNA polymerase III transcription, including *Brf1, Tbp, Bdp*, genes of RNA polymerase III subunits and TFIIIC subunits, and RNA polymerase III suppressor *Maf1*, was not significantly changed, we supposed that IDO shRNA treatment altered transcription of the specific tRNA gene expression but not global tRNA gene transcription. The role of these specific tRNA genes and their potential derivatives and the transcription regulatory mechanism were addressed in the present study.

231. Development of an Ion-Pairing LC-MS/MS for the Quantitation of an Antisense Oligonucleotide (ASO) and its Four Deconjugated ASOs in Human Plasma

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A bioanalytical method was developed for the quantitation of a 20-nucleotide partially 2'-O (2 methoxyethyl) (2' MOE) modified antisense oligonucleotide (ASO) conjugated to a cluster of three GalNAc sugar molecules via a 5'-Trishexylamino linker (THA-GalNAc cluster) (fully conjugated ASO), as well as its deconjugated species, in human plasma by ion-pairing liquid chromatography tandem mass spectrometry (IP-LC-MS/MS). After subcutaneous dosing, various enzymatic cleavages lead to first removal of the GalNAc sugars followed by removal of the entire linker cluster: minus one GalNAc (-1GalNAc); minus two GalNAc (-2GalNAc); minus three GalNAc (-3GalNAc)); and unconjugated ASO (full-length ASO without a THA-GalNAc cluster). Using a 50-μL aliquot of plasma, samples were prepared with an initial phenol/chloroform liquid-liquid extraction followed by a solid phase extraction. Chromatographic separation was performed with a gradient elution on a Waters Acquity UPLC system, using an ACQUITY UPLC® Oligonucleotide BEH C18 column. Mobile phase A and B are mixture of hexafluoroisopropanol (HFIP), triethyl amine (TEA), and methanol. Analysis was performed on a Waters Xevo TQ-S mass spectrometer equipped with electrospray ionization (ESI) source and operated in negative, multiple reaction mode (MRM). Fully conjugated ASO concentrations were quantitated using its respective calibration curve and unconjugated ASO concentrations were quantitated using an unconjugated ASO calibration curve. Standards for the partially conjugated ASOs species (-1GalNAc, -2GalNAc, -3GalNAc) were not available and were therefore quantitated using the fully conjugated ASO calibration curve. Using an unconjugated control ASO as an internal standard (IS), analyte concentrations were calculated by Masslynx Version 4.1 and Assist LIMS Version 6 (PPD) with a linear regression and the least squares method (with $1/x^2$ weighting) over a quantitation range of 1.00 nM to 200 nM for both the fully conjugated ASO and unconjugated ASO in human plasma. Analysis was successfully conducted for selective human plasma samples collected from a double-blind, placebo-controlled, dose-escalation Phase 1 study to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of this ASO when administered subcutaneously to healthy volunteers. Detailed discussions will be provided regarding development of LC-MS/MS based methods for the quantitation of ASOs in biological matrices, from sample preparation, chromatography development using ion-pairing reagents, to LC-MS/MS analysis.

232. RNA Editing Via Endogenous ADARs Using Stereopure Oligonucleotides

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Recruiting endogenous RNA editing enzymes using chemically modified oligonucleotides holds great promise for treating human disease. PRISM™ is Wave Life Sciences' proprietary discovery and drug development platform, which enables us to target genetically defined diseases with stereopure oligonucleotides—those in which the chiral configuration of a backbone (e.g., *R*p or *S*p configuration at phosphorothioate) is precisely controlled at each linkage. PRISM combines our unique ability to construct these stereopure oligonucleotides with a knowledge of how the interplay among oligonucleotide sequence, chemistry and backbone stereochemistry impacts activity. Here, we report the application of PRISM for the optimization of stereopure oligonucleotides for adenosine deaminase acting on RNA (ADAR)-mediated RNA editing. We demonstrate that stereopure oligonucleotides can co-opt endogenous ADAR

enzymes to potently edit endogenous transcripts in multiple primary human cell types *in vitro*. Stereopure oligonucleotides were more efficient than traditional, stereorandom oligonucleotides. Stereopure oligonucleotides elicited efficient editing in primary human hepatocytes when delivered under gymnotic (i.e., free uptake) conditions or using an *N*-acetylgalactosamine (GalNAc) conjugate. Finally, we demonstrate ADAR-mediated editing of multiple RNA transcripts with several different stereopure oligonucleotides *in vitro* in primary human hepatocytes, which validates that our technology is applicable to multiple sequences. These preclinical investigations in primary human cells illustrate the versatility of PRISM and its suitability for the development of stereopure oligonucleotides for RNA editing.

233. Lowering the Pathogenic Exon 1 HTT Fragment by AAV5-miRNA Gene Therapy

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Huntington disease (HD) is a genetic neurodegenerative disease caused by a trinucleotide repeat in the huntingtin gene (HTT). Despite its monogenic cause, HD pathology is highly complex and targeting the leading pathogenic mechanisms is relevant for developing disease-modifying therapies. It has recently been demonstrated that apart from the mutant full-length HTT protein, exon 1 HTT fragments generated by aberrant splicing are very prone to aggregate and contribute to HD pathology. These findings suggest that approaches capable of reducing the expression of the highly pathogenic exon 1 HTT protein might achieve a greater therapeutic benefit than targeting only the full-length mutant HTT protein. We have developed an engineered microRNA targeting exon 1 HTT (miHTT), delivered via adeno-associated serotype 5 virus (AAV5). AAV5-miHTT treatment has been demonstrated to lower mHTT in several rodent and large animal models by reducing full-length HTT and reversing HD phenotype. In this study, we evaluated the ability of AAV5-miHTT to reduce the exon 1 HTT mRNA in knock-in mice. Polyadenylated exon1 HTT mRNA was successfully detected in Q175FDN striatum and cortex by 3'RACE and qPCR. After intrastriatal administration of AAV-miHTT treatment, dose-dependent lowering of both full-length HTT and mis-spliced exon 1 HTT mRNA were detected in striatum and cortex in Q175FDN mice. These results suggest a new aspect to the potential therapeutic value of the first RNAi-based gene therapy for HD disease to enter clinical trials.

234. An *In Vitro* Human Hepatocyte Assay for the Evaluation of the Efficacy of GalNac-Conjugated siRNA

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Efficient and target-specific knockdown of target RNA expression are key considerations for oligonucleotide therapeutic development. Due to the abundance of asialoglycoprotein receptor (ASGPR) on hepatocellular membranes and its receptor-mediated endocytosis of the ligand N-acetylgalactosamine (GalNAc), GalNAc-conjugated siRNA represents a promising modality to specifically deliver to hepatocytes for the correction of aberrant RNA expression. For many siRNA targets, sequences differences across preclinical species prohibit use of animal models in the prediction of clinical outcomes. Consequently, development of this modality requires preclinical approaches that enable researchers to assess the efficiency of both targeted cell delivery as well as the magnitude and duration of the desired effects on target RNA expression in a species-relevant system. Primary human hepatocytes, an in vitro system used extensively in the evaluation of human-specific drug properties in the development of small and large molecule drugs, may represent an alternative preclinical model for the evaluation of GalNAc-conjugate delivery as well as mRNA silencing activity. We have recently developed cryopreserved human hepatocytes (999Elite Cryopreserved Human Hepatocytes) that can be cultured for over 30 days while retaining hepatic gene expression. We report here a successful proof-of-concept (POC) study using the 999Elite hepatocytes in the evaluation of GalNAc-conjugated siRNA-mediated target RNA knockdown. In the POC study, the hepatocytes were treated with 1 μM of GalNAc-conjugated siRNA targeting human hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA for 4 hrs, followed by 3x weekly medium change for 41 days, with quantification of viability (WST assay) and target mRNA on days 0, 1, 2, 3, 4, 8, 12, 16, 20, 26, 33 and 41. HPRT1 mRNA expression in the siRNA-treated cultures decreased to 21% relative to pre-dose (day 0) on day 2, <10% from days 2 to 12, <20% from days 16 to 33, and further increased to 64% by day 41. No decrease in HPRT1 mRNA expression was observed in PBS vehicle-treated cell cultures. Our results suggest that long-term cultured human hepatocytes represent a species-relevant cost- and time-efficient experimental tool to aid in GalNAc-siRNA candidate screening for efficacy and duration of target RNA expression effects.

235. Antisense Oligonucleotide Mediated Increase of OPA1 Expression Using TANGO Technology for the Treatment of Autosomal Dominant Optic Atrophy

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Purpose: Autosomal dominant optic atrophy (ADOA) is one of the most commonly diagnosed optic neuropathies. This optic nerve disease is associated with structural and functional mitochondrial deficits that lead to degeneration of the retinal ganglion cells and progressive, irreversible loss of vision. 65-90% of ADOA patients carry mutations in *OPA1* and most mutations lead to haploinsufficiency. *OPA1* encodes a mitochondrial GTPase with a critical role in mitochondrial fusion, ATP synthesis and apoptosis. Currently, there is no approved disease-modifying treatment for ADOA patients. Here, we employ TANGO (Targeted Augmentation of Nuclear Gene Output), a novel therapeutic approach, that uses antisense oligonucleotides (ASOs), to increase the endogenous expression of *OPA1*. TANGO targets a naturally-occurring, alternatively spliced exon that has an in-frame stop codon whose inclusion results in a non-productive *OPA1* transcript that

is degraded by nonsense-mediated mRNA decay. This alternatively spliced exon is conserved in human, non-human primates and rabbit. **Methods:** We designed and tested ASOs to prevent the inclusion of the alternatively-spliced exon with the goal of increasing productive *OPA1* mRNA and protein. ASOs were administered either by lipid-based transfection or gymnotic delivery to HEK293 or human fibroblast cells and RNA/protein was extracted to assess the impact on *OPA1* mRNA splicing, and changes in mRNA and protein expression. ASOs were also administered to wild-type rabbits by intravitreal injection and retinae were collected to evaluate the impact of ASOs on *OPA1* mRNA splicing. **Results:** *In vitro* screens identified ASOs that decreased the non-productive, alternatively spliced *OPA1* transcript and increased expression of *OPA1* mRNA and protein. Intravitreal injection of lead ASOs in wild-type rabbits were well tolerated and led to a dose-dependent reduction in non-productive *OPA1* mRNA levels after 15 days. Ongoing studies are evaluating the effect of ASOs on mitochondrial function in ADOA patient fibroblasts and *OPA1*-haploinsufficient HEK293s. **Conclusions:** These results indicate that TANGO can be used to potentially address ADOA caused by *OPA1* haploinsufficiency by using ASOs to increase endogenous *OPA1* mRNA and protein expression levels. This approach leverages the *OPA1* wild-type allele and can be applied in a mutation-independent manner to treat ADOA.

236. Development and Optimization of Logic Gated Small Interfering RNAs for Operation Inside Mammalian Cells

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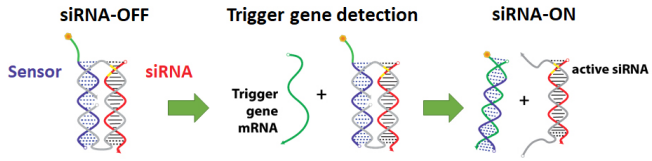
In 1900, Paul Ehrlich put forth the concept of a pharmacologic "magic bullet", a combination of a disease selective chemical agent conjugated to a non-selective toxin that could target drug activity to disease causing cells without harming healthy tissues in the body. For the past two decades, various researchers in DNA and RNA nanotechnology have proposed ideas for riboswitch regulated magic bullets in which the activities of oligonucleotide drugs are switched ON or OFF according to the presence or absence of specific biomarkers in tissues or cells. Although elegant in concept, practical implementation of these "logical therapeutics" for mammalian cells has proven elusive. We have now developed a **conditionally activated small interfering RNA (Cond-siRNA)** in which an RNA sensor regulates the RNAi activity of a conjugated siRNA post-transfection into mammalian cells. The sensor switches ON RNAi activity when it base-pairs to an RNA transcript from a designated "trigger gene". In cells without trigger gene expression, the sensor keeps RNAi activity OFF. The identities of the trigger and target genes are encoded by two entirely independent, easily programmable RNA sequences, giving the Cond-siRNAs the ability to target a specific population of cells for the silencing of an arbitrary gene. We will present experimental data for Cond-siRNAs with different trigger and target gene combinations in different mammalian cell lines, explain the design principles and optimizations that enable easy programmability and correct functioning in mammalian cells, and demonstrate an early example of therapeutic application development in the treatment of cardiac hypertrophy. As small, chemically modified, unimolecular RNA complexes, Cond-siRNAs are compatible with existing methods of siRNA delivery and practical for development into clinically viable RNAi drugs. By making possible post-delivery targeting of RNAi activity to specific populations of disease driving cells, they could enable paradigm shifting development of RNAi drugs that can safely target pleiotropic genes whose dysregulated activities drive the progression of many chronic diseases lacking effective treatments today.



237. Development of a Minimized Exons 45-55 Skipping Cocktail for the Treatment of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive neuromuscular disorder characterized by progressive, body-wide muscle weakness and degeneration. It is the most common inherited neuromuscular disorder worldwide, with 1:3,500-1:5,000 males affected, and there is currently no cure for it. DMD is mostly caused by large out-of-frame deletions of the *DMD* gene that lead to dystrophin loss in muscle. Exon skipping uses antisense oligonucleotides (AOs) to exclude out-of-frame exons from the *DMD* transcript to restore the reading frame and allow for the production of truncated, partially functional dystrophin. While promising, current exon skipping approaches have limited patient applicability since they only target single exons. We aim to develop an AO formulation capable of skipping *DMD* exons 45-55, which due to the region being in a mutation hotspot, could treat 47% of all patients. We designed phosphorodiamidate morpholino oligomer (PMO) AOs targeting the human *DMD* exons 45-55 region, one PMO per exon except for exon 48 which required two. We have shown that this 12-PMO cocktail restored up to ~15.9% dystrophin of normal levels in immortalized DMD patient-derived muscle cells with various mutations. Presently, we found that we can reduce the number of PMOs in this cocktail and yet retain appreciable levels of exons 45-55 skipping. Immortalized patient-derived myotubes carrying an exon 52 deletion were transfected with a 5-PMO cocktail at 3 days post-differentiation. At 2 days post-treatment, 34% exons 45-55 skipping on average was observed by RT-PCR in the treated cells, which was not significantly different from the 25% skipping by the full cocktail. On average, Western blot showed 21% dystrophin of normal levels upon treatment with the 5-PMO cocktail; 13% dystrophin was observed for

the full cocktail. As we plan to deliver this minimized cocktail *in vivo*, we have tested peptides that can be conjugated to the PMOs to increase their muscle uptake. A promising peptide is DG9, which we previously identified through a zebrafish-based screen of cell-penetrating peptides. When conjugated to an exon 51-skipping PMO, a single retro-orbital injection of DG9-PMO in hDMDdel52;*mdx* mice enhanced exon 51 skipping and dystrophin rescue by up to 7-fold and up to 4-fold, respectively, across skeletal muscles and the heart compared to the naked PMO 1 week post-treatment. Widespread dystrophin-positive fibers were observed by immunostaining in these tissues as well. Overall, we have developed a minimized exons 45-55 skipping cocktail that effectively restored dystrophin production *in vitro*. Future work will test the efficacy of the DG9-conjugated version of this cocktail *in vivo*.

238. Engineering More Efficient Therapeutic miRNAs for FSHD Gene Therapy

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Background: Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common muscular dystrophies affecting 870,000 people worldwide. The disorder leads to weakness in skeletal muscles, most commonly of the face, shoulder girdle, and upper arms. There is no effect on lifespan, but the disease can be debilitating and there is currently no treatment. FSHD is a dominant disorder caused by derepression of the myotoxic gene DUX4. Previously our lab demonstrated proof-of-principle for an RNA interference-based gene therapy. We engineered 36 microRNAs capable of targeting and inhibiting DUX4 to varying degrees. When packaged into AAV and delivered to DUX4-expressing mice, these miRNAs protected the muscles from DUX4-induced damage. However, some miRNAs also led to muscle toxicity potentially due to improper processing. **Objectives:** We hypothesized that the efficacy and safety of our *DUX4*-targeted miRNAs could be improved by optimizing the sequences immediately flanking the mature miRNA. To test this, we created new versions of our toxic and non-toxic *miDUX4* miRNAs. The aim is to design highly efficient *DUX4*-targeted miRNA to use as gene therapy product for FSHD. **This is beneficial to our gene therapy strategy as it will allow us to use a reduced therapeutic dose, which could lead to safer outcomes. Methods:** We tested the inhibition efficiency of the new *miDUX4* constructs using the Dual-Luciferase Reporter assay and western blotting. We assessed *miDUX4* processing using qPCR. **Results:** The original *miDUX4* constructs have flanking regions 40-50 nucleotides long at both the 5' and 3' ends. Our lead sequence is called *miDUX4*. First, we engineered seven *miDUX4* constructs with various 5' and 3' flanking sequences and tested them *in vitro* using Dual-Luciferase Reporter (DLR) Assay to compare their ability to reduce *DUX4* expression. A construct called *miDUX4F*, containing one short and specific flanking sequence at the 3' end, had the best silencing efficiency, with 40-fold greater potency than our original *miDUX4* sequence. Since the flanking sequences of *miDUX4* impacted its silencing efficiency, we hypothesized that incorporating the same pro-silencing flanking sequences into other non-functional or toxic miRNAs might enhance their targeting efficiency and reduce the dose required to elicit gene silencing. Using the DLR assay, none of the other miRNAs showed any enhanced efficiency to silence *DUX4*. **Conclusions:** By testing multiple flanking sequences on all of our *DUX4*-targeted miRNAs, we were able to enhance the inhibition efficiency of only one miRNA (*miDUX4*). Our results show that enhancing the inhibition efficiency of a miRNA does not only depend on the 5' and 3' flanking sequences but is also specific to the miRNA sequence. Thus, although flanking sequences play an important role in miRNA processing and efficiency, in our work there was no universal approach that could be translated to any sequence. Empirical testing remains important. Future studies will focus on testing our best lead *miDUX4* candidates *in vivo* using our published tamoxifen-inducible DUX4 mouse model.

239. Identification of Top Anti-HIV-1 RNAs for Use in HIV-1 Gene Therapy

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Background: Available anti-HIV-1 drug therapies have improved the quality of life for people living with HIV-1 (PLWH). However, the drugs must be taken daily and in combination for the entire life of an infected person. To date the only confirmed cases of an HIV-1 cure include Timothy Brown (also known as the 'Berlin Patient') and more recently the 'London Patient'. Both patients received a hematopoietic stem cell transplant (HSCT) from an individual with natural resistance to HIV-1. Due to the high incidence of fatal graft vs host disease following allogeneic HSC transplant, and the low incidence of known HIV-1 resistant donors, this treatment option is inaccessible to the vast majority of PLWH. An alternative option to achieve a similar outcome is to use a person's own cells and modify them to become HIV-1 resistant during an autologous HSCT. Several anti-viral RNAs from different classes have been identified as potential candidates for combination anti-HIV-1 gene therapy via autologous HSCT; however, there have not been a lot of studies comparing different candidate genes and even less to compare different expression strategies for the top candidates. **Objectives:** Our objectives were to compare the safety and efficacy of top anti-HIV-1 RNAs from different classes and with different expression strategies. The goal of this work was to identify the best candidates to be used in combination gene therapy to treat HIV-1 infection. **Methods:** Genes expressing anti-HIV-1 ribozymes, decoy/aptamer RNAs, short hairpin (sh) RNAs and U1 interference (U1i) RNAs were compared for efficacy in a HIV-1 co-transfection assay in HEK293T cells and following HIV-1 infection of transduced SupT1 cells. A sensitive competitive growth assay in SupT1 cells was used to determine whether the expression of the genes gave cells either a growth advantage or disadvantage compared to cells that were not transduced. The top candidates were also compared when expressed from different RNA polymerase III promoters (U6, 7SK and H1). **Results:** Following co-transfection with an HIV-1 molecular clone, anti-HIV-1 shRNAs and U1i RNAs were several fold more potent at inhibiting HIV-1 production compared to a ribozyme and several RNA decoys/aptamers. The shRNAs and U1i RNAs were also more effective at inhibiting HIV-1 replication in transduced SupT1 cells.

Expression of anti-HIV-1 shRNAs from the 7SK and U6 promoters resulted in more efficacious inhibition of HIV-1 production compared to the H1 promoter; however, some shRNAs had a negative impact on cell growth when expressed from the U6 and 7SK promoters. All promoters produced RNAs with different start and end sites, with the U6 promoter providing the highest percentage of expected transcripts. **Conclusions:** By directly comparing different RNAs using the same assays we have identified several promising candidates for HIV-1 gene therapy. Our results suggest that shRNAs and U1i RNAs are the most efficacious groups of anti-HIV-1 RNAs. We also show that both the U6 and 7SK promoters produce more potent shRNAs compared to the H1 promoter, but that the high expression from these promoters can sometimes lead to negative impacts on transduced cells. Overall the results from our studies should help in the selection of safe and effective combinations of anti-HIV-1 genes for use in HSCT therapy for HIV-1 infection.

240. Translatable Biomarkers in Gene Therapy for Huntington Disease: Innovative Approaches and Learnings from Pre-Clinic to the Clinic

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Huntingtin (HTT)-lowering therapies hold great promise to slow-down or halt neurodegeneration in Huntington disease (HD). From the different approaches under development, gene therapies using adeno-associated viral vectors (AAVs) are typically administered locally into the brain region of interest, after which they expand to interconnected regions though different mechanisms. A single administration should be sufficient to ensure long-term persistence of the therapeutic transgene (i.e. lowering agent), especially in non-dividing cells such as neurons. We have developed AAV5-miHTT, a recombinant AAV-based gene therapy expressing an engineered microRNA that specifically binds to HTT exon1, resulting in lowering of both full-length and exon1 HTT mRNA expression. Now on its way to clinical development, AAV5-miHTT has demonstrated safety and efficacy in small and large animal studies, with remarkable brain-wide spread and long-term persistence, through retrograde and anterograde transport of the AAV followed by extracellular spread of the therapeutic miRNA, and without off-target effects. Adequate translational measures to evaluate the safety, efficacy and durability of HTT lowering mediated by AAV5-miHTT in patients are much needed. To this end, we assessed the response of candidate biofluid and imaging biomarkers in preclinical models (from rodents to minipigs and non-human primates) to AAV5-miHTT administration. In cerebrospinal fluid (CSF), assessments included pharmacokinetic (miHTT expression) and pharmacodynamic (HTT protein, NFL) measures. Imaging biomarkers included volumetric magnetic resonance imaging (vMRI) and magnetic resonance spectroscopy (MRS). Many of these measures have the potential to follow-up safety and efficacy of HTT-lowering therapies in general. However, because of the unique properties of gene therapy approaches in contrast to other HTT-lowering therapies, a tailored biomarker panel for HTT-lowering gene therapies in HD patients may be needed. Data supporting this customized selection will be presented, in light of the known mechanism of action of AAV5-miHTT.

241. Secreted Therapeutics: Monitoring Durability of microRNA-Based Gene Therapies in Huntington's Disease

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MicroRNA-based gene therapies are emerging as a potential approach to lower huntingtin (HTT), the disease-causing protein of Huntington disease (HD). We have developed an engineered microRNA targeting human huntingtin (miHTT), delivered via adeno-associated serotype 5 virus (AAV5). AAV5-miHTT treatment has demonstrated an efficient lowering *in vitro* and *in vivo* in the brain in different HD animal models. One of the current translational challenges is the need of biomarkers indicative of the long-term expression and therapeutic efficacy of miHTT in the brain, where the therapy is directly infused. Recently, extracellular vesicles (EVs) have been identified as carriers of RNA species and proteins directly secreted from cells and present in all biofluids, becoming potential sources of biomarkers for diagnosis. In this study, we investigated the detection of EV-associated miRNAs in biofluids as suitable measurements to monitor the expression and endurance of AAV-delivered miRNAs in the brain. We used iPS-derived neurons from HD patients to confirm the secretion of mature miHTT molecules within EVs. Moreover, EV-enriched miHTT levels were strongly correlated with viral dose and transgene expression in neuronal cells. We further investigated the transability of extracellular miHTT to monitor the expression and durability of miHTT *in vivo*. Quantifiable miHTT levels enriched in EVs were detected in the cerebral spinal fluid in non-human primates up to six months and in HD transgenic minipigs up to 2 years after a single intrastriatal injection. Altogether, these results confirm the long-term expression of AAV-delivered miRNAs and support the potential role of EV-associated miRNAs as a novel translational pharmacokinetic marker in gene therapies for central nervous system (CNS) disorders.

242. Selection, Engineering and In Vitro Characterization of CD3-Specific DNA Aptamers

Anna Miodek, Cecile Bauche, Renaud Vaillant, Frederic Mourlane

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A number of therapeutic strategies that modulate T cell immunity by targeting CD3, the signalling component of the T cell receptor, are widely used clinically as immunosuppressive agents in transplantation (OKT3), autoimmune type I diabetes and psoriasis (teplizumab and

otelixizumab). More recently, bispecific therapies retargeting the cytotoxic activity of effector T cells by binding to CD3 to tumors expressing tumor-associated antigen have demonstrated striking activity in patients across different cancers (blinatumomab). aratinga. bio TNP has been focusing on the development of CD3-specific aptamers to generate new targeting agents to specifically deliver anti-cancer therapeutics to T lymphocytes. Using SELEX technology, we have isolated DNA aptamers binding to CD3 eγ or CD3 ed protein complexes expressed on human and murine T lymphocytes. By combining biophysical and on-cell binding methods, CD3-specific interactions with nanomolar range affinities were reported with the best cross-specific candidates. Isolated aptamers recognized an epitope on CD3 which is different from the epitopes covered by OKT3 and other T-cell activating antibodies. Accordingly, these CD3 aptamers did not induce any activation of primary human T lymphocytes which is of particular interest for the development of inert targeting agents without any immunomodulating properties. All isolated aptamers shared a minimal conserved motif predicted to be a G quadruplex structure that was sufficient to mediate specific interactions with the CD3 target. Sequence engineering of the core sequence with site-specific abasic sites or base substitutions resulted in improved affinity, specificity and serum stability. These encouraging in vitro results indicate that the lead CD3 aptamers have highly promising properties with a great potential for further preclinical and clinical development of CD3-targeting therapeutics.

243. Development of DNA Vaccine for Precision Medicine

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Immunotherapy and precision medicine become two important issues in the treatment of cancer. However, the high cost of immune therapy, such as checkpoint antibodies and CAR-T therapy, are difficult to expand patient use. Whole genome sequencing has made personalized medicine possible. Target therapy against specific mutation results in regression of tumor and less side effects. Although most somatic mutations in tumor are patient-specific. Some mutations occur at a frequency higher than 2% in cancer patients. The DNA vaccine is an affordable platform due to the low cost and high safety. Several immunogenic neo-epitopes, which cause T cells immunity, have been identified in B16F10 melanoma. We designed multiple neo-epitope DNA vaccine based on the identified mutation peptide fragment in pcDNA3.1 plasmid. Furthermore, the codon usage bias and tRNA copy number were considered to optimize DNA vaccine. We first indicated that the peptides of DNA vaccine were expressed in vitro. In the B16F10 melanoma orthotopic mouse model, the mice were received 4 times treatment of DNA vaccine. The multiple neo-epitope DNA vaccine inhibited the tumor growth in mouse models. Additionally, Immunohistochemistry staining demonstrated that infiltrating CD4+ T cells and CD8+ T cells were increased in tumor microenvironment. The FASC analysis also indicated that the CD4+ T cells, CD8+ T cells and activated dendritic cells were increased after DNA vaccine treatment. Our data suggested that the DNA vaccine attenuated tumor growth through promotion of immunity in B16F10 melanoma orthotopic mouse model. This study approach to discovering cancer vaccine may shed unique insight into the treatment with cancer. Moreover, this study established the important basis for developing a low-cost DNA vaccine and nucleic acid therapy targeting to eradicate tumor in the future.

244. Synthesis of Low Immunogenicity RNA with High-Temperature In Vitro Transcription

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The use of synthetic RNA for therapeutics requires that the in vitro synthesis process be robust and efficient. The technology used for the synthesis of these in vitro-transcribed RNAs, predominantly using phage RNA polymerases (RNAPs), is well established. However, transcripts synthesized with RNAPs are known to display an immune-stimulatory activity in vivo, that is often undesirable. Previous studies have identified double-stranded RNA (dsRNA), a major byproduct of the in vitro transcription (IVT) process, as a trigger of cellular immune responses. Here we describe the characterization of a high-temperature IVT process using thermostable T7 RNAPs to synthesize functional mRNAs that demonstrate reduced immunogenicity without the need for a post-synthesis purification step. We identify features that drive the production of two kinds of dsRNA byproducts—one arising from 3' extension of the run-off product and one formed by the production of antisense RNAs—and demonstrate that at a high temperature, T7 RNAP has reduced 3'-extension of the run-off product. We show that template-encoded poly-A tailing does not affect 3'-extension but reduces the formation of the antisense RNA byproducts. Combining high-temperature IVT with template-encoded poly-A tailing prevents formation of both kinds of dsRNA byproducts generating functional mRNAs with reduced immunogenicity.

245. Abstract Withdrawn

246. Screening DNA Vectors for Enhanced Nonviral Gene Delivery to Human Mesenchymal Stem Cells

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Human mesenchymal stem cells (hMSCs) are under intense research for applications in cell therapeutics due to their unique properties, however, the intrinsic therapeutic properties of hMSCs could be greatly enhanced by gene delivery. Viral gene delivery is efficient but suffers from design and safety concerns that limit its therapeutic potential. Conversely, nonviral gene delivery, while safer compared to viral methods, suffers from inefficiency, especially in hMSCs. To address the shortcomings of nonviral gene delivery to hMSCs, our group has demonstrated that pharmacological 'priming', or the addition of compounds to the culture media to modulate the cellular response to transfection, can significantly increase transfection compared to a vehicle control. However, even with priming, hMSC transfection is inefficient, possibly due to a lack of understanding of the key attributes underlying successful transfection in hMSCs. Research has shown that

many factors can contribute to the success of a nonviral gene delivery system, including the delivery vehicle and associated priming, but also the DNA vector, promoter, and DNA vector bacterial elements. However, the effects of these key plasmid attributes on transfection in hMSCs have yet to be investigated. For this work, our objective was to investigate three DNA vectors (plasmid DNA (pDNA), mini-intronic plasmid (MIP), and minicircle DNA (MC)) and two promoters (cytomegalovirus (CMV) and elongation factor 1 alpha (EF1α)), to determine parameters that promote high transgene expression and transfection efficiency in hMSCs. MIP and MC vectors were selected due to their smaller vector size and reduced bacterial elements, as both parameters have been shown to increase transgene expression compared to conventional plasmids. Furthermore, strong promoters (i.e. produce rapid and high levels of transcripts), like CMV, have been shown to lead to transgene silencing, therefore, the endogenous EF1α promoter was selected as its weaker promoter activity compared to CMV may lead to less transgene silencing. Finally, most conventional plasmids contain an F1 origin of replication (F1 Ori) for packaging of single-stranded DNA into phages. The F1 Ori is non-essential for propagation of plasmids in bacteria; therefore, we removed the F1 Ori from a conventional plasmid in order to elucidate its role in the transfection process. Screening the DNA vector constructs identified MIP as a potent vector for increasing transgene expression in hMSCs, as MIPs increased transgenic luciferase activity by as much as 35-fold compared to a conventional plasmid with the same promoter (Table 1). Furthermore, removal of the F1 Ori increased transgene production by almost 3-fold compared to a conventional plasmid with the same promoter. These data suggest that removal of bacterial elements may be key to enhancing transfection in hMSCs, possibly by limiting cytosolic sensing of bacterial elements which can lead to cytotoxicity, however, MCs, which contain no bacterial elements, dramatically decreased transfection under control of the CMV promoter while marginally increasing transfection when under control of the EF1a promoter. Together, these data show that hMSCs transfection depends on DNA vector bacterial elements as well as the promoter used and suggest that these factors can be further tuned to design an efficient nonviral gene delivery system for hMSCs.

Table 1: DNA Vectors and Transgene Expression

Vector	Transgene	Promoter	Bacterial Elements	Luciferase Activity Fold-Change Relative to pEGFPLuc-CMV	Luciferase Activity Fold-Change Relative to pEGFPLuc-EF1α
pEGFPLuc-CMV	Fusion protein of EGFP and luciferase	CMV	pUC origin of replication, F1 origin of replication, Kanamycin resistance marker	1	7.6
pEGFPLuc-CMV No F1			pUC origin of replication, Kanamycin resistance marker	2.3	18
MC.EGFPLuc-CMV			None	0.01	0.6
MIP.EGFPLuc-CMV			pUC origin of replication, RNA-OUT selectable marker	6.6	50
pEGFPLuc-EF1α		EF1α	pUC origin of replication, F1 origin of replication, Kanamycin resistance marker	0.1	1
pEGFPLuc-EF1α No F1			pUC origin of replication, Kanamycin resistance marker	0.4	2.9
MC.EGFPLuc-EF1α			None	0.2	1.9
MIP.EGFPLuc-EF1α			pUC origin of replication, RNA-OUT selectable marker	4.5	35

Fold-changes are presented as averages of triplicate values

247. Abstract Withdrawn

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

248. High Dose Hematopoietic Stem Cell Therapies, like MGTA-456, Enable Complete Neural, Peripheral, and Skeletal Disease Cross-Correction Through Rapid and Robust Engraftment

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Background. Allogeneic hematopoietic stem cell transplant (HSCT) is a potentially curative approach to halt disease progression of select inherited metabolic disorders (IMDs). For IMDs that affect the central nervous system (CNS), donor-derived cells, like microglia, cross-correct defects via production of normal enzyme levels. The typical cell dose used in HSCT can be sub-optimal and, in gene therapy applications, copy number has been shown to be variable. We developed MGTA-456, a high dose cell therapy that led to rapid neutrophil recovery and 100% engraftment in patients with malignant and non-malignant diseases including IMDs (Wagner et al Blood 2017; Orchard et al AAN 2019). The impact of cell dose on disease correction, however, is unknown. Here, we show that fast and robust hematopoietic recovery via high cell dose therapies, like MGTA-456, leads to rapid and complete CNS, peripheral, and skeletal disease resolution in a murine model of Hurler syndrome. We further show that correction of CNS defects requires donor engraftment in the brain and that the high dose cell therapy, MGTA-456, leads to higher numbers of microglia and greater enzyme production. ***Results.*** In wild-type mice, transplant of increasing doses of bone marrow cells led to a dose-dependent increase in brain microglial engraftment at 1-16 weeks post-HSCT (26-fold for $10x10^6$ cells vs $0.3x10^6$ cells, $p<0.01$). In *Idua*$^{-/-}$ mice, a model of Hurler syndrome (Figure A), high cell doses led to an improvement in CNS disease endpoints, such as IDUA enzyme production (n=5 mice, $p<0.01$) and reduced substrate accumulation (n=5, $p<0.01$). Notably, we observed normalization of behavioral activity to wild type levels at 4 and 16 weeks post-HSCT using a rotarod performance test (Figure B-C). Low dose HSCT, however, did not improve disease endpoints at 4 or 16 weeks. Histopathological analysis revealed a significant reduction in CNS tissue vacuolization of high dose treated animals. Notably, transplant of $10x10^6$ cells into mice conditioned with a myeloablative dose of treosulfan, an agent that does not permit brain microglial engraftment (Figure A), did not correct CNS defects (Figure B-C), suggesting that donor engraftment in the CNS is required for disease correction. In the periphery, reduction of substrate to wild type levels was observed as early as 1 week post-HSCT with $10x10^6$ cells (n=5-10, $p<0.01$). Moreover, high dose HSCT, but not low dose HSCT, restored skeletal architecture to wild-type levels, as assessed by microcomputed tomography of skulls, femurs, and tibiae (not significant vs wild-type, n=5 mice). Relative to low cell dose therapies, transplant of MGTA-456, a high cell dose therapy with two normal *IDUA* gene copies, led to robust, long-term immune recovery (n=88 animals), 60-fold greater microglial engraftment as early as 2 weeks post-HSCT (Figure D, n=8, $p<0.001$), and >600-fold higher IDUA enzyme levels (Figure E, n=2 donors, $p<0.001$). Mechanistically, brain microglia are derived from CD34+CD90+ cells, which are present at high numbers in MGTA-456. ***Conclusions.*** We show that high dose HSCT leads to improved disease correction via robust donor hematopoietic engraftment in a murine model of Hurler syndrome. The high dose cell therapy, MGTA-456, may rapidly and durably resolve CNS, peripheral, and skeletal abnormalities associated with IMDs, and is being studied in a Phase 2 clinical trial.

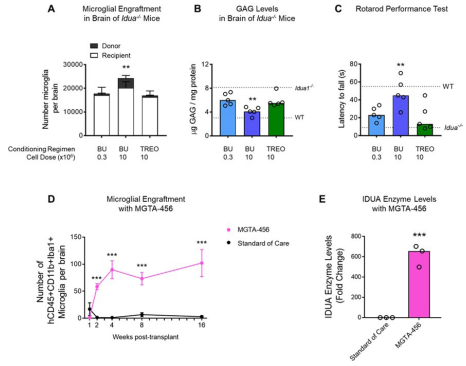

Figure: Durable resolution of CNS defects in *Idua*$^{-/-}$ mice via high cell dose transplantation at 16 weeks post-HSCT. *Idua*$^{-/-}$ mice were conditioned with busulfan (BU) or treosulfan (TREO) and transplanted with $0.3x10^6$ or $10x10^6$ cells. **(A)** Number of donor and recipient microglia were measured by flow cytometry at 16 weeks post-HSCT. **(B)** Levels of glycosaminoglycan (GAG) substrate levels was determined at 16 weeks post-HSCT. **(C)** Neurological behavior was determined using a rotarod performance test. At 16 weeks post-HSCT, time on rotarod was measured daily for 3 days after a brief training period. Data are presented from the first experimental day. Bars represent mean ± SEM. Dashed lines indicate average values for non-transplanted wild type (WT) or *Idua*$^{-/-}$ mice. **(D)** Levels of hCD45+CD11b+Iba1+ microglia were determined by flow cytometry 1-16 weeks after transplant of unexpanded cord blood CD34+ cells, the standard of care, or MGTA-456 into busulfan-conditioned NSG mice (n=8 mice). Line graphs represent mean ± SEM. **(E)** IDUA enzyme levels were determined from unexpanded cord blood cells or MGTA-456 using a fluorometric assay. Bars represent median and each symbol represents an individual cell donor (n=3). For panels A-C, statistics are presented relative to low cell dose. For panels D-E, statistics are presented relative to the unexpanded cohort. *p<0.05, **p<0.01, ***p<0.001, and ns = not significant.

249. Therapeutic Correlation of Neurological Deficits and Brain Inflammation with Windows of Treatment by AAV-Mediated Gene Therapy in Gaucher Disease Mice

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The early-onset, irreversible pathological changes in the central nervous system (CNS) are major obstacles in treating neuropathic lysosomal storage diseases, including neuropathic Gaucher disease (nGD). GD is caused by mutations in *Gba1* gene encoding acid β-glucosidase (GCase), resulting in defective function of GCase and subsequent substrate accumulation in visceral organs and CNS. Current therapeutic options have minimal effect on the CNS manifestations of GD. We aimed to investigate the correlation of dynamic changes in CNS pathology and functional deficits with temporal, spatial windows of treatment by injection of adeno-associated viral vector (AAV) which has emerged as the most effective tool for *in vivo* gene delivery. We utilized a mouse model of nGD carrying V394L mutation in GCase and knocking out of Saposin C (4L;C*), which started to display detectable behavioral abnormities by age of day 45 with median survival of 56.5 days. We intravenously injected AAV2/9 overexpressing human GCase (ssAAV9-hGCase-ires-GFP) into 4L;C* mice at neonatal (postnatal day 2-3, AAV-Neo), adolescent (day 21, AAV-Ado) and adult (day 42) stages, resulting in varying impact on median survival rates (from >=157 days to 71 days to unimproved 57 days, respectively). In neonates, administration of AAV at dose of 5×10^{12} vg/mouse provided significant therapeutic benefits to 4L;C* mice as demonstrated in several aspects with sustained and various GCase expression in serum, viscera organs and brain. Firstly, treated mice maintained normal body-weight growth similar to their normal littermates (4L;Het) until

the age of 170 days. Secondly, short-term memory deficits found in age-matched 4L;C* mice by repeated open-field test was normalized in treated mice at day 50. Thirdly, the motor-neuron dysfunctions detected in untreated 4L;C* mice, such as hindlimb clasping and abnormal gait pattern, were ameliorated in AAV-Neo group with dramatical delay of onset and reduced severity. Fourthly, severe CNS pathology detected in untreated 4L;C* was significantly improved or normalized in different brain regions of AAV-neo mice as demonstrated with immunohistology analysis for GFAP and CD68. Finally, abnormally higher frequency of activated microglia and CNS immune infiltrates (myeloid/macrophage, lymphoid/T cells) found in untreated 4L;C* were completely rescued or near-normalized to the levels in normal littermates as quantified by flow cytometry analysis. Compared to AAV-Neo, AAV-Ado mice (receiving same dose of AAV) exhibited significantly higher GCase in liver (2-fold), serum (2-fold) and lung (~1.3-fold), similar in spleen and brain, but much less in heart. However, much less extension of lifespan was observed in AAV-Ado mice with no improvement in gait pattern, less severity in hindlimb clasping test, and partial normalization in memory deficits as detected in untreated age-matched mice. Interestingly, various degrees of pathological reduction were detected in different brain regions of AAV-Ado mice, which were associated with different alteration in microglia and subpopulations of CNS immune infiltrates. Together, the data demonstrated diversifying correlation of survival with neurological dysfunctions, CNS pathology and brain inflammation status in association with windows of treatment by AAV-mediated *in vivo* gene therapy in nGD mice.

250. Mouse Models of, and AAV Gene Therapy for, Propionic Acidemia

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Propionic Acidemia (PA) is a rare inherited metabolic disorder caused by a deficit in propionyl-CoA carboxylase (PCC) activity. The mitochondrial localized PCC enzyme is responsible for the conversion of propionyl-CoA to D-methylmalonyl-CoA. PCC is composed of six alpha and six beta subunits encoded by the *PCCA* and *PCCB* genes. Pathogenic mutations in the PA patient population are found in the *PCCA* or *PCCB* genes at equal frequencies. Patients diagnosed with PA typically present in the early newborn period with a metabolic crisis, which can be fatal if not promptly recognized and treated. The current management of PA relies upon dietary restriction of branch chain amino acid precursors with carnitine supplementation during intercurrent infections and stress. Despite vigilant monitoring and aggressive medical management, patients can suffer from metabolic decompensations, hyperammonemia, pancreatitis, sudden death from ventricular arrythmia, strokes of the basal ganglia, poor growth, and cytopenias. The severe disease burden, high rates of morbidity and mortality, and poor quality of life experienced by PA patients has led to the use of elective liver transplantation as a surgical treatment for PA to restore PCC activity and ameliorate or eliminate disease related symptoms. The use of expanded newborn screening to detect PA patients in the neonatal period adds another dimension of urgency to the development of new therapies for PA. We have therefore generated new mouse models of PA that accurately replicate the severe form of the disease and we have developed systemic AAV gene therapies using vectors that could be immediately translated for patient use. The new disease alleles of *Pcca* and *Pccb*, which includes a milder *Pcca* (A134T) knock-in mutation, were generated using CRISPR Cas-9 gene editing and bred to homozygosity. Homozygotes for *Pcca* and *Pccb* mutant alleles (except Pcca A134T) displayed neonatal lethality and increased 2-methylcitrate, similar to severely affected PA patients. Next, a series of AAV cassettes, designed to express the human orthologues of PCCA or PCCB from either constitutive or liver specific promoters, were prepared and pseudoserotyped with an AAV8 or AAV9 capsid. $Pccb^{-/-}$ or $Pcca^{-/-}$ mice were treated at birth and showed improved survival, persistent transgene expression and a reduction in plasma levels of 2-methylcitrate. In addition, $Pcca^{-/-}$ males treated with AAV9-CBA-PCCA were fertile. In summary, we have created new murine models of severe PA caused by Pcca or Pccb deficiency, developed candidate AAV vectors that express the human PCCA or PCCB orthologues, and tested them for efficacy in the mutant mice. Several vectors are highly efficacious and could be advanced to the clinic. Our results provide the preclinical data to enable systemic AAV gene therapy for patients with propionic acidemia.

251. Swine Biomedical Models to Enhance Translation of Cell and Gene Therapies

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There are an estimated 10-20,000 genetically inherited diseases in the human condition. Our ability to treat these genetic diseases is limited by several factors. Most notable, is that many novel therapeutics effective in standard research models are not effective in patients. Cell and gene therapies have been incredibly successful in rodents, but the difference in size, physiology, and delivery methodology make translation to patients a considerable challenge. The pig, physiologically and anatomically, is widely considered one of the closest matches to humans. Like mice, and unlike non-human primate models, pigs can be effectively engineered using gene-editing technology to model a variety of genetic diseases to the nucleotide level. These models not only give us a platform for testing therapies intended to replace loss of function disease alleles, but also to use gene editing to directly repair mutant alleles using human therapeutic reagents. We will showcase three swine models designed for gene therapy testing: alpha-1 antitrypsin deficiency (PI*ZZ allele), phenylketonuria (PAH^{R408W}), and dilated cardiomyopathy ($RBM20^{R636S}$). We will also showcase our custom development pipeline to create models unique to an individual company's specific diseases or genetic target, in order to produce models that best serve the needs of our customers.

252. Choroid Plexus-Targeted AAV Gene Therapy for Global Brain Treatment of Alpha-Mannosidosis

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The choroid plexuses (CP) are highly vascularized structures that project into the ventricles of the vertebrate brain. The polarized epithelia of the CP produce cerebrospinal fluid by transporting water

and ions into the ventricles from the blood and normally secrete a large number of proteins. We demonstrated that lateral ventricle administration of recombinant adeno-associated virus serotype 5 (rAAV5) resulted in selective gene transfer to the CP epithelia and rescued a mouse model of Menkes disease, a lethal infantile disorder of copper transport. While optimal rescue of Menkes mouse models requires an AAV serotype with broader neuronal tropism (viz., AAV9), we are assessing the feasibility of selective CP transduction with rAAV gene therapy vectors for treatment of lysosomal storage disease (LSD). There are no ideal therapeutic options currently available, especially for the CNS manifestations of LSDs. CSF-directed enzyme replacement has shown promise for several LSDs but requires repeated intrathecal administration due to short enzyme half-lives. Stem cell transplantation has also been carried out for other LSDs in which there is neurological involvement, however this approach requires bone marrow ablation and can be associated with significant morbidity and mortality. In contrast, rAAV-mediated gene transfer of missing lysosomal enzymes to CP epithelia would be safer and should enable continuous synthesis and secretion of enzymes directly into the CSF with penetration to cerebral and cerebellar structures. This is because 1) the CP epithelia are post-mitotic and do not turnover, and 2) rAAV transduction of non-dividing cells results in sustained episomal (non-integrating) transgene expression. CSF flow extends into the subarachnoid fluid compartment from where proteins can ultimately reach the entire brain via Virchow-Robin spaces, the paravascular, CSF-filled canals surrounding perforating arteries in the brain parenchyma. The metabolic cross-correction phenomena in which lysosomal enzymes from enzyme-competent cells can be taken up by enzyme-deficient cells (via mannose-6-phosphate receptors) affords another key advantage to the CP-targeting approach. Vector leakage from the CSF to the blood may enable off-target rAAV transduction of peripheral organs, especially the liver, with the consequence of enhanced systemic lysosomal enzyme levels, a further advantage. We propose therefore to use rAAV gene transfer to harness the potential of the choroid plexus as a long-term source of lysosomal enzyme production after a single treatment. We are combining rAAV gene therapy experiments in two well-established animal models of a prototypical LSD, Alpha-mannosidosis (AMD), with an ongoing human natural history protocol of AMD. AMD is caused by deficiency of lysosomal alpha-mannosidase (LAMAN) that results in chronic neurocognitive abnormalities that prohibit independent living in adulthood. We compared CSF-directed rAAV1, 4, 5, 6 & 9 in the mouse model of AMD. Recombinant AAV1 and rAAV6 showed the most robust transduction of CP in mouse brain, consistent with these serotypes' impressive transduction of CPE in nonhuman primates. Of note, rAAV1 and rAAV6 are closely related, their capsid sequences differing by only six amino acids. We found global brain restoration (olfactory bulb, cortex, cerebellum, brainstem) of LAMAN enzyme activity comparable or in excess of AMD heterozygote levels with both serotypes. Future mouse and cat AMD model preclinical experiments will focus on further direct comparisons between rAAV1 and rAAV6, which appear to be the most promising choroid plexus-targeting candidate serotypes for future clinical translation.

253. Defining Clinical Outcome Parameters for Cobalamin C (*cblC*) deficiency, a Common and Severe Disorder of Vitamin B12 Metabolism

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Combined methylmalonic acidemia and homocystinuria *cblC* type (*cblC*) is the most common inborn error of intracellular cobalamin (vitamin B12) metabolism caused by pathogenic variants in *MMACHC*. The majority of affected *cblC* patients present in infancy with severe symptoms including failure to thrive, anemia, developmental delay, progressive vision loss and, historically, neonatal mortality. Current guidelines recommend parenteral hydroxocobalamin (OHCbl), but lifelong daily injections are required, optimal dosing is debated, and the effects on long term clinical outcomes remain to be determined. Early identification by newborn screening has improved survival yet recent studies suggest the ocular and neurocognitive manifestations persist despite conventional management. Through a dedicated natural history protocol(clinicaltrials.gov ID: NCT00078078), we evaluated 45 individuals with infantile onset *cblC* (ages 2-35 years, median 10.9y). 24/45 (53%) were homozygous for the common *MMACHC* c.271dupA variant. The study population included patients diagnosed symptomatically (N=26, 58%) or pre-symptomatically following newborn screening (N=17, 38%) or family-based screening (N=2, 4%). The average age of diagnosis in the symptomatic group was 5.8 months (range 0.2-55) vs the pre-symptomatic group 0.3 months (range 0-0.8). The majority of the symptomatic group presented with failure to thrive and encephalopathy (81%) and in 50%, visual abnormalities. In our cohort, developmental delay/intellectual disability (DD/ID) IQ≤70 or impaired adaptive functioning (22/45, 49%), moderate to severe vision loss (32/45, 71%), hypotonia (28/45, 62%), microcephaly (10/45, 22%) and epilepsy (16/45, 36%) were frequently observed. To assess the effects of newborn screening, we compared outcomes in the symptomatic vs pre-symptomatic groups. DD/ID, microcephaly and epilepsy were more prevalent in the symptomatic group ($p<0.05$). Furthermore, pre-symptomatic diagnosis was associated with higher cognitive function (IQ $p=0.003$, ABC $p=0.022$), increased head circumference ($p=0.02$) and decreased prevalence of epilepsy ($p=0.063$). However, a significant percentage of pre-symptomatic *cblC* patients had DD/ID ≤70 and/or impaired adaptive functioning despite early identification and treatment (5/19, 26%). Retinopathy was common in both groups (symptomatic 21/26, 81%; pre-symptomatic 14/19, 74%). We also observed a positive correlation of homocysteine with age ($R^2=0.2381$, $p<0.0001$) suggesting poor metabolic control in adults. In conclusion, our data support the concept that pre-symptomatic diagnosis improves, but does not eliminate, disease complications specifically DD/ID and retinopathy. This suggests earlier intervention with more effective therapies will be needed to improve clinical outcomes in *cblC*. Preliminary work using mouse models of *cblC* establishes systemic AAV gene therapy as a potential future

therapeutic approach. The careful delineation of the natural history of *cblC*in the post newborn screening era provides the foundation for future systemic and/or ocular AAV gene therapy trials.

254. Abstract Withdrawn

255. Disease Correction by Intraparenchymal or Cisternal Delivery of a Modified AAV8 Capsid Expressing Codon Optimized NAGLU for Mucopolysaccharidosis Type IIIB Mice

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Mucopolysaccharidoses IIIB (MPSIIIB) is an autosomal recessive lysosomal storage disease caused by mutations in N-acetyl-glucosaminidase (NAGLU), resulting in aberrant retention of heparan sulfate within lysosomes and progressive central nervous system (CNS) degeneration. We previously demonstrated effective disease improvement using AAV5-NAGLU intraparenchymal injection at 6 sites. Additionally, we demonstrated AAV8 provides a broader transduction area in the MPS IIIB mouse brain compared to AAV5, 9 or rh10. A triple-capsid mutant (tcm) modification designed to reduce ubiquitination and degradation of AAV8 further enhanced expression and distribution. **Hypothesis:** The tcmAAV8 vector carrying a codon optimized NAGLU (coNAGLU) will improve disease outcomes in the MPS IIIB mouse model. **Methods:** *Naglu*$^{-/-}$ animals received AAVtcm8-coNAGLU at post-natal day 3 (P3) by either intracranial six site (IC6) or cisterna magna (ICM) injection at a total dose of 1.8x10E10 vg. Wild-type or heterozygous littermates (normal), *Naglu*$^{-/-}$, IC6+*Naglu*$^{-/-}$ (IC6) and ICM+*Naglu*$^{-/-}$ (ICM) animals were observed for survival, behavioral, and hearing (auditory brainstem response; ABR) assessments and biochemical and histological examination. **Results:** IC6 or ICM vector administration resulted in elevation of NAGLU enzymatic activity and improvement in histologic indices of disease. Compared to normal, an increase in vector-mediated NAGLU expression was observed in forebrain (IC6: 227 fold, ICM: 9 fold), hindbrain (IC6: 62 fold, ICM: 7 fold), cerebellum (IC6: 15 fold, ICM: 15 fold), and spinal cord (IC6: 3 fold, ICM: 12 fold). Histological examination of lysosomal distention revealed significant reduction in animals receiving IC6 or ICM when compared to *Naglu*$^{-/-}$ (LAMP-1 staining area percentage; cortex: Normal-0.1 [p<0.0001 vs no tx], *Naglu*$^{-/-}$no tx-3.6, IC6-0.3 [p<0.0001 vs no tx], ICM-0.2 [p<0.0001 vs no tx]; cerebellum: Normal-0.2 [p<0.0001 vs no tx], *Naglu*$^{-/-}$-no tx 2.2, IC6-0.6 [p=0.0002 vs no tx], ICM-0.3 [p<0.0001 vs no tx]). Significant improvement of hearing deficits was observed as measured by auditory brainstem response (ABR) threshold at 8 kHz (Normal: 60 dB SPL [p<0.001 vs no tx], *Naglu*$^{-/-}$no tx: 87 dB SPL, IC6: 66 dB SPL [p<0.05 vs no tx], ICM: 52 dB SPL [p<0.0001 vs no tx]) and 16 kHz (Normal: 63 dB SPL [p<0.01 vs no tx], *Naglu*$^{-/-}$no tx: 85 dB SPL, IC6: 73 dB SPL [p=0.4631 vs no tx], ICM: 58 dB SPL [p<0.001 vs no tx]). Extension of median lifespan was observed in IC6 and ICM animals compared to *Naglu*$^{-/-}$(Normal 76% at 537 days [p<0.0001 vs], *Naglu*$^{-/-}$ no tx 322 days, IC6 72% at 537 days [p<0.0001 vs no tx], ICM 84% at 537 days [p<0.0001 vs no tx]). **Conclusions:** Neonatal treatment of MPS IIIB mice with tcm8-coNAGLU either by IC6 or ICM injections results in supranormal NAGLU activity and normalization of lysosomal storage, hearing and survival out to at least 1.5 years. Acknowledgements: Sanfilippo Children's Research Foundation, Cure Sanfilippo, Lacerta Therapeutics and NIH/NINDS R01NS102624

256. *In Vivo* Suppressor tRNA Mediated Readthrough Therapy for Nonsense Mutations

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Genetic diseases are caused by a variety of mutations and changes to the genome. A nonsense mutation introduces a premature termination codon (PTC) in the mRNA and accounts for ~11% of mutations associated with human diseases. Since the PTC stops translation before a full-length protein is produced, the truncated protein may be degraded or not be as effective. PTC readthrough is a method to restore protein expression at the translational level. Modified tRNAs that can decode a stop codon are known as suppressor tRNAs, and have therapeutic potential as readthrough agents. However, their *in vivo* therapeutic efficacy has been understudied compared to other readthrough agents such as aminoglycosides. In this study, we aim to develop an *in vivo* suppressor tRNA therapy delivered by AAV vectors for a lysosomal storage disease known as mucopolysaccharidosis type I (MPS-I). We first screened a panel of suppressor tRNAs for their UAG stop codon readthrough activity in HEK293 cells using a GFP reporter with a Y39X (UAC-->UAG) mutation, and a dual-luciferase reporter with a nonsense mutation (UGG-->UAG) underlying MPS-I, respectively. Both assays consistently identified suppressor tRNAs that can efficiently induce readthrough and restore reporter protein expression. The suppressor tRNAs were packaged into lentiviral vectors and used to infect fibroblast cells from an MPS-I patient with homozygous *IDUA-W402X* mutation (UGG-->UAG) that abrogates iduronidase (IDUA) activity. One of the suppressor tRNAs was able to restore IDUA activity to 0.7% of the normal level, which is above the targeted therapeutic threshold of 0.5%. This suppressor tRNA was packaged into AAV and systemically delivered to a mouse model of MPS-I harboring the *Idua-W392X* mutation (UGG-->UAG) analogous to the human mutation. Sustainable restoration of serum and liver IDUA activity of 1-3% of the normal level was observed up to 13 weeks post-injection and counting. Further assessment on the therapeutic efficacy is ongoing. Furthermore, we are employing ribosome profiling to assess global readthrough that may underlie potential toxicity. The functional suppressor tRNAs identified in this study could be potentially used to treat other diseases caused by a UAG PTC. Compared to gene replacement and CRISPR-based gene editing, the small gene size of suppressor tRNA is highly amenable to AAV vector delivery, and lack of foreign protein expression has a favorable immunological profile.

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257. rAAV Gene Therapy for the Treatment of Citrullinemia Type I (CTLN-1) in Immature Mice

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Citrullinemia type I (CTLN-1; OMIM# 215700) is a rare autosomal recessive genetic disorder caused by different mutations in the *ASS1* gene, which encodes Argininosuccinate Synthase 1 (ASS1; EC 6.3.4.5), the third enzyme of the urea cycle. The lack of this enzyme prevents hepatic processing and elimination of excess nitrogen as urea, resulting in accumulation of ammonia and urea cycle byproducts, such as citrulline, in blood. In the neonatal presentation of CTLN-1, serum ammonia increases quickly to neurotoxic levels, leading to encephalopathy. Current therapies are based on hyperammonemia crisis control by limiting dietary protein intake in combination with the administration of nitrogen scavengers. Early restoration of hepatic expression of the missing protein represents the most attractive strategy for the treatment of this disease. For that purpose, Vivet Therapeutics has developed a rAAV vector expressing ASS1 under the control of a liver-specific promoter, designated as VTX-804. Therapeutic efficacy of VTX-804 was evaluated in 1- and 3-week-old CTLN-1 mice ($ASS1^{fold/fold}$), an age at which the liver is growing and hepatocytes proliferating. A group of animals that received since birth nitrogen scavenging agents (sodium phenylbutyrate and sodium benzoate) supplemented with L-Arginine was included. Animals received $5x10^{13}$ vg/kg of VTX-804 i.v. Five weeks after vector administration serum ammonia levels were reduced significantly to healthy WT levels. Three weeks later (8 weeks after treatment), the therapeutic effect disappeared in animals treated at 1 week of age but remained significant in mice treated at 3 weeks of age. In addition, citrulline levels were also reduced in 3-week-old treated animals. Furthermore, while CTLN-1 mice show a lower body weight than WT mice and are infertile, treated mice at 3 weeks of age normalized body weight, and became fertile, producing viable offspring. Independently of the age at treatment, all groups receiving VTX-804 presented a better survival than the untreated CTLN-1 mice. Moreover, 3-weeks-old treated mice improved the survival of animals receiving only nitrogen scavengers. Mice were sacrificed 8 weeks after vector administration and the number of rAAV viral genomes and *hASS1* mRNA expression in the liver were analyzed. CTLN-1 mice treated at 3 weeks of age presented 6 times more rAAV genomes and 11 times higher *hASS1* mRNA expression levels than mice treated at 1 week of age. Altogether, our data shows that a single dose of VTX-804 administered to CTLN-1 mice at 3 weeks of age, improves overall health status, and significantly reduces the blood accumulation of both, ammonia and citrulline, for up to 8 weeks after vector administration.

258. Developing Body-Wide Gene Therapies for Treatment of Progerias

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Progeroid syndromes result from single gene mutations that cause accelerated onset of aging-like features in one or more of the body's organ systems. Gene therapies hold promise for treating these diseases, but application of such therapies is hampered at present by the fact that there are no known gene delivery vectors that have sufficient breadth of tropism and uniformity of expression across tissues to effectively complement disease-causing gene mutations across all of the different cell types that can be affected. Here, we tackle this problem by engineering novel adenoassociated virus (AAV) vectors that combine multiple expression control modalities. In particular, our vector approach combines self-complementary AAV genomes, driven by a strong, compact and non-silencing promoter, with in vivo validated, codon optimized protein coding sequences and liver-specific microRNA target sequences in the 3' UTR to reduce overexpression in the liver. We further produced and delivered our gene therapy vector as a combination of AAV serotypes (AAV9 and PHP.eB) to simultaneously deliver the transgene to both the brain and peripheral organs. Using this system, we have documented relatively uniform transduction and transgene expression across each of the major tissues of the mouse body and verified transgenic expression of AAV-delivered cargo in a mouse model of Wolfram Syndrome II, a progeroid disease characterized by sensorineural, metabolic and hormonal defects. Studies are currently underway to evaluate the utility of genetic complementation therapy via this approach for rescuing disease-relevant progeroid features in these animals.

259. Insertion of Short Double-Stranded Oligonucleotides Using CRISPR/Cas9 as a Therapeutic Approach for Glycogen Storage Disease Type 1a

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Glycogen storage disease type Ia (GSDIa) is a rare autosomal recessive disease that can lead to life threatening hypoglycemia and progressive liver and kidney damage. GSDIa is caused by loss of function mutations in the glucose-6-phosphatase-α (G6PC) gene. G6PC, an integral membrane protein primarily expressed in the liver, kidneys, and intestines, enzymatically cleaves phosphate from glucose-6-phosphate to liberate glucose to stabilize blood sugar levels. Here, we explore CRISPR/Cas9-based strategies to correct the prevalent R83C mutation in G6PC by utilizing non-homologous end joining (NHEJ) repair mechanisms and short double-stranded DNA oligonucleotides (dsODNs). We identified guide RNAs (gRNAs) that

cleave G6PC exon 2 genomic DNA near the site of the mutation and for each gRNA, designed dsODNs to introduce the R83C mutation as a proxy for correction. Each dsODN, when transcribed to RNA, consists of a 3' splice site (a branch point sequence, a polypyrimidine tract, and a terminal YAG) and an exonic region containing protein coding sequences. Insertion of the dsODN creates a new version of G6PC exon 2 that has tandem 3' splice sites and a single 5' splice site. As predicted by the exon definition model of splice site pairing, the newly introduced 3' splice site is proximal to the 5' splice site and spliced to G6PC exon 1, resulting in the protein coding sequences from the dsODN being included, in frame, in the final mRNA. The introduction of Cas9 mRNA, a single-stranded gRNA, and the dsODN into primary human hepatocytes (PHHs) *in vitro* via transient transfection and into mice *in vivo* via lipid nanoparticles routinely led to over 10% of mRNA isolated from both mice and PPHs containing the R83C mutation as measured by Next-Generation Sequencing. Of note, studies in mice have suggested that restoring approximately 4% of G6PC activity could prevent the hypoglycemic seizures that occur in GSDIa patients. The dsODN insertional technique described here bypasses the need for the homology-directed recombination machinery and functions in both dividing and non-dividing cells, making this technique an intriguing potential means to correct a number of genetic diseases in cells and tissues that have low rates of homologous recombination.

260. Development of Surrogate Endpoints for Clinical Outcomes in Propionic Acidemia

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INTRODUCTION: Propionic acidemia (PA) is a severe, pediatric-onset inborn error of metabolism associated with significant mortality and multiorgan morbidity. There are no FDA-qualified biomarkers or FDA-approved genomic therapies for PA. To support the development of novel PA therapies, we implemented a framework to identify and validate biomarkers that could serve as surrogate endpoints in pre-clinical studies and clinical trials. METHODS: We evaluated PA patients through a dedicated natural history study (The Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia, ClinicalTrials.gov ID NCT02890342) at the NIH clinical setting. To identify variables associated with PA outcomes, we developed a PA database comprised of > 400 clinical, laboratory and imaging parameters. Associations of clinical outcome parameters with prior-art and novel biomarkers are routinely reanalyzed using univariate selection analysis. In this work, we used a subset of 12 variables representative of the PA disease characteristics (height z-score, full scale IQ (FSIQ), alanine aminotransferase, optic nerve atrophy, sensorineural hearing loss, estimated glomerular filtration rate (eGFR), left ventricular ejection fraction (LFEV%), white cell blood count (WBC), red blood cell count (RBC), platelet count, complete and incomplete protein as a % of RDA) to evaluate their association with a novel biomarker based on the *in vivo* oxidation of 1-^{13}C-propionate. Baseline VCO_2 production (ml/min) was measured by an open-circuit indirect calorimetry cart. After a single enteral bolus of 1-^{13}C-propionate, we measured $^{13}CO2$ enrichment in expired total CO_2 using isotopic ratio mass spectroscopy. A set of healthy volunteers (n=19) served as controls. RESULTS: We enrolled 40 participants with PA, ages 2-52 years (mean age 16 years, 45% females, 5 individuals after liver transplant), of whom 33 completed the stable isotope study. Participants with biallelic null variants (missense variants, indels resulting in frame shift variants, or microdeletions) in *PCCA* or *PCCB* had lower 60-minute 1-13C-propionate oxidation (median = 2.3%) compared to those with at least one missense variant (median = 14%, $P < 0.0001$). In non-transplanted participants, 1-^{13}C-propionate oxidation correlated with height z-scores ($R^2 = 0.18$, $P < 0.05$), FSIQ ($R^2 = 0.49$, $P < 0.0001$), alanine aminotransferase ($R^2 = 0.16$, $P < 0.05$), sensorineural hearing loss ($P < 0.05$), and incomplete protein intake as % of RDA ($R^2 = 0.16$, $P < 0.05$). Oxidation was normal in liver transplant recipients. CONCLUSIONS: 1-^{13}C-propionate oxidation was significantly different between participants with severe vs milder genotypes and was associated with several clinically important outcomes. Normal 1-^{13}C-propionate oxidation in transplanted patients support the role of *in vivo* stable isotope studies as a surrogate endpoint in clinical trials aimed at restoring hepatic propionate oxidation.

261. Neurometabolic Phenotype Precedes Symptoms Onset in Canavan Disease and Restoration of ASPA Expression Shifts Glial Precursor Pools

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Canavan disease (CD) is a rare monogenic disorder caused by mutations in the aspartoacylase (ASPA) gene, hallmarked by spongy degeneration of white matter in the central nervous system (CNS) and elevation of N-acetylaspartate (NAA), the only known metabolite of ASPA. NAA is catabolized to L-aspartate and acetate and it has been postulated that acetate is required for myelin lipid synthesis. In CD patients, symptoms, such as lack of head control, macrocephaly, muscular hypotonia, and seizures, first present weeks to months after birth. The Nur7 mouse model is commonly used in CD research and shows initial symptoms and neuropathological changes around post-natal day 13-14. Interestingly, brain pathology and molecular changes are not detected prior to postnatal day 10. Given the developmental increase in ASPA expression and the proposed contribution of NAA to myelination, we hypothesize that infantile CD patients might be asymptomatic because NAA is not required that early in brain function. Thus, it is currently not known if molecular characteristics of Canavan disease precede symptom onset. To determine if Canavan disease displays a characteristic disease metabolic pattern in early postnatal age, we used whole brain metabolomics to analyze brains of wild-type and Nur7 mice at postnatal day 7. We detected a total of 646 metabolites and 162 were statistically significantly different. Among those metabolites was NAA, which was 2-fold elevated in the ASPA deficient brain, suggesting NAA may already play an early role in brain development. If NAA has biological significance in supplying building blocks for myelin lipids, myelin lipids should be reduced in Nur7 mice. Contrary

to our expectations, a significant number of lipids involved in myelin synthesis were significantly increased in brains of ASPA deficient Nur7 mice, suggesting that NAA might not be the only source for myelin synthesis early in brain development. Using rAAV to re-introduce ASPA expression at postnatal day 1 allowed for complete normalization of the neurometabolic phenotype. We next hypothesized that NAA may be required to maintain or recruit glial precursor cells, which could explain the disconnect between increase of myelin lipids and loss of ASPA function and CNS white matter pathology. We therefore used RNAscope to quantify cell markers associated with different stages of oligodendrocyte and astrocyte development in juvenile mice which are symptomatic at the time of treatment and fully recovery after gene therapy. Our preliminary data suggest that ASPA deficient mice, which show substantial white matter degeneration, exhibit increased expression of the mature oligodendrocyte marker myelin basic protein (MBP) despite the loss of myelin on electron microscopy (EM). In contrast, re-constituting ASPA expression seems to be associated with reduction of PDGFRalpha oligodendrocyte precursor markers and restoration of myelination on EM, which might suggest that NAA metabolism is associated with shifts in oligodendrocyte precursor pools required for myelination.In conclusion, our data suggest that CD specific changes in brain metabolomics of Nur7 mice precede the manifestation of symptoms and that the contribution of NAA as a building block for myelination might have less biological relevance in early stages of brain development. Furthermore, we demonstrate that glial lineages respond to ASPA gene correction with restoration of myelination and motor function. We are currently finalizing our efforts to conclusively understand the connection of NAA and glial cell lineage control.

262. Long-Term Safety and Efficacy of the PS Gene Editing System for Mucopolysaccharidosis Type I

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Mucopolysaccharidosis type I (MPS I) is a lysosomal disease leading to growth delay, skeletal abnormality, neurodegeneration, and premature death. MPS I results from the deficiency of alpha-L-iduronidase (IDUA) and subsequent accumulation of glycosaminoglycan (GAG). Previously, we designed a PS gene editing system based on the CRISPR technology. Through i.v. administration of two AAV8 vectors, the PS system integrates a promoterless therapeutic cDNA sequence into the albumin locus of hepatocytes. To evaluate the long-term safety and efficacy, the PS system was injected into neonatal and adult MPS I mice (n=8 to 14 each group) at three different doses. Kaplan-Meier survival analysis showed that the survival of treated MPS I mice was significantly increased than controls ($p<0.01$). Plasma IDUA activities reached approximately 622-fold of wildtype levels and were sustained for 10 months. More importantly, IDUA activity in the brain increased to 75% of wildtype levels, and GAG accumulation levels in these tissues, including the brain, were normalized ($p<0.0001$). Fear conditioning showed that treated MPS I mice had improved memory and learning ability ($p<0.05$). Also, histological analysis showed efficacy reflected by the absence of foam cells in the liver and of vacuolation in neuronal cells. As to safety, no treatment-related adverse events, signs of toxicity (clinical signs, gross or microscopic findings), or increased tumor risk were observed. A very low level of antibody against IDUA proteins was detected by ELISA only at 10 months post-dosing. Albumin production was reduced only in the high dose group at 1 month post-dosing, but returned to normal after 10 months. AAV copy number and Cas9 mRNA level were significantly reduced from 1 month to 10 months post-dosing ($p<0.001$). In addition, the PS system showed efficient gene editing at the target locus of human hepatocytes, and no off-target effects were detected through the unbiased off-target analysis GUIDE-Seq (sensitivity=0.1% indel frequency). In summary, these results showed the long-term safety and efficacy of the PS system in treating MPS I disease and pave the way for advancing this therapy to the clinical trial stage. Additionally, as a therapeutic platform, the PS system has the potential to treat other lysosomal diseases.

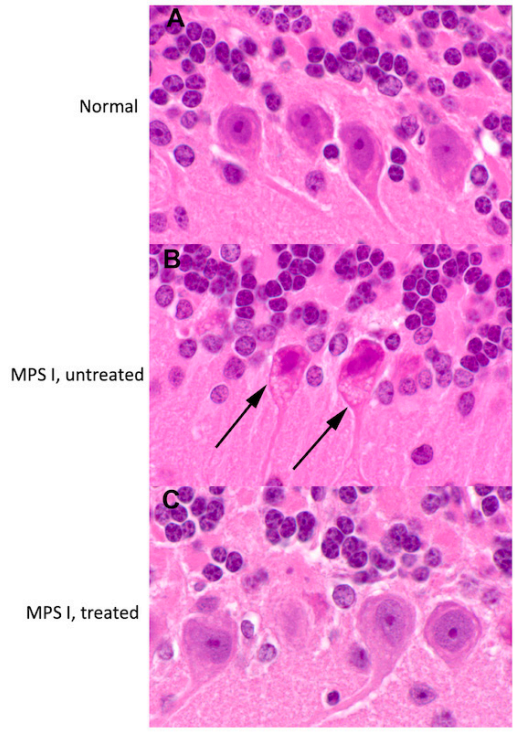

Figure 1. H&E staining of cerebellum of treated and control mice. Panel A shows tissues from normal mice, Panel B from untreated MPS I mice, and panel C from treated MPS I mice. The Purkinje neurons in untreated MPS I mice have cytoplasmic cellular vacuolation (arrows), which is absent in treated MPS I and normal mice.

263. DNA-Encoded Glutamine Synthetase Enzyme as Ammonia-Lowering Therapeutic for Hyperammonemia

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Hyperammonemia is a life-threatening metabolic condition attributed to excessive blood ammonia levels which can lead to irreversible brain

damage if left untreated. It can be caused by various pathological conditions including acute or chronic liver diseases, inherited metabolic disorders, premature birth, drugs, among others. Current pharmacotherapeutic approaches available for hyperammonemia patients are inefficient and have been associated with major side-effects. As yet, hemodialysis has been the most effective strategy for removing ammonia. However, hemodialysis is not available in all communities and is risky in terms of the complications that can arise. Here, we developed a novel DNA-encoded glutamine synthetase enzyme therapy using direct in vivo gene delivery for the treatment of hyperammonemia. In an ammonium acetate-induced hyperammonemia model, we achieved a 30.5% decrease in blood ammonia levels 15 min post-administration of ammonium acetate, with DNA-encoded glutamine synthetase treated group. In addition, substantial reduction in mortality was observed in acute liver injury model with glutamine synthetase treated mice. A comparison of the secreted vs. intracellular DNA-encoded glutamine synthetase enzyme demonstrated similar increases in survival in the acute liver injury model, with 40% mortality in the secreted enzymes and 30% mortality in the intracellular enzymes, as compared to 90% mortality in the control group. Direct *in vivo* delivery of DNA-encoded glutamine synthetase demonstrated important ammonia-lowering potential. Such DNA-encoded enzymes could be valuable ammonia-lowering therapeutics for treating hyperammonemia.

264. AAV9-Mediated Gene Therapy for NGLY1 Deficiency and Assessment of GNA Biomarker Changes in a Rat Disease Model

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N-glycanase 1 (NGLY1) deficiency is a rare genetic disorder known to affect > 60 individuals worldwide. Patients display heterogeneity in disease presentation and severity but share 5 core features: (hypo) alacrima, elevated liver transaminases, peripheral neuropathy, developmental delay, and a hyperkinetic movement disorder. As there is currently no treatment for NGLY1 deficiency, we are pursuing several therapeutic modalities, including AAV9-mediated NGLY1 gene therapy (GS-100). The development of GS-100 requires 1) a reliable biomarker for NGLY1 deficiency consistent with a lack of NGLY1 enzymatic activity, and 2) an animal disease model that exhibits both systemic and CNS/PNS disease hallmarks. NGLY1 is a cytosolic deglycosylase involved in the endoplasmic-reticulum-associated protein degradation (ERAD) pathway. It cleaves *N*-glycans from the asparagine residues of misfolded proteins at the GlcNAc-Asn bond. In NGLY1's absence, this GlcNAc-Asn bond is left intact, which could lead to the cytoplasmic accumulation of Asn-glycan metabolites like GlcNAc-Asn (GNA). To determine whether patients have increased levels of GNA and related metabolites relative to a control population, we conducted multiple semi-quantitative analyses of metabolites in NGLY1 deficient patient plasma. This study identified significantly increased GNA abundance (Z-score > 3) in NGLY1 patient plasma. Preliminary quantitative analysis confirmed that GNA levels in patient plasma are 4.3 fold higher than in controls. Using an oligosaccharide-focused analysis, we also discovered that GNA is elevated (difference in population mean Z scores > 5.4) in patient urine. This was accompanied by elevation in the relative abundance of a modified version of GNA, Neu5Ac1Hex1GlcNAc1-Asn (NHGNA), consistent with the hypothesis that plasma proteins can turn GNA into modified moieties. Elevated GNA and NHGNA were also recently reported in dried blood spots and urine samples, respectively, from NGLY1 patients. Based on this data, we predict cytosolic GNA (and NHGNA) accumulation is a characteristic of NGLY1 deficiency, correlating directly with a lack of NGLY1 activity. Although *Ngly1* knockout mice are perinatal lethal, we recently obtained a *Ngly1* knockout (*Ngly1*$^{-/-}$) rat model. Approximately 25% of these homozygous animals survive beyond weaning. Characterization of these surviving knockouts reveals decreased initial body weight and abnormalities in rotarod and grip strength tests. Longitudinal assessments of these phenotypes, as well as GNA biomarker changes in blood, urine and CSF are currently ongoing (data to be presented).In order to establish proof of concept for GS-100 gene therapy, cohorts of study animals (wildtype and *Ngly1*$^{-/-}$ rats) were treated with a single intravenous dose of an AAV9 vector expressing human NGLY1 at age 5 weeks. GNA levels in serum/urine were monitored weekly and CSF was collected at 5 weeks post-administration, the study's end, for comparison with untreated wildtype and *Ngly1*$^{-/-}$ controls (data to be presented). The GNA biomarker, linked tightly to NGLY1's enzymatic activity, combined with an NGLY1 disease rat model that exhibits early onset of motor neuron defects consistent with patient phenotypes are critical to determining the *in vivo* efficacy of GS-100 gene therapy. These data will guide GS-100 development toward the clinic as well as other treatment modalities for this devastating genetic disorder.

265. Minimal Protein from DNA Mini Circles Provides Therapeutic Benefit in CPS1 Deficiency

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Carbamoyl phosphate synthetase 1 (CPS1) deficiency is a rare autosomal recessive disorder characterized by the loss of function of hepatic CPS1, the protein responsible for catalyzing the first and rate-limiting step of the urea cycle. CPS1 deficiency patients with neonatal onset typically develop severe hyperammonemic symptoms shortly after birth, including vomiting, coma, and cerebral edema, which often lead to death despite aggressive treatment. No curative options are available to these patients except liver transplantation, which suffers from low donor availability and the need for long-term immune suppression. To address the unmet need for novel therapeutic options, we previously generated and characterized a Cre/LoxP-based conditional mouse model of CPS1 deficiency that recapitulates the human phenotype and demonstrated its subsequent correction using both adenovirus and adeno-associated virus gene therapies. To improve the gene delivery system and circumvent the steep challenges of viral

approaches, we turned to DNA mini circles (MCs). MCs represent a powerful alternative to other viral and non-viral strategies: i) they may be easily re-administered; ii) there is a reduced chance of immune rejection due to lack of viral proteins; iii) there is decreased silencing compared to parent plasmids subsequent to the removal of bacterial DNA elements; and iv) they are expressed much more rapidly than viruses. MCs may also be produced and purified significantly more rapidly than their viral counterparts, an important aspect for eventual industrial-scale production and clinical translation. Therefore, to investigate their potential to treat CPS1 deficiency, we generated MCs containing human codon optimized CPS1 driven by the CMV enhancer/chicken b-actin (CAG) promoter. MCs were complexed to Polyplus *in vivo* jetPEI-Gal, a PEI-based polymer conjugated to galactose, and injected retro-orbitally into adult Cps1-deficient mice. In a dose escalation study, mice received 130-190μg MC complexed at a nitrogen/phosphate ratio of 8 spread out into 4-5 injections over a 2.5-week period, beginning 3 days after the initial injection of liver-specific promoter-driven AAV-Cre recombinase. Though mice receiving less MC DNA succumbed to disease after 30 days, mice receiving 170μg or more survived to the study endpoint of 6 weeks, with untreated mice (AAV-Cre alone with no MCs administered) perishing by day 21. MC-treated mice had an average plasma ammonia level of 790.1μM, higher than wild type controls (300.8μM) but lower than untreated controls (1643.0μM). Immunohistochemical analysis of the liver showed high CPS1 expression in periportal hepatocytes that drops sharply further into the hepatic parenchyma, highlighting the low total protein levels in the tissue. Further, gene expression in treated mice was lower than expected relative to wild types, with an average vector copy number of 14.8 per diploid genome. Altogether, the data show that therapeutic protein levels may be more closely linked to their abundance in periportal hepatocytes rather than total lobular abundance, in contrast to what has been previously suggested by our and other's work. With optimization, MC treatment may provide a potential new therapeutic option for the treatment of carbamoyl phosphate synthetase deficiency, allowing for simplified and fast-acting administration, which are essential for early treatment of this rapidly progressing inborn error of metabolism.

266. Employing the PS System for β-Galactosidase Expression and Metabolic Correction to Treat GM1-Gangliosidosis

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GM1-gangliosidosis is a progressive neurological disease caused by a deficiency in the lysosomal enzyme β-galactosidase (β-gal, E.C. 3.2.1.23). Reduction or loss of β-gal activity results in the accumulation of GM1 ganglioside in the cell, primarily affecting the central nervous system. This leads to progressive neurodegeneration and deterioration of motor function and, in the severe infantile form, death before three years of age. A major limitation of current approaches for treating GM1 gangliosidosis is utilizing episomal expression of the β-gal encoding gene, *GLB1,* which results in vector dilution as the body mass increases (e.g. as an infant grows to adulthood). An alternative approach is to integrate the *GLB1* transgene into the genome and utilize an endogenous promotor to drive the expression of the transgene. In the current study, a novel AAV-mediated gene therapy, designated PS-905, is engineered to integrate a promoter-less *GLB1* cDNA sequence into the albumin locus, driving expression from the endogenous albumin promotor. Three doses (low, middle, high) of PS-905 were injected intravenously into affected neonatal β-gal deficient mice (β-gal$^{-/-}$). Thirty (30) days following injection, β-gal enzyme activity in plasma was increased 20-fold and 4-fold in mice receiving high and middle dose, respectively, compared to heterozygous mice. At 7 months post-injection, β-gal enzyme activity was 12-fold in the high dose mice and at heterozygous levels in the middle dose mice. Performance on the accelerating rotarod at 4 months of age showed that the β-gal$^{-/-}$ mice that received the high dose displayed significantly improved motor function and coordination compared to untreated β-gal$^{-/-}$ mice ($p<0.05$). Further testing at 6 months of age with the beam walk showed that the high dose β-gal$^{-/-}$ mice had significantly less hind-limb slips compared to untreated β-gal$^{-/-}$ mice ($p<0.05$ on all 4 test days). Neurocognitive testing at 6 months with the Barnes maze also showed improvement of memory and learning in the high-dose β-gal$^{-/-}$ mice on day 3 of testing compared to untreated β-gal$^{-/-}$ mice ($p<0.01$). Biochemical analysis of tissues revealed the presence of β-gal enzyme activity in the spleen and heart, in addition to a significant increase in β-gal enzyme activity in the liver of high dose β-gal$^{-/-}$ mice ($p<0.0001$). Additionally, ganglioside quantification utilizing HPLC-MS/MS showed a significant reduction of GM1 ganglioside in the liver ($p<0.0001$) and heart ($p<0.05$) in high dose β-gal$^{-/-}$ mice. Overall, preliminary analysis of this novel gene editing system reveals that PS-905 appears to be a safe and potent means to produce a functional β-gal enzyme, provide a therapeutic level of β-gal enzyme activity, and improve the motor and cognitive function in β-gal$^{-/-}$ mice.

267. mRNA Therapy for Treatment of OTCD

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Ornithine Transcarbamylase Deficiency (OTCD) is the most prevalent Urea Cycle disorder, affecting about 1 in 50,000 people. It is an x-linked trait that predominantly affects boys, and is subclassified into early onset or late onset forms, reflecting total or partial OTC activity loss, respectively. Symptoms result from the accumulation of ammonia in the blood leading to vomiting, seizures, and coma. Early onset disease presents within 1 day of life and is usually fatal within 1 week. Late onset cases can have elevated basal plasma ammonia levels or even be completely asymptomatic. However, these patients, defined as having between 2-25% wild-type OTC activity, are subject to sudden bouts of metabolic decompensation at any point in their lives that could lead to coma and death. Standard of care consists of removing excess ammonia via hemodialysis, a lifetime low-protein diet, and use of ammonia scavenging drugs such as sodium phenylbutyrate (Buphenyl). In some

cases, liver transplantation is performed and is considered curative. AAV8-based gene replacement and mRNA-based therapies are being pursued in Phase I/II clinical studies. We have previously reported the effect of an optimized OTC-mRNA construct in Spf-ash mice. In this metabolic decompensation model of late onset disease, we showed in single and multiple dose studies that a codon optimized OTC-mRNA maintained body weight and increased survival by lowering the plasma ammonia levels that spike following a high protein diet challenge. Early onset of OTC has typically been modeled by OTC knockout mice. Unfortunately, these animals die within 24 hours after birth and it is technically challenging to dose early and often enough to rescue them. While we have observed some rescue of these pups, ranging from 2-14 days, a better model for late onset disease is needed. Here, we report the characterization of a novel tamoxifen inducible OTC knock out mouse model and compare the ability of a codon optimized OTC-mRNA sequence, with and without half-life extending modification, to rescue this model as well as Spf-ash mice. The inducible mouse model uses adult mice treated for 5 consecutive days with tamoxifen. Endogenous OTC mRNA was decreased 95% by the first timepoint assessed (7 days). Endogenous protein and activity levels tapered off more gradually, with both decreased 90% by day 21, which is coincident with body weight loss and rise in plasma ammonia levels. The model is 100% penetrant, with animals dying between 25-30 days. To test the efficacy of our optimized mRNA, we dosed animals on day 18. We found that both mRNAs increased survival by 7 days. Percent body weight was likewise maintained for about a week, with the modified mRNA treated animals trending better. For comparison, we tested the same mRNAs in Spf-ash mice. Modification of the optimized OTC-mRNA sequence showed no difference in expression or activity 2 days post dosing, but improved activity was observed 7 days post dosing. When challenged with a high protein diet, codon optimized OTC-mRNA treated animals maintained body weight for 7 days while the modified mRNA counterpart maintained body weight 10 days longer. There was also a trend toward improved survival, with 4/6 codon optimized OTC-mRNA treated animals surviving to day 29, whereas all animals survived in the modified mRNA treated group. These studies demonstrate our inducible knock out animals have a much more severe phenotype than Spf-ash mice, consistent with early onset disease. Importantly, our optimized OTC-mRNAs work in multiple animal models representing both early and late onset OTCD.

268. In Vivo HSC Gene Therapy of Hemophilia A in Mice

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We developed an *in vivo* hematopoietic stem cell (HSC) transduction approach that involves HSC mobilization from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAd5/35++ vectors target human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive Sleeping Beauty transposase (SB100x) and transgene marking in peripheral blood cells can be increased by *in vivo* selection. Here we directed transgene expression to HSC-derived erythroid cells using β-globin regulatory elements. We hypothesized that the abundance and systemic distribution of erythroid cells can be harnessed for high-level production of therapeutic proteins. We first demonstrated that our approach allowed for sustained, erythroid-lineage specific GFP expression and accumulation of GFP protein in erythrocytes. Furthermore, after *in vivo* HSC transduction/selection in hCD46-transgenic mice, we demonstrated stable supraphysiological plasma concentrations of a bioengineered human factor VIII, termed ET3. High-level ET3 production in erythroid cells did not affect erythropoiesis. We then performed a therapy study in $hCD46^{+/+}/F8^{-/-}$ hemophilia A mice. In addition to the HDAd5/35++ vector that expressed ET3 from an erythroid specific beta-globin mini LCR, we also included vectors expressing factor from the ubiquitously active PGK promoter and the monocyte/macrophage-specific (3.4kb) CD68 promoter, based on the hypothesis that factor VIII expression in tolerogenic cells would inhibit the development of inhibitor antibodies. Mobilized $hCD46^{+/+}/F8^{-/-}$ mice were injected with A) 50% LCR vector + 50% SB vector, B) 45% LCR +15% PGK vector + 40% SB vector, or C) 450% LCR + 15% CD68 vector +40% SB vector and in vivo selection was started 4 weeks later. At week 13 after in vivo transduction, anti-ET3 antibody titers >10,000 were found in 1 out 9 (A), 5 out of 13 (B), and 3 out of 8 (C). ET3 mRNA levels in total blood cells were comparable for group A and B, but significantly lower for group C. Antibody titers >10,000 inhibited factor VIII activity measured by chromogenic assay, aPTT assays and tail bleeding assays. In summary, phenotypic correction was achieved in 85% of hemophilia A mice injected with vector containing the ET3 gene under the β-globin LCR. Attempts to express ET3 in potentially tolerogenic cells, using the PGK and CD68 promoters, were not successful.

269. Comparison of the Efficacy of Three Viral Vectors for Decreasing Sulfatide Levels in Central and Peripheral Nervous System in a Mouse Model of Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is a rare lysosomal storage disorder (LSD) with no available treatment. We designed multiple recombinant lentivirus vectors (LVs) and adeno associated virus vectors (AAVs) and tested their ability to engineer expression of human arylsulfatase A (ARSA) in a mouse model of MLD. We performed efficacy trials of three vector types (LV, AAV9 and AAVrh10) in both *ex vivo* cell culture studies and in vivo animal models of MLD. MLD mice injected with LV, AAV9 and AAVrh10 into the lateral ventricle demonstrated a decrease in sulfatide accumulation in the CNS indicating an increase in ARSA activity 3 months following treatment. MLD mice injected IV with AAV9 and AAVrh10 demonstrated increased ARSA activity and lipid reduction in the spinal cord and sciatic nerve at 3 months following injection. No negative effects of vector treatment were apparent in mouse tissue or cell lines following treatment with vector serotypes. Data presented represents a novel

study whereby three viral vectors are compared based on efficacy of treating both the CNS and PNS disease pathologies associated with MLD.

270. High Incidence of Tumor Formation in Successful Treated MLD Model Mice by Systemic Administration of AAV9 Vector Expressing ASA

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by the deficiency of arylsulfatase A (ASA) and characterized by severe neurological symptoms due to widespread demyelination in the both central and peripheral nervous systems. Recently, we have succeeded to treat ASA knockout MLD model mice by systemic neonatal gene delivery of type 9 single stranded adeno-associated viral (AAV) vector expressing ASA (ssAAV9/ASA). We also treated adult (12-weeks old) MLD model mice using type 9 double stranded (ds) AAV9/ASA.These treated mice were analyzed at age 1.5 year. Immunohistochemical analysis showed efficient ASA expression was detected in the brain. Alcian blue stainingand quantative analysis of sulfatide contents by biochemical assay showed decrease of the amount of stored sulfatide in these treated mice compared to non-treated MLD mouse. In the behavior test, improvement in their ability to traverse narrow balance beams test were observed. However, high incidence tumor formation was detected in these treated MLD model mice. About 93% (12/13) of ssAAV9/ASA (2 x 10^{12} vg/body) injected, 75% (3/4) of dsAAV9/ASA (2 x 10^{12} vg/body) injected neonatal MLD mice and 50% (2/4) of dsAAV9/ASA (1 x 10^{13} vg/body) injected adult MLD mice exhibited liver or lung tumors. Moreover, 100%(8/8) of ssAAV9/GFP (2 x 10^{12} vg/body) injected control neonatal and 75% (3/4) of dsAAV9/GFP (1 x 10^{13} vg/body) adult MLDmice also exhibited liver tumors. On the other hand, only 11% (2/18) of non-injected MLD mice exhibited liver tumors. Small amount of dsAAV9/ASA vector (2 x 10^{11} vg/body) injection result in decrease the tumor formation (50%; 10/20). These data indicate that systemic administrationof AAV vector has a possibility of tumorigenesis in both neonatal and adult mice. Since the efficiency of tumor formation is depending on the dose of AAV vector, it important to decrease the vector doses to treat by systemic administration of AAV vectors.

271. Lipid Nanoparticle mRNA Therapy Improves Survival in a Mouse Model of Classic Maple Syrup Urine Disease

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Deficiency of the branched-chain alpha-keto acid dehydrogenase (BCKDH) enzyme complex results in a rare metabolic disorder called maple syrup urine disease (MSUD). Similar to other metabolic diseases, MSUD can range in severity. The neonatal onset seen in the classic form of MSUD is the most severe form of the disease. Current treatment options for MSUD are limited, and patients must typically follow a carefully monitored, restricted diet, with the potential for undergoing liver transplantation. Based on our previous success in using lipid nanoparticle (LNP) mRNA therapy in a mouse model of a less severe, intermediate form of MSUD, we evaluated the same treatment approach in a mouse model of classic MSUD. The classic MSUD mouse model is deficient in the mouse E2 subunit of BCKDH and demonstrates a lethal neonatal phenotype; in this case, knockout (KO) mice do not survive past the second day of life. We evaluated the use of LNPs to deliver mRNA encoding three BCKDH subunits (E1α/E1β/E2) in E2 KO mice. We administered newborn mice with LNP-encapsulated mRNA on days 0 and 3 of life. Starting on day 7, mice received weekly or biweekly intravenous administrations of LNPs. This treatment extended survival in this severe model of classic MSUD to a mean survival of 5 days in a group of 22 treated E2 KO mice. Some mice survived to 11, 13, 15, and 40 days. Therefore, we conclude that LNP mRNA therapy may be a treatment option for both classic and intermediate forms of MSUD.

272. Morquio Syndrome Type A Treatment with Non-Viral Vector

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Background: Morquio A (mucopolysaccharidosis IVA) is a rare congenital metabolic disease caused by the deficiency of the lysosomal enzyme, galactosamine 6 sulfatase. This pathology mainly affects the skeletal muscle system, causing an unsuitable development in the entire bone musculature during growth (skeletal dysplasia), affecting also other systems such as visual, auditory, cardiovascular and respiratory. Pulmonary compromise is usually the main reason for mortality of these patients. The drug currently used to treat this pathology is the Elosulfase alpha, route of administration is by parenteral perfusion. The main limitations of this therapy are the high doses that are required to exert the clinical effect (2 mg/Kg), its low effectiveness and the immunogenicity generated over time, the main reason to interrupt the enzyme replacement therapy. In this work, the enzyme was immobilized in nanostructured lipid systems in order to increase the efficacy. **Materials and Methods:** Double emulsion process w / o / w, in the first emulsion w / o the enzyme is protected by a biodegradable gel and the lipid part is formed by a mixture of different lipids, combined short, medium and long chain lipids. The second w / o / w emulsion includes stabilizing components for the lipid surface and polyethylene glycol structures. The nanostructure lipid carriers (NLCs) were obtained by ultracentrifugation, subsequently lyophilized

with the help of cryoprotectants. **Results and Discussion**: Once the formulation was characterized, internalization studies were carried out on chondrocyte cells (cell line TC28a2), where different techniques were used, proteomic techniques through 1D electrophoresis gels, the bands obtained were sequenced through the MALDI-TOF/TOF for protein identification, enzymatic activity techniques and imaging techniques, confocal microscopy and electron microscopy. Subsequently, the proteomic techniques to study fibroblast pathological cells of patients Morquio A, the metabolic pathway is reconstituted once the drug was internalized with the NLC, reaching the lysosomes (TEM), the reduction of GAG within the cells is also studied.Studies in tissues from healthy donors and in tissues from diseased patients demonstrate their internalization in the cells and even if the pharmacological effect is improved by applying a dose 2000 times lower, it also increases the cellular enzymatic activity.

Cardiovascular and Pulmonary Diseases

273. Cardiac Direct Reprogramming Gene Therapy for Ischemic Injury

Laura M. Lombardi, Huanyu Zhou, Christopher A. Reid, Jin Yang, Elena C. Leon, Tawny Neal Qureshi, Chetan Srinath, Emily R. Nettesheim, Aliya Zeng, Emma Xu, Zoe Duclos, Jun Liu, Samantha Jones, Charles Feathers, Tae Won Chung, Neshel Getuiza, Frank Jing, William S. Prince, JianMin Lin, Timothy Hoey, Kathryn N. Ivey

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Heart failure is a major cause of death, with an estimated prevalence of 38 million patients worldwide. Although many treatment strategies have been proposed, the inability of cardiomyocytes to regenerate has been the main therapeutic roadblock. Direct cardiac reprogramming has the ability to convert non-myocytes into working myocardial cells, thereby generating induced cardiomyocytes at the site of injury. Cardiac reprogramming has been shown to improve cardiac function after myocardial infarction (MI) in rodents, however reprogramming of human cells has proven much more complex. We have successfully developed a first-in-class single vector for the advancement of human direct cardiac reprogramming into the clinic using an optimized human reprogramming cocktail, cassette and viral capsid. We achieved an optimized reprogramming cocktail comprised of three factors by screening and multiple rounds of cassette engineering to maximize the conversion of human cardiac fibroblasts (hCFs) into cardiomyocytes (CMs), *in vitro*. Additionally, viral capsid engineering increased hCFs transduction efficiency, such that our lead candidate efficiently reprograms hCFs, *in vitro*, yielding cells that exhibit calcium transients and express CM-specific genes. Critically, our lead candidate also infects and converts resident non-myocytes into CMs *in vivo*, improving cardiac function in rodent MI models. The clinical cassette has been further improved to selectively express the cocktail components for the greatest product safety. Current and future studies will assess the optimal timing of viral vector delivery, route of administration, and dose-dependent activity in various animal models.

274. CRISPR-Based Transcriptional Activation of the IL-10 Gene in the Donor Lung for Transplantation

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Introduction: Lung transplantation (LTx) is the only life-saving treatment for end-stage respiratory failure. One of the major issues in LTx has been a low utilization of donor lungs; currently only 20% of donated lungs have been transplanted. This problem stems from lung injury and inflammation in the donor lungs. We aspire to address this issue by modulating characteristics of the donor lung as a whole organ using gene therapy. The progress of CRISPR/Cas9 technology has made it possible to manipulate gene expression by targeting the genome in a highly flexible manner. Besides, the development of ex vivo lung perfusion has strengthened the potential of gene therapy by providing the donor lung with physiological conditions and has made this a clinically relevant treatment. These advances have motivated us to optimize donor lungs for transplant by inducing beneficial features through CRISPR/Cas9-based gene therapy. In this study, we propose to develop a CRISPR/Cas9-based anti-inflammatory gene therapy for LTx. To prove the concept, we aimed to achieve targeted activation of the IL-10 gene, a potent anti-inflammatory cytokine, in rat lungs via trans-airway administration of adenoviral vectors. **Methods**: The adenoviral vector expressing Cas9 activator (dSaCas9-VPR) and either one or two effective guide RNAs (the single gRNA group and two gRNA group, respectively), were assessed for the IL-10 gene activation in vitro in comparison with the no gRNA group (the adenovirus expressing Cas9 activator alone). To investigate the targeted IL-10 gene activation in the lung, Lewis rats were divided into three groups: the single gRNA group, the two gRNA group, and the diluent group (buffer without virus). They were then administered adenoviruses via airway at a dose of 1E8 PFU/rat (Fig B). Immunosuppression simulating clinical LTx was applied pre- and post-viral administration to reduce vector-induced inflammation. Treated lungs were analyzed after 72 hours for the IL-10 gene activation, lung function, and inflammation. **Results**: A time-dependent increase of the rat IL-10 protein in cell culture supernatant was observed in both the single and two gRNA groups (Fig A). The two gRNA virus induced potent activation compared to the single gRNA group (76300±1273 pg/ml and 3290±311 pg/ml at 48 hours, respectively). The IL-10 protein in the no gRNA group stayed undetectable over time. In the rat study, the two gRNA group showed increased expression of the rat IL-10 gene compared to the diluent group (relative expression: 17.5±9.2 vs 1.2±1.0, p=0.0008), whereas the single gRNA group showed no increase (3.0±0.8, p>0.1) (Fig C). There was no significant increase in the expression of TNFa, IL-6, and IL-1b, which represent inflammatory cytokines, in the two gRNA group compared to the diluent group. Lungs in all groups showed P/F ratios of over 450 and consistent Wet/Dry ratios without significant macroscopic change (Fig D), demonstrating that the treated lungs had minimal vector-induced inflammation. **Conclusion**: We have achieved targeted activation of the IL-10 gene using CRISPR/Cas9-based transcriptional modulation in rat lungs. The treated lungs were considered acceptable

for transplant, lacking major signs of vector-induced inflammation. This progress will provide a framework for our goal of optimizing donor organs to deliver more lungs to patients.

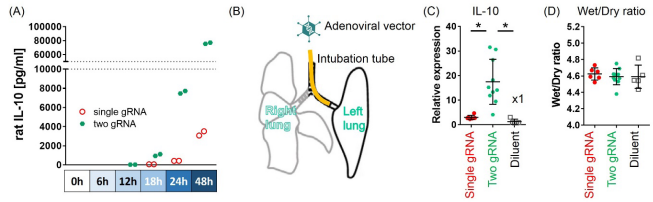

Figure. Targeted activation of the endogenous IL-10 gene via CRISPR/Cas9-based transcriptional modulation in vitro and in the rat lung in vivo. (A) Rat lung epithelial cell line was transduced with adenovirus expressing Cas9 activator with one or two gRNAs designed to activate the IL-10 gene in the rat genome. The supernatant was sampled at each timepoint and processed to measure the rat IL-10 protein using ELISA. (B) Adenoviral vectors were administered to the rat lung through airway. (C) The expression of the rat IL-10 gene in the lung tissue was measured using qPCR. (D) Wet/Dry ratio of the treated lungs showed no significances between groups.

275. Abstract Withdrawn

276. Assessment of AAV Tropisms for Patient-Derived Airway Organoids

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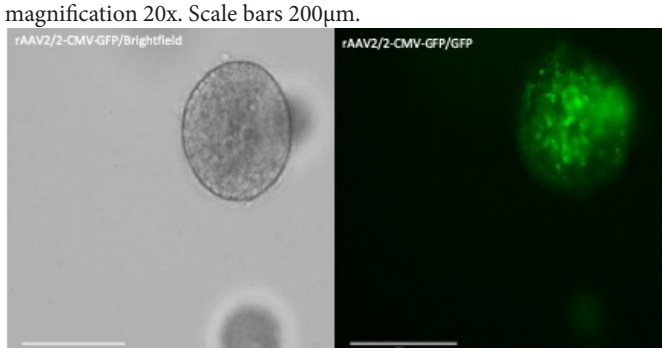
Airway epithelium plays a critical role in preserving lung homeostasis, including maintaining the air conduit and regulating immune responses against pathogens. Dysfunction of the epithelium leads to debilitating conditions such as cystic fibrosis, COPD, ciliary dyskinesia, and asthma. Development of gene therapy vectors targeting the major airway cell types, such as ciliated cells or progenitor basal cells, will be valuable in treating such lung diseases. Given the continually emerging varieties of AAV serotypes for cell-type targeting, a human-relevant airway *in vitro* model that allows cost-effective, high throughput screening to identify candidate vectors would be highly beneficial. Recently, a 3D culture model that forms airway organoids from patient resected tissue was established, where progenitor basal cells self-organize into properly differentiated and functional airway cell types recapitulating key features of the airway epithelium (Sachs *et al.* (2019), EMBO J, 38:4). Our aim is to utilise this *in vitro* model to elucidate recombinant AAV transduction profile and assess the airway cell tropisms of various AAV serotypes in patient-derived airway organoids. Three human airway organoid lines were generated using resected human lung tissues from three donors. Characterization of these airway organoids for cell-specific mRNA markers using RT-qPCR (0.1 ng cDNA input) showed marker levels (n=3, mean ± SD) for basal cells (KRT5) at 0.78 ± 0.013 fold, ciliated cells (FOXJ1) at 0.90 ± 0.016 fold, club cells (SCGB1A1) at 0.64 ± 0.002 fold, and goblet cells (MUC5AC) at 0.89 ± 0.016 fold, relative to the HPRT endogenous control, respectively. Immunohistochemistry using protein markers was used to further validate the presence of basal (KRT5), ciliated (β-tubulin), club (SCGB1A1), and goblet (MUC5AC) cells. Our findings correlate with the observations of others when generating stable airway organoid cultures. Importantly, beating cilia and mucus were observed as early as day 12 after each passage of the organoids. Organoids (n = >35), with an average diameter of 200μm, were microinjected with AAV2/2 vectors expressing the eGFP transgene from the CMV promoter at an approximate MOI of 10,000 (total of 3.75E9 Genome Copies). After 48 hours, eGFP fluorescence was detected (Figure 1) indicating that these organoids were successfully transduce. FACS analysis of the airway organoid cells transduced with various AAV serotypes is underway to assess cell-specific vector tropisms. Findings from these studies will inform the utility of these patient-derived organoids to identify candidate AAV serotypes for efficient, airway cell-specific targeting and the utility of airway cell-specific regulatory elements to improve basic and translational understanding of AAV-based gene therapy in the human airway epithelium.

Figure 1. Representative images of live rAAV transduced-organoids taken under the brightfield and GFP channels. Microscope magnification 20x. Scale bars 200μm.



277. Lentiviral Vector Pseudotyped with Sendai Virus F and HN Proteins Uses Sialylated Glycan Receptors to Efficiently Target Human Airway Cells

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We are developing a lentiviral vector pseudotyped with the F and HN coat proteins from Sendai virus (Murine Respirovirus) in order to target lung cells. The F/HN pseudotype was shown to facilitate efficient transduction of a range of cells in the murine lung (Alton et al. 2017 Thorax 72:137). Whereas Sendai virus receptor targeting utilises various subtypes of ubiquitous sialylated glycans, it is not yet known how well this targeting will translate to the human lung, due to differences in the distribution of these subtypes between species. In collaboration with Oxford Biomedica, two alternative influenza-based pseudotypes, predicted to differ in sialylated glycan subtype targeting, have been developed for comparison with F/HN. Here, we investigate transduction efficiency and cell type targeting by pseudotyped HIV-based vectors in human airway air-liquid interface (hALI) cultures derived from human Bronchial Epithelial Cells (hBEC) to assess dependency on sialylated glycan receptors. Human ALI cultures were treated 6-8 weeks post airlift with equivalent doses of recombinant HIV vectors expressing eGFP, pseudotyped with glycoproteins from Sendai virus (F/HN), influenza virus (HA/NA), or Vesicular Stomatitis Virus (VSVg) as a negative control. After 14 days, transduced hALI cultures were imaged and the percentage area of eGFP fluorescence above background calculated. At a dose of 7.5e7 Transducing Units (TU) the F/HN pseudotype resulted in high levels

of eGFP (18.5 - 51.6% area eGFP; n=4) whereas VSVg pseudotyped vector showed no transduction (0-0.8% area eGFP; n=4), presumably due to lack of access to basolateral VSVg receptors. The F/HN vector was significantly more efficient at transducing hALI cultures (7.5e6 TU; n=4) than either of the HA/NA influenza-based pseudotypes tested (Kruskal-Wallis, F/HN vs HA/NA, P=0.0333 and p<0.0001), with stable EGFP expression persisting for more than 4 months. Specific cell-type targeting was determined in hALI cryosections with colocalization of native eGFP fluorescence and antibodies against β-Tubulin (ciliated cells), Mucin 5Ac (goblet cells), and Cytokeratin 5 (basal cells); the F/HN, and both HA/NA Influenza pseudotypes, were confirmed to target all three cell types. Consistent with previous published literature, no club cells (Uteroglobin antibody) were detected in the hALI cultures derived from hBEC (n=4). Lectins specific for α2,3 and α2,6 subtypes of glycan sialylation were used to stain for the receptor distribution in hALI cultures, revealing an abundant distribution of α2,6 (and a subtype of α2,3) in hALI cultures (n=4), representative of the distribution in human airways described in the literature. To confirm that F/HN and HA/NA pseudotyped vector cell entry is dependent on sialylated glycans, hALI cultures were pre-treated with sialidase to cleave sialic acid, prior to vector administration. Transduction, quantified by native eGFP fluorescence, was significantly reduced for all F/HN and HA/NA vectors (Kruskal-Wallis, Sialidase pre-treatment vs no pre-treatment, p<0.0001 (F/HN), P=0.0194 and P=0.0022 (HA/NA); n=4). These data confirm the distribution and importance of sialylated glycans in the efficient transduction of airway cells, and studies are underway to characterise the specific sialylated glycans responsible for targeting of hALI cultures. These data should allow us to predict whether these pseudotyped vectors are likely to be efficient in the human airways during clinical studies.

278. Amelioration of Lipid Profile in LDLR-Deficient Mice after Intramuscular Administration of a HD-AD Vector Expressing a LDLR/TF Chimeric Protein

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Familial hypercholesterolemia is an inherited disorder mainly due to mutations in the LDL receptor gene, characterized by premature onset of cardiovascular disease due to high plasma LDL. Homozygous FH patients do not always respond to conventional therapies and have often a poor prognosis. Therefore, more effective therapeutic strategies, such as gene therapy, are still of main interest. We developed a safe and effective gene therapy strategy based on liver expression of a secreted chimeric protein composed of the extracellular portion of the LDLR linked to a transferrin dimer using a helper-dependent adenoviral (HD-Ad) vector; this chimeric protein binds LDL and removes them from the bloodstream through the transferrin receptor (TfR). Intravenous administration of this HD-Ad vector ameliorated the lipid profile and a reduced aortic atherosclerosis in LDLR-deficient mice. To improve this strategy, we generated a HD-Ad vector for a muscle-restricted expression of the mLDLR/mTF chimeric protein using a muscle-specific promoter and intramuscular administration of the vector. We observed expression of the chimeric protein in vitro after infection of myoblast C2C12 cells with our vector and an amelioration of lipid profile after intramuscular administration in LDLR-deficient mice. In summary, we developed an innovative strategy for FH therapy based on the expression of a secreted chimeric protein after intramuscular administration of an HD-Ad vector. This approach reduces risks due to systemic administration of viral vectors and is applicable to other genetic diseases; collection of additional efficacy and safety data will further define its applicability in clinical settings.

279. Efficacy of Cardiac Reprogramming via Gene Therapy in Rat with Chronic Heart Failure

Jin Yang, Aliya Zeng, Laura Lombardi, Huanyu Zhou, Christopher Reid, Emma Xu, Samir Elmojahid, Elena Leon, Tawny Qureshi, Chetan Srinath, Emily Nettesheim, Zoë Duclos, Jun Liu, Samantha Jones, Charles Feathers, Tae Won Chung, Frank Jing, William Prince, Kathryn Ivey, Timothy Hoey, JianMin Lin

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Myocardial infarction (MI) results in ischemic cell death of heart muscle cells caused by prolonged inadequate supply of oxygen-rich blood to the heart. Current therapies for heart failure after MI are limited and non-curative. In comparison to treatment immediately post-MI, there is a greater unmet medical need for patients with severe reduced ejection fraction (HFrEF) at the chronic stage post-MI. We have developed a reprogramming therapeutic in a single AAV expressing cardiac reprogramming factors that enables conversion of cardiac fibroblasts into functioning myocardial cells. In murine acute MI models, delivery of viral vectors at the injury site at the time of MI has shown improved cardiac function. It is of interest to determine whether the reprogramming approach can be efficacious in the setting of chronic MI where viral vector delivery occurs later, after the period of fibroblast proliferation and fibrotic scar formation. In these studies, we established an MI model in rats and assessed AAV vector delivery at various times after MI. The optimized model allows direct intramyocardial injections at the infarct region at later time points after the acute stage. In this model, the injected AAV vector mediates expression in cardiac fibroblasts in the ischemic zone. AAV-mediated gene delivery of the reprograming cocktail significantly improved LV function when delivered fourteen days after cardiac infarct. There were no test-article related arrhythmias or other safety findings observed in the efficacious dose range. Currently, we are evaluating our lead reprogramming therapeutics in the rat chronic MI setting and aiming to advance this approach to clinical studies.

280. Engineering Novel rAAV Vectors with Enhanced Cardiac Tropism

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Gene therapy is an emerging treatment option for both acquired and inherited cardiac disorders. Particularly, recombinant adeno-associated virus (rAAV) serotype 9 has become the workhorse vector for gene transfer to cardiomyocytes due to its ability to transduce the heart following systemic delivery. While AAV9 can achieve moderate transduction of the heart, the majority of vector traffics to the liver. Moreover, in order to achieve therapeutic levels of transduction in the heart, high systemic doses are required, potentially leading to systemic inflammation and in turn, toxicity. Thus, the development of an AAV capsid with improved cardiac tropism is critical for safe and efficacious cardiac gene therapy. We has developed a proprietary capsid evolution platform generating highly diverse rAAV capsid libraries with over 100 million unique variants. Using Tenaya's custom-made libraries, we performed multiple rounds of directed evolution in human pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and in a murine animal model in order to identify variants with enhanced cardiomyocyte tropism. Evolved variants displayed significantly improved cardiac transduction and decreased liver tropism compared to AAV9 when systemically delivered in rodents. Importantly, top performing novel variants demonstrated similar or improved neutralizing antibody evasion compared to AAV9. Together, these data support the use of our novel rAAV vectors for cardiac gene therapy applications.

281. Abstract Withdrawn

282. *In Vitro* Pharmacology of KB407, an HSV-1-Based Gene Therapy Vector, for the Treatment of Cystic Fibrosis

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Cystic fibrosis (CF), the most common inherited genetic disorder in the United States, is caused by mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR). In recent years, drug development has largely focused on small molecule modulators of specific CFTR dysfunctions, thereby treating only a subset of the CF population harboring the mutations responsive to these drugs. Unfortunately, an effective monotherapy targeting molecular correction of the genetic defect underlying CF, and thus, appropriate for the treatment of all CFTR mutations, remains lacking. To this end, we have engineered KB407, a replication-defective HSV-1 gene therapy vector encoding full-length human CFTR for the treatment of CF. Here, we establish that KB407 possesses a robust safety and efficacy profile in multiple relevant *in vitro* CF models. KB407 efficiently transduced *CFTR*-deficient human small airway epithelial cells and produced functional CFTR, as assessed by qRT-PCR, western blot, immunofluorescence, and rhodamine 6G dye uptake analyses. Furthermore, KB407-mediated functional restoration of CFTR was demonstrated in clinically relevant 3D patient-derived intestinal organoids (PDOs) cultured from four CF patients (two distinct *CFTR* nonsense mutations, two F508del mutations). Transduction with KB407, even at low doses, rescued the cystic phenotype in treated PDOs to levels at or above positive control small molecule intervention. Taken together, these data indicate that KB407 capably infects relevant airway epithelia, efficiently produces functional human CFTR, and molecularly corrects multiple *CFTR* defects, supporting application of KB407 as a novel, broadly applicable gene therapy for the treatment of CF.

283. Codon Optimization of Cystic Fibrosis Transmembrane Conductance Regulator to Enhance Therapeutic Gene Expression

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Cystic fibrosis (CF) is a common, life-shortening disease caused by mutations in cystic fibrosis transmembrane conductance regulator (*CFTR)*, which encodes an anion channel. CFTR is expressed in many organ systems, but respiratory complications are the most common cause of death. Recent advances in treatments include small molecules that rescue protein function. Unfortunately, these are mutation-class specific and are ineffective for some mutations and not all patients tolerated the medications. Thus, the need remains to develop mutation agnostic treatments, such as *CFTR* gene addition. Lentiviral vectors are attractive for this purpose because they can accommodate large genes like *CFTR*. They transduce non-dividing cells, and they integrate into the host's genome, providing long-term expression. Previous proof of principle studies demonstrated that lentiviral delivery of *CFTR* to the airways of CF pigs partially rescued phenotypic defects including Cl^- transport, airway surface liquid (ASL) pH, and bacterial killing ability. In order to advance lentiviral gene therapy and achieve full correction of CF phenotypes in the airways, we investigated the effects of *CFTR* codon optimization on transgene expression and function. Since airway epithelial cells are interconnected through gap junctions, we hypothesized that a small number of cells expressing high levels of CFTR could perform sufficient anion transport to rescue CF phenotypes. To test the therapeutic potential of *CFTR* codon optimization, we first compared three different *CFTR* cDNA sequences in a model cell line. Each codon optimized *CFTR* (co*CFTR*) sequence was cloned into the pcDNA3.1 expression vector and electroporated into Fischer rat thyroid (FRT) cells. FRT cells do not express endogenous CFTR and can form a polarized epithelium. One week later we measured transepithelial Cl^- transport and found that each codon optimized sequence provided a different degree of improved *CFTR* expression and function as evidenced by Cl^- transepithelial transport. To confirm this was not a cell line specific effect, we also measured mRNA and protein production in transfected HEK293 cells. Based on results from these cell line experiments we selected the *coCFTR* sequence that resulted in the highest increase in transepithelial Cl^- current compared to WT CFTR and tested it in CF primary human airway epithelial cells using lentiviral vectors. We observed a dose-dependent increase in transepithelial Cl^- transport with coCFTR compared to WT

CFTR at all vector doses tested. In future experiments we plan to test the therapeutic potential of these vectors *in vivo* in CF animal models. Our findings suggest that codon optimization of *CFTR* could be used to increase protein expression, and function. This may provide a useful reagent for developing a gene therapy vector that would benefit all *CFTR* mutations.

284. A Human Air-Liquid Interface Cell Culture Model for Surfactant B Deficiency

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Surfactant protein B (SP-B) deficiency is a severe, rare genetic interstitial lung disease which causes alveolar collapse and respiratory distress syndrome in newborn babies. The majority of cases result in loss of life within the first few months due to the unstable disease conditions, lack of curative therapy and donor organs for lung transplantation. Correction of this genetic defect has been reported using non-viral gene therapy applications in mouse models of this disease, but the therapy is transient and relatively inefficient. We propose to utilize our recombinant lentiviral gene therapy platform based on simian immunodeficiency virus (SIV) pseudotyped with Sendai virus glycoproteins F and HN (rSIV.F/HN) (Thorax 2017;**72**:137-147) to express secreted SP-B in the alveoli to prevent lung collapse and increase survival. However, there is a lack of a well-characterized and physiologically relevant *in vitro* models of human lung parenchyma, which is a crucial limitation in the development and assessment of advanced therapy medicinal products for SP-B deficiency. Here, we selected two human lung adenocarcinoma cell lines, A549 and H441, to investigate their potential to serve as an *in vitro* model for surfactant deficiencies. We successfully established a human surfactant air-liquid interface (hSALI) culture model based on H441 cells to recapitulate the key characteristics of human alveolar cells in primary culture. In order to establish hSALI cultures, H441 cells were grown in transwells with culture media supplemented with dexamethasone and insulin-transferrin-selenium. Seven days post-airlift (i.e. removal of culture media from the apical side of the transwells) hSALI cultures were able to develop pseudostratified cellular layers (as evidenced by immunofluorescence staining of fixed-frozen sections) with functional barrier properties (transepithelial electrical resistance 249.3±25.1Ωcm^2 and potential difference 1.35±0.13mV). The electrical resistance and potential difference were stable up to at least 28 days post airlift. Formation of tight junctions was also confirmed via immunocytochemistry against junction proteins such as E-cadherin, zonula occludens-1, and claudin 4. Furthermore, expression of surfactant proteins A, B, and C, a unique characteristic of alveolar type II cells, was observed. RT-qPCR analyses demonstrated a 10,000-fold increase in SP-A and SP-B RNA levels and a 100-fold increase in SP-C RNA in hSALI cultures compared to that of H441 cells grown in submerged culture. In addition, expression of SP-B and SP-C proteins were confirmed via immunoblotting and immunocytochemistry. We anticipate that human SALI cultures could serve as an important *in vitro* model for research into the human alveolar epithelium, especially in the areas of pediatric genetic disorders and in the future testing of inhalation biopharmaceuticals. Further work is being undertaken regarding generation of a human *SFTPB* knockout model and investigation of resulting phenotypic changes in hSALI cultures.

285. Somatic Genome Editing in Murine Lung via AAV Delivery of CRISPR

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We are developing a somatic genome editing platform via AAV delivery of CRISPR into murine lungs to facilitate rapid generation of mouse models to study lung biology. We identified that AAV6 can transduce alveolar type II (AECII) cells at a very high efficiency and distal airway epithelial cells at a moderate efficiency. We next evaluated *in vivo* editing efficiency in these cell types in Rosa-LSL-tdTomato reporter mice using SaCas9/gRNA system delivered via an all-in-one AAV vector. Moderate editing efficiency of LSL sites was observed. We also evaluated the *in vivo*editing efficiency of SpCas9/gRNA system delivered via dual AAV vectors to Rosa-LSL-tdTomato mice, in comparison to AAV delivery of the same gRNAs to SpCas9 transgenic mice. Whereas no detectable editing of gene of interest (Gene X) was observed in AECII cells in Rosa-LSL-tdTomato mice, high editing efficiency was achieved in SpCas9 transgenic mice as determined by significant protein knockdown (kd) of Gene X. The result indicated that, in non-SpCas9 animal models, SpCas9 expression plays the key role in determining the somatic editing efficiency. A split-cas9 system delivered by self-complementary AAV is explored to enhance the SpCas9 expression level in AECII cells to achieve a higher editing efficiency in non-SpCas9 animal models. Our study thus has established a somatic editing platform for highly efficient gene kd in SpCas9 transgenic murine lungs, which can facilitate the investigation of the biological roles of the gene of interest *in vivo*.

Neurologic Diseases

286. CRISPR/Cas9-Mediated Allele Specific Disruption of *PSEN1* Carrying the M146L Mutation

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Objectives Mutations in the presenilin 1 gene (*PSEN1*) cause early-onset forms of familial Alzheimer's disease (AD). Carriers of the *PSEN1*^{M146L} mutation display increased generation of the more aggregation-prone amyloid beta form with 42 amino acids

(Abeta42) and thereby a simultaneous increase in the Abeta42/Abeta40 ratio. These alterations are evident not only in the brain, but also in peripheral cells, such as fibroblasts. Our objective was to evaluate, on fibroblasts from AD mutation-carriers, if the CRISPR/Cas9 system could effectively disrupt the mutated allele whilst leaving the wild-type allele intact. Apart from investigating if the physiological cellular ratio of Abeta42/Abeta40 could be normalized, we also wanted to evaluate if CRISPR/Cas9 could restore mutation-associated conformational changes in the presenilin 1 protein complex. **Methods** Human fibroblasts from AD patients heterozygous for the *PSEN1*^{M146L} mutation were transfected with a plasmid expressing PSEN^{M146L} guide RNA and Cas9 protein via nucleofection. Abeta40 and Abeta 42 levels were measured using ELISA. Editing efficiency was assessed through Sanger and Next Generation sequencing and the CRISPResso2 software. Presenilin 1 complex conformation was evaluated via Förster resonance energy transfer by fluorescence lifetime imaging (FRET-FLIM). **Results** Through CRISPResso2, we found robust intel formation in the DNA from PSEN1^{M146L} guideRNA+Cas9 treated human *PSEN1*$^{M146L/WT}$ fibroblasts, but not in PSEN1$^{WT/WT}$ fibroblasts. No indels were formed upon treatment with control vectors. The induced disruption of the mutated allele in the targeted fibroblasts was paralleled by a significant reduction in the Abeta42/Abeta40 ratio. Possible CRISPR/Cas9-related effects on the presenilin 1 protein conformation are currently being assessed. **Conclusions** By utilizing the CRISPR/Cas9 system, we could demonstrate a selective disruption of the mutated *PSEN1*^{M146L} allele and a concomitant shift in the Abeta42/Abeta40 ratio in human AD patient fibroblasts. We believe that this system has the potential to be developed as a tool for future gene therapy against certain forms of familial AD.

287. Gene Suppressing Therapy for Pelizaeus-Merzbacher Disease Using AAV Harboring *PLP1*-Targeting Artificial miRNA

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Copy number increase or decrease of certain dosage-sensitive genes may cause genetic diseases with distinct phenotypes, conceptually termed genomic disorders. The most common cause of Pelizaeus-Merzbacher disease (PMD), an X-linked hypomyelinating leukodystrophy, is genomic duplication encompassing the entire proteolipid protein 1 (*PLP1*) gene. Although the exact molecular and cellular mechanisms underlying *PLP1* duplication, which causes severe hypomyelination in the central nervous system, remain largely elusive, *PLP1* overexpression is likely the fundamental cause of this devastating disease. Here, we investigated if adeno-associated virus (AAV)-mediated gene-specific suppression may serve as a potential cure for PMD by correcting quantitative aberrations in gene products. We developed an oligodendrocyte-specific *Plp1* gene suppression therapy using artificial miRNA under the control of human CNP promoter in a self-complementary AAV (scAAV) platform. A single direct brain injection achieved widespread oligodendrocyte-specific *Plp1* suppression in the white matter of wild-type (WT) mice. AAV treatment in *Plp1* transgenic mice, a *PLP1* duplication model, ameliorated cytoplasmic accumulation of Plp1, preserved mature oligodendrocytes from degradation, restored myelin structure and gene expression, and improved survival and neurological phenotypes. In addition, we verified that the AAV delivery by intraventricular injection and ultrasound-targeted microbubble destruction enhances the gene delivery in our mice model. Together, we provide evidence that AAV-mediated gene suppression therapy can serve as a potential cure for PMD resulting from *PLP1* duplication and possibly for other genomic disorders. For future clinical application, development of delivery systems with broader distribution may lead to better therapeutic outcome.

288. An Experimental Animal Model of Bietti Crystalline Dystrophy

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Purpose: Bietti crystalline dystrophy (BCD) is an autosomal recessive inherited retinal disease caused by mutation cytochrome P450, family 4, subfamily V, member 2 (CYP4V2) gene and the etiology and development mechanism of this disease is still unclear. The CYP4V3 gene is the mouse ortholog of human CYP4V2. We established CYP4V3$^{-/-}$ mice as an experimental animal model for exploring the pathogenesis of BCD. Methods: The CYP4V3$^{-/-}$ mice were generated by CRISPR/Cas9 technology. The morphologic and functional characteristics of eye fundus were assessed by fundus imaging, optical coherence tomography (OCT), full-field electroretinogram (ERG). Hematoxylin-eosin staining and immunofluorescence were used to investigate the histologic features. Results: The CYP4V3$^{-/-}$ mice are viable and fertile and have no obvious physiological defects. In addition, this model displayed age-related progression of BCD that mimics the human ocular phenotype. The fundus photographs of CYP4V3$^{-/-}$ mice showed crystalline scattered over the fundus and the crystalline became more and more obvious with age. OCT demonstrated atrophy of the outer retinal layers and ERG showed no response at the age of 12 months. The photoreceptor damage, retinal pigment epithelium (RPE) cells atrophy and inflammatory activation of the fundus was displayed by the histologic analysis. Due to the slow disease progression, we carried out the light damage and got significant effects. Conclusion: We constructed this CYP4V3-/- mice as a preclinical model for BCD through a long-term follow-up study on the natural course of the eye characteristics. And this model may be used for providing significant research basis and new ideas for the treatment of BCD.

289. C9ORF72 Variant-Specific RNA Interference Rescues C9-ALS/FTD Molecular Hallmarks *In Vivo* and *In Vitro*

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Amyotrophic Lateral Sclerosis (ALS) is a terminal neurodegenerative disease that affects upper and lower motor neurons, causing progressive muscle weakening and respiratory failure. A hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{30+}$ in the intronic region of C9ORF72, contributes to 40% of familial and 10% of total ALS cases. Up to 50% of patients with this expansion also develop Frontotemporal dementia (FTD). Several hypotheses have emerged to explain the mechanism behind this HRE, including haploinsufficiency, RNA binding proteins sequestration in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide production. Both C9ORF72 ALS and FTD are aggressive diseases with no treatments to significantly slow disease progression or extend life expectancy. We are developing an AAV-RNAi gene therapy approach, using artificial microRNAs packaged in AAVrh10, to specifically target C9ORF72 mRNA variants that contain the HRE (variant (V) 1 and V3). Hereby we preserve the most abundant variant, V2, which does not have the HRE, in order to avoid haploinsufficiency. We found significantly lower levels of V1 in treated primary neurons of C9ORF72 BAC-transgenic (Ranum) mice compared to untreated groups, while V2 levels remained unaffected. We next treated adult C9ORF72 BAC-transgenic mice through striatal injections and found also a decrease in V1 mRNA containing the HRE, but not in V2 which does not contain the HRE, in the striatum. Future experiments will test the efficacy of this therapeutic approach to prevent the formation of toxic dipeptides and RNA foci formation and eliminate existing toxic dipeptides and RNA foci, to ultimately rescue motor neuron pathology in ALS.

290. Abstract Withdrawn

291. Therapeutic Genome Editing Approach to Replace Large DNA Sequences in the Murine Abca4 Gene

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Stargardt disease, caused by mutations in the ABCA4 gene, is the most common juvenile maculopathy, leading to an accumulation of toxic degradation products in the retina and underlying RPE. Since the cDNA of the ABCA4 gene exceeds the packaging capacity of a single AAV vector, the development of AAV-based gene addition therapy is hampered. Genome editing represents a therapeutic alternative. By employing highly specific endonucleases, such as the CRISPR-Cas9 system, and a template DNA containing the correct sequence at the target site, this approach has the potential to correct disease causing mutations in a wide variety of disease entities. To test this treatment approach, we aim at inserting a CMV+exon1/2 minigene into the 5' DNA region of the Abca4 KO mouse gene, which lacks parts of the promoter region and exon 1. To develop the strategy, the murine NIH-3T3 cell line was used. In total, 10 gRNA target sites upstream and downstream of the promoter-exon1 region of the murine Abca4 gene were tested for their activity in inducing double strand breaks (DSB) via the Streptococcus progenies (sp)CRISPR-Cas9 system, using a custom-made bioluminescence resonance energy transfer (BRET) based non-homologous enjoining (NHEJ) assay. Template DNA was designed to include the CMV promoter and fused exons 1 and 2, and homologous regions within the promoter region and exon 2, in order to enable in frame integration. A second template DNA contained the CMV promotor, the GFP cDNA in frame with exon 2, in order to enrich/count edited cell by FACS. Four gRNAs showed significant activity at their target sites and were used to test template integration. Use of the reporter gene template resulted in up to 4% of GFP positive cells. Integration of the therapeutic template was verified by PCR covering the border region of the modified zone. Replacing large fragments of DNA in mutant genes might result in the availability of one therapeutic approach targeting a large number of mutations. Efficiency and safety of the approach needs to be confirmed in subsequent in vivo experiments in the Abca4 KO mouse model.

292. Leveraging the Proteasome to Clear Tau with Novel Bifunctional Intrabodies

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In both sporadic and familial cases of Frontal Temporal Dementia (FTD), tau hyperphosphorylation, misfolding, aggregation, and prion-like propagation occur. Many immunotherapies targeting tau focus on halting extracellular secretion and subsequent templated propagation of proteopathic seeds. However, these methods do not prevent intracellular accumulation and aggregation, which can be toxic and is upstream from pathogenic transfer. Our hypothesis is that targeting the degradation of monomeric tau protein through the cell's normal protein clearance pathways will reduce available tau, thus reducing cellular toxicity and neurodegeneration. Using recombinant antibody technologies, we have produced bifunctional intracellular antibody (intrabody) reagents that *(1)* specifically bind tau and prevent its aggregation and *(2)* specifically target tau into the cell's normal protein clearance pathways. To create a bifunctional intrabody that will promote tau degradation, we have taken the novel approach of fusing the intrabody to a proteasomal PEST degron derived from mouse ornithine decarboxylase (ODC) (indicated as 'mPEST'). We engineered 17 human anti-tau-mPEST bifunctional intrabodies selected against tau amino acids 151-441 derived from a non-immunized human phage display library. We screened the initial 17 constructs and identified several that successfully lowered tau inside ST14A cells. Of these, we selected three candidate anti-tau-mPEST intrabodies that reduced GFP-tagged tau levels ~90% (clones 1, 4 and 5), for further study. While these tau lowering bifunctional intrabodies did not show overt signs of toxicity in ST14A cells, it is important to test efficacy in disease relevant human iPSC-derived neurons. 3D organoids provide an excellent model system to test candidate therapeutics including examination of gene therapy transduction levels and performance in a complex cellular setting that resembles *in vivo* aspects of brain

tissue. To ensure PEST degron target engagement in human cells, we co-transduced 2-month old 3D forebrain organoids with lentivirus encoding GFP-R5L-2N4R-tau and anti-tau-PEST intrabodies or empty lentivirus control and verified tau reduction by live confocal time lapse imaging following 21 days of transgene expression. Anti-tau-mPEST intrabodies counteracted morphological signs of *apoptosis* (*cellular* shrinkage, membrane blebbing) compared to empty lentivirus control. Anti-tau-PEST intrabodies (1, 4, and 5) significantly ($p<0.05$) reduced endogenous tau levels compared to untreated control and empty virus control. The long-term safety and efficacy of anti-tau-mPEST intrabodies is currently being evaluated in 2D and 3D cortical neurons derived from patient-specific iPSCs with *MAPT* mutations N279K, V337M, R406W and their respective isogenic controls. These data provide the groundwork to conduct future translational studies *in vivo* using gene therapy approaches.

293. Abstract Withdrawn

294. Analysis of Viral Vector Targeting in the Cochlea after Noise Induced Damage Using Different Delivery Routes

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Noise-induced hearing loss (NIHL) is one of the most prevalent disabilities for which there is no effective therapeutic treatment. Auditory insult via noise exposure damages the sensory elements of the ear, leading to metabolic and/or mechanical damage to hair cells and degeneration of spiral ganglion neurons (SGN). To improve hearing in NIHL patients, the inner hair cell-SGN synapses must be maintained or regenerated. Though effective compounds for the treatment of NIHL have been suggested, challenges in delivery hinder the translation of these therapies to a clinical setting. In this study, we examined the efficacy of different AAV viral vectors and promoters carrying a GFP transgene, administered via different delivery routes, in targeting the IHCs and SGNs. Vector transduction efficacy was compared in the presence or absence of noise injury. To induce auditory injury, mice were exposed to 100 dB sound pressure level (SPL) octave band noise for 2 hours. Five adult mice, both male and female, were used for each injection route and vector. Injections were performed at various time points pre- and post-noise exposure to determine whether noise exposure influenced vector transduction. Age-matched, uninjected mice were processed alongside experimental mice. Mice were sacrificed three weeks post injection and cochleae were assigned a random number. Cochleae were processed and analyzed for GFP expression and intact synapses in samples by immunofluorescence. Our results demonstrate that AAV vectors are effective in targeting cells in both healthy and noise-damaged cochleae via multiple injection routes, at varying timepoints of injection post noise exposure. Using gene therapy techniques, transgenes or therapeutic compounds can be efficiently delivered to the cochlea. Our findings open a novel avenue of therapeutic treatment for auditory injury and cochlear disorders.

295. Abstract Withdrawn

296. Engineered Zinc Finger Protein Transcription Factors Efficiently and Specifically Reduce Mouse and Human Prion Expression

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Prion disease is an invariably fatal neurodegenerative disorder caused by misfolding of the cellular prion protein, PrP, encoded by the *PRNP* gene. Progression is relentless and rapid, with a median time of <12 months between first clinical symptoms and death for most subtypes. While the best-known examples involve transmissible forms acquired through dietary routes, most cases are either sporadic or result from one of a known set of dominant heterozygous mutations in *PRNP*. There are currently no approved therapies or clinical trials to evaluate disease-modifying therapies for the prevention or treatment of prion disease. Abundant preclinical evidence suggests that lowering endogenous PrP levels by 50% either genetically or at the mRNA level (for example using antisense oligonucleotides) can approximately double the lifespan of prion-infected mice. Moreover, *Prnp* null animals are completely resistant to prion inoculation. A single-administration, AAV-based prion lowering strategy has the potential to provide important therapeutic benefits, including the robust, selective, and sustained elimination of neuronal PrP for the lifetime of an at-risk individual. We therefore investigated whether zinc finger protein transcription factors (ZFP-TFs) could be designed to achieve efficient lowering of prion expression. We fused the human KRAB repression domain to a panel of ZFPs (n=192 per species) designed to target regions surrounding either the mouse *Prnp* or human *PRNP* transcriptional regulatory elements. These ZFP-TFs were tested in mouse Neuro2A or human SK-N-MC cells across a 300-fold dose range. We identified dozens of ZFP-TFs capable of reducing prion mRNA levels by 50% to >99% with saturating dose-response profiles. Approximately 36 candidates from each design set were then manufactured as AAV6 and tested for on- and off-target activity in either primary mouse cortical neurons or human iPSC-derived neurons. Several ZFP-TFs lowered prion levels by >99% for up to 32 days in culture, including some that had no detectable off-target activity as assessed by transcriptome-wide specificity profiling. We also screened variants of the parental ZFP-TFs in which key non-specific DNA phosphate contact interactions were eliminated by replacing a conserved arginine residue of the ZFP backbone. Many of the resulting variants maintained their on-target activity while exhibiting substantially improved off-target behavior,

thereby providing an accelerated route to lead candidate identification. These results demonstrate that engineered ZFP-TFs can potently and specifically reduce endogenous mouse and human prion expression. Planned *in vivo* studies to investigate ZFP-TF efficacy in the brain will also be presented.

297. MR Imaging Probes for Non-Invasive Evaluation of Gene Therapy

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Adeno-associated virus (AAV) gene therapy has shown transformative outcomes in treating many enzymatic deficiencies in animal models and preliminary data in patients is promising. However, much of this success has been achieved after dose escalations in patients, where decisions to increase the dose were made after patients treated with lower doses exhibited clinical signs of deterioration. This is in part because there is not a non-invasive way to evaluate transduction and expression of the AAV encoding therapeutic protein. Development of such a technology, could enable rapid dose escalation and potentially redosing of patients before disease progresses. Here we describe an in-vivo magnetic resonance imaging (MRI) probe that non-invasively measures enzymatic activity of a therapeutic protein expressed by AAV in real time. This gadolinium based agent has an exposed arm that is the enzymatic target of the therapeutic, which, upon cleavage, results in MRI signal enhancement. We have previously shown that GM1 mice (β-gal$^{-/-}$) treated with AAV9 encoding β-gal (GLB-1), results in global distribution, with highest β-gal levels in the CNS after intracranial (IT) delivery and in the liver after intravenous (IV) administration. Similarly, IT administration of β-gal-responsive contrast agent, 1 month after IT administration of AAV (thalamus, unilateral, 1E10 vg), resulted in strong MRI signal enhancement in CSF and brain parenchyma (n=1). Kinetics of signal enhancement increased until ~1-1.5 hours, after which it plateaued with a 30% increase in signal. The same pattern of enhancement was present after IT administration of the agent in wild type mouse (n=1), however; the enhancement was absent in the β-gal$^{-/-}$ mouse (n=1). Intraperitoneal administration of the contrast agent in an AAV9 treated β-gal$^{-/-}$ mouse (IV, 3e11 vg, 1 month), resulted in strong enhancement of liver and internal organs (n=1), while a wild type mouse showed mild enhancement (n=1). These preliminary data suggest that the detection limits of this agent extend from ~normal levels of β-gal to the super physiologic levels (~50 fold over normal) achieved after gene therapy. Enzymatic assays using a fluorogenic substrates will be used to correlate the magnitude of contrast enhancement in MR images to further assess sensitivity. This contrast agent can be modified to interact with the active sites of other enzymes and could be extended to other AAV gene therapies, enzyme replacement therapy and/or CRISPR based technologies.

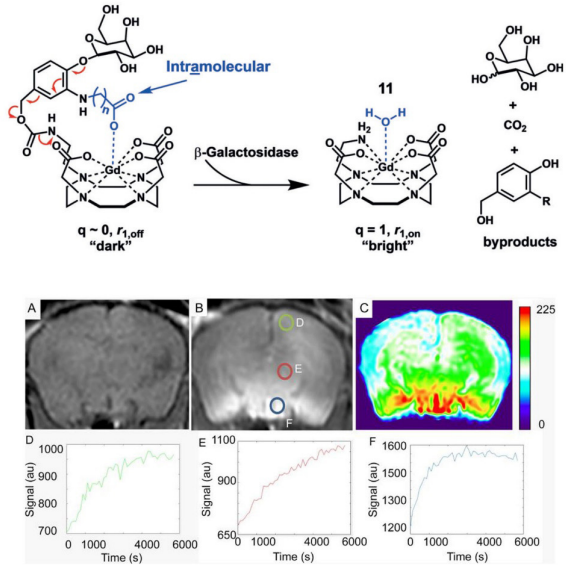

Figure: Top: Activation of *β-gal responsive* contrast agent**. Bottom:** ***Kinetics of signal enhancement by contrast agent.*** Representative brain MR images of an AAV treated β-gal$^{-/-}$ mouse (A) pre-contrast. B) post-contrast image showing areas (D-F) where signal changes were quantified over time. Note scale y-axis is greater for F, because this area has the greatest exposure to CSF backflow after IT injection. (C) Heat map of signal enhancement shows the greatest enhancement in the ventral aspect of the brain.

298. Intra-Cisterna Magna Injection of an AAV Vector with the GLUT1 Promoter Transduces Endothelial Cells and Neurons in Broad Areas of Pig Brain without Toxicity

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Introduction: Glucose transporter 1 deficiency syndrome (GLUT1DS, OMIM #606777) is caused by haplo-insufficiency of *SLC2A1*, the gene encoding GLUT1. Heterozygous mutation of *SLC2A1* results in impaired hexose transport into the brain, leading to irreversible neurologic disorders. Previously, we generated a tyrosine-mutant AAV9/3 vector in which *SLC2A1* was expressed under the control of

the human intrinsic GLUT1 promoter (AAV-GLUT1). AAV-GLUT1 administration improved the motor function and cerebrospinal fluid-glucose levels of heterozygous knock-out murine *Glut1* (GLUT1$^{+/-}$) mice. In preparing for a first-in-human trial, AAV-GLUT1 was injected into the cisterna magna of an infantile hairless pig, and the distribution of exogenous GLUT1 in the central nervous system and off-target effects in other organs were examined.**Methods:** AAV-GLUT1 (1.63×10^{12} vector genomes [vg]/kg, total 4.01×10^{13} vg) was injected into a 5-month-old female hairless pig (weight, 20 kg). A lumbar puncture was performed and the catheter was ascended via the subarachnoid space up to the cisterna magna; then, AAV-GLUT1 was injected slowly into the cisterna magna. At 4 weeks after intrathecal injection, the pig was euthanized and the brain and other organs were removed. Vector genome levels were detected by quantitative PCR using genomic DNA isolated from tissue. The distribution of exogenous GLUT1 protein in the brain and other organs was examined by immunohistochemistry. **Results:** After AAV-GLUT1 injection, the vector genome was detected in the brain: cerebral cortex (mean vg quantity: 4,089-33,950 vg/pig diploid genome [vg/dg]), hippocampus (9,575 vg/dg), pons (11,296 vg/dg), medulla oblongata (20,987 vg/dg), and cerebellum (23,797 vg/dg). Lower vector genome levels were detected in the thalamus (1,197 vg/dg), hypothalamus (1,501 vg/dg), and striatum (1,059 vg/dg). The vector genome was also detected in other organs: liver (16,187 vg/dg), kidney (8,673 vg/dg), and spleen (5,578 vg/dg), while it was detected at a moderate level in the spinal cord (558-2,725 vg/dg) and at a very low level in the ovary (24 vg/dg). Immunohistological staining showed exogenous GLUT1 expression in the endothelial cells and neurons of the cerebral cortex, a part of the white matter, hippocampus, pons, and cerebellum. GLUT1 expression was also detected in the spinal cord, liver, kidney, spleen, a part of the pulmonary alveolus, and smooth muscle of the uterus. Exogenous GLUT1 was not detected in the heart, ovary, and pancreas. Neither significant inflammation nor cell degeneration was observed. **Conclusions:** After intra-cisterna magna injection of AAV-GLUT1, the vector genome was distributed in the cerebral cortex, hippocampus, and cerebellum. Although the vector genome was also detected in systemic organs, no significant toxicity was observed. Therefore, we consider intra-cisterna magna injection of AAV-GLUT1 to be a safe and feasible approach for gene therapy of GLUT1DS.

299. Gene Replacement Therapy for *SURF1*-Related Leigh Syndrome Using AAV9

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Mitochondria undertake the most fundamental cellular energy processes, and their dysfunction often lead to diverse and variable multisystem dysfunctions. Mitochondrial disorders are by and large inherited disorders caused by mutations in both nuclear and mitochondrial DNA. Currently, no specific pharmaceutical drugs have been shown to effectively treat mitochondrial disorders in large-scale clinical trials. Therapies for most patients with mitochondrial disorders are limited to preventing or treating the complications. *SURF1*-related Leigh syndrome (LS) (also known as Charcot Marie Tooth disease type 4K) is an early onset neurodegenerative disorder characterized by reduction in the assembly factor of complex IV, resulting in disrupted mitochondrial function. Here, we hypothesized that functional gene replacement strategy could restore mitochondrial functions in LS caused by *SURF1*(surfeit locus protein 1) loss-of-function mutations. The knockout (KO) of *SURF1* in mice reduced complex IV/cytochrome *c* oxidase (COX) activity in multiple tissues, such as liver, brain and muscle. We used broad central nervous system (CNS)-directed delivery (intrathecal administration, or a combination of intrathecal and intravenous administration) of the *SURF1* gene into *SURF1* KO mice to ameliorate the mitochondrial dysfunctions, in a manner that is compatible with human translation. A codon-optimized version of the human *SURF1* (h*SURF1*) was packaged within self-complementary adeno-associated virus serotype 9 (AAV9) viral vectors, which have been shown to induce robust expression in CNS. Our data indicate that 4 weeks after intrathecal administration into juvenile mice, AAV9/h*SURF1* vectors were able to partially and significantly rescue the COX activity in the liver, muscle and cerebellum, but not cerebrum of the brain. However, a combination of intrathecal and intravenous administration significantly increased the COX activity in cerebrum. The toxicity of the vectors was evaluated through intrathecal administrating into wildtype (WT) mice at a maximum feasible dose. No deleterious effects on weight, survival, or body condition have been observed up to 5 months following gene transfer. Given that this approach is demonstrating safety and efficacy of AAV9/*hSURF1* in mice following intrathecal administration, we propose this as a potential treatment worthy of further development for *SURF1*-related LS patients. Further, this approach might be amenable as a potential treatment for patients with other related forms of LS or other mitochondrial disorders.

300. Fusion of RVG or gh625 Peptides to Iduronate-2-Sulfatase for the Treatment of Hunter Disease

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Mucopolysaccharidosis type II (MPSII or Hunter disease) is a lysosomal storage disease caused by a mutation in the IDS gene, resulting in heparan sulfate (HS) and dermatan sulfate (DS) accumulation. This leads to skeletal and cardiorespiratory disease with severe neurodegeneration in two thirds of suffers. Enzyme replacement therapy is ineffective at treating neurological disease, as intravenously-delivered IDS is unable to cross the blood-brain barrier (BBB). Haematopoietic stem cell transplant is also unsuccessful, presumably due to insufficient IDS enzyme production from transplanted cells engrafting in the brain. We have previously demonstrated that a haematopoietic stem cell gene therapy (HSCGT) strategy, using a lentivirus containing IDS fused to ApoEII (LV.IDS.ApoEII), can correct neuropathology in MPSII mice 6 months post-transplant. Peptides designed to bind receptors for proteins that normally transit the blood brain barrier, such as the ApoEII peptide, are of significant interest in HSCGT as they improve the ratio of peripheral to CNS enzyme distribution after HSCGT. Here we present data using two different peptide sequences RVG (rabies virus glycoprotein) and gh625 (Herpes virus glycoprotein derived peptide), fused to IDS and delivered via

HSCGT, in MPSII mice 6 months post-transplant. HSCGT with LV.IDS.RVG and LV.IDS.gh625 was compared to LV.IDS.ApoEII and LV.IDS in MPSII mice. Wild-type mice and un-treated MPSII mice were used as controls. Levels of IDS enzyme activity in the brain and peripheral tissues were lower in LV.IDS.RVG and LV.IDS.gh625 treated mice than in LV.IDS.ApoEII and LV.IDS treated mice, despite comparable vector copy numbers. Microgliosis, astrocytosis and lysosomal swelling were partially normalised in MPSII mice treated with LV.IDS.RVG and LV.IDS.gh625. Skeletal thickening was normalised by both treatments to wild-type levels. Although reductions in skeletal abnormalities and neuropathology are encouraging, given the low levels of enzyme activity compared to LV.IDS and LV.IDS.ApoEII, the RVG and gh625 peptides are unlikely to be ideal candidates for HSCGT in MPSII, and are inferior to the ApoEII peptide that we have previously demonstrated to be more effective at correcting MPSII disease than IDS alone.

301. Abstract Withdrawn

302. Assessment of miQURE™ Efficacy and Safety in SCA3 Neurons

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The miQURE™ platform is an engineered microRNA-based, AAV-delivered approach suitable to apply for multiple clinical indications including potential treatment of the neurodegenerative disorder Spinocerebellar ataxia type 3 (SCA3). SCA3 is caused by the expansion of a CAG triplet repeat in the *ATXN3* gene. By specifically downregulating ATXN3, AAV-miATXN3 is expected to alleviate the toxicity induced by the mutant ataxin-3 protein. The off-target effects of the miQURE™ platform are expected to be low, due to lack of passenger strand production and expression levels comparable to endogenous small RNAs due to the polymerase II promoter. To investigate potential off-target effects of AAV5-miATXN3, induced pluripotent stem cell (iPSC) -derived neuronal cultures from two SCA3 patients and astrocytes were transduced with different concentrations of AAV5-miATXN3. These cells were selected because they represent the most disease relevant cell type and will be the target of AAV5-miATXN3 in potential future therapeutic application. RNA and protein were isolated 10 days after AAV5-miATXN3 treatment, and RNA sequencing performed to assess potential differential expression of genes besides ATXN3. In the AAV5-miATXN3 transduced neurons and astrocytes, a clear dose-dependent expression of the therapeutic agent (miATXN3) could be seen. Additionally, mature miATXN3 molecules detected in the culture medium were associated with extracellular vesicles. The levels of extracellular vesicles-enriched miATXN3 strongly correlated with AAV5 dose and miATXN3 expression, suggesting the potential therapeutic spread of the engineered miATXN3. Next to a strong dose dependent increase in miATXN3 expression, a clear ATXN3 knockdown was seen, confirming target engagement. Small RNA sequencing showed that mature miATXN3 was processed correctly without off-target passenger strand production. No cellular microRNAs were dysregulated, indicating that endogenous-RNAi machinery was unaffected by miATXN3 overexpression. Finally, gene expression analysis was performed on cells treated with formulation buffer, control AAV5 and with the functional AAV5-miATXN3, allowing for detection of miATXN3 specific up- or downregulation of genes besides ATXN3. In conclusion, AAV5-miATXN3 is efficiently processed in human iPSC-derived neurons and dose-dependently lowers ataxin-3, with no observed toxicity or off-target effects in gene expression and regulation. The data provide further evidence for the safety and efficacy of AAV5-miATXN3 and supports the continuation of the development of the miQURE™-based ataxin-3-lowering gene therapy for SCA3.

303. Intracerebroventricular Delivery of AAV9 Gene Therapy Prevents Central and Retinal Neuron Loss, Preserving Vision in CLN6-Batten Disease Mice

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Batten disease is a family of rare, fatal, neuropediatric diseases presenting with memory/learning decline, blindness, and loss of motor function. Recently, we reported the use of an AAV9 mediated gene therapy that prevents disease progression in a mouse model of CLN6-Batten disease (*Cln6*^{nclf}), resulting in full lifespans for treated animals. Despite the success of our viral mediated gene therapy, the dosing strategy used was optimized for neuronal delivery to the brain and currently the therapeutic potential of this viral vector in correcting vision related phenotypes remains unknown. Here, we examine whether cerebrospinal fluid (CSF) delivery of scAAV9.CB.CLN6 is sufficient to preserve visual deficits in *Cln6*^{nclf} mice. We show that CSF distribution of scAAV9.CB.CLN6, obtained through a neonatal intracerebroventricular (ICV) route of administration, completely prevents classic Batten disease pathology in the visual processing centers of the brain, preserving neurons of the superior colliculus, thalamus, and visual cortex. Importantly, ICV delivery of scAAV9.CB.CLN6 also leads to expression of CLN6 throughout the retina and preserves photoreceptors typically lost in *Cln6*^{nclf} mice. Lastly, with multiple visual centers preserved, ICV administration of scAAV9.CB.CLN6 maintains visual acuity in *Cln6*^{nclf} mice as measured by optokinetic tracking. Taken together, we provide the first instance of ICV mediated gene delivery benefiting visual function by targeting both central and retinal neurons in a model of Batten disease.

304. Development of Surgical Targeted Delivery Procedures for Effective Gene and Cell Therapy Delivery to the Central Nervous System

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Preclinical development of gene and cell therapies is a complex and customizable process which requires a strategic technical approach and sound knowledge of the ever-changing regulatory landscape. With the promise of sustained, curative treatment for a variety of genetic diseases, many programs are on accelerated timelines which makes efficient preclinical study execution imperative to product success. Here, we utilized our dedicated staff of study directors and surgeons to pilot technical approaches to targeted delivery systems for gene and cell therapies in rodents and large animals. Gene therapy is a promising approach for treatment of central nervous system (CNS) disorders, but clinical success has been limited due to the physiological challenges of transport across the blood brain barrier. Therefore, targeted delivery systems are needed to achieve efficacy and sustained gene expression. To meet this need, we developed MRI stereotaxic delivery methods for targeted brain injections and fluoroscope guided intrathecal injections. The MRI stereotactic pilot work used anesthetized Macaques mounted in an MRI-compatible stereotaxic frame. The monkeys then underwent pre-operative MRI to determine target coordinates for a dosing cannula. Target enhancement of zeroing locations were achieved, and bilateral craniotomies performed. Cannulea were inserted per target coordinates with positive pressure application. Contrast agent was infused in the target brain region at a controlled rate, then dose site and distribution were confirmed via post-surgical MRI. Results show the contrast agent was localized at the target coordinates. For intrathecal delivery, fluoroscopic imaging was used on anesthetized rats to guide injection of a contrast agent into the subarachnoid space between L3-L4 using a 33-gauge needle. Animals were recovered and observed post-surgery for limb impairment or other neurological effects. Results show successful delivery of contrast agent to the subarachnoid space and dispersion in the CSF. No marked neurological signs were observed during the recovery period. To better support cell therapies for spinal injury indications, we piloted a thoracic laminectomy and dura closure procedure in rats, targeting the ninth and tenth thoracic vertebra. A dorsal midline incision over the lumbar thoracic junction was made on anesthetized animals. The laminectomy was performed from T9-T10 and four saline injections were made 1mm below pia mater at the T9-T10 and T10-T11 junctions. The dura, musculature and subcutaneous tissue was closed, and the animals observed for neurological signs during a recovery period. Animals recovered well and no neurological signs were observed. In conclusion, we have successfully developed safe and effective methods for targeted delivery to the CNS. All three pilot methods discussed herein have since been used successfully on numerous regulated and non-regulated studies and have been expanded in some cases for specialized requirements and MRI targeting of other deep brain structures. With the unique concerns associated with cell and gene therapies, it is crucial to find a development partner with deep knowledge, experience and technical abilities, while keeping to translational study designs to help guide programs successfully to the clinic and to patients who need them.

305. Gene Therapy with a Synthetic AAV Vector Rescues the Audiovestibular Dysfunctions in a Mouse Model of Recessive Hearing Loss

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Congenital hearing loss is the most common sensory deficits at birth. About two to four per thousand babies are born with hearing loss, of which two-thirds are found to have genetic defects. Most congenital hearing loss is caused by degeneration or loss of cochlear hair cells and/or their associated spiral ganglion. For mild to moderate hearing loss, hearing amplification such as hearing aids is used to restore hearing. For severe hearing loss caused by loss of sensory hair cells, cochlear implants are surgically implanted into the inner ear to stimulate the auditory nerve. However, the degree of hearing restoration depends on the residual functional auditory nerves. Recently, several novel adeno-associated virus (AAV) serotypes, such as Anc80L65, have been proved as a promising delivery system for restoring the function of inner ear sensory cells, and thus a promising biological treatment for severe hearing loss that cannot benefit from ordinary hearing amplification such as hearing aids and cochlear implants. We have previously demonstrated a mouse model with a common recessive missense *PJVK* mutation in the Asian population. The *Pjvk* mutant mice exhibited both hearing and balance dysfunctions. In addition to the sensory cells of the inner ear, pathological changes were also observed in the neurons of auditory/vestibular nerves. We delivered *PJVK* gene into the inner ear of Pjvk mutant mice using the Anc80L65 vector at the newborn stage. At the adult stages, the treated mice showed both improved auditory and vestibular functions. These findings suggest that the Anc80L65-mediated gene therapy is an efficient delivery system for simultaneously introducing genes to both the sensory cells of the inner ear and the neurons of the auditory/vestibular pathways.

306. Evaluation of CSF-Injected AAV.PHP.eB vs AAV9 for Optimal CNS Delivery of Intrabodies to Treat Parkinson's Disease and Synucleinopathies

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Synucleinopathies, such as Parkinson's disease (PD) and Dementia with Lewy Bodies (DLB), are misfolded protein diseases that present with progressive pathologies and symptomatology with advanced disease duration. Successfully protecting brain regions with therapeutics designed to relieve proteopathic spread may be effective in slowing or halting disease. We have previously demonstrated antibody fragments

raised against alpha-synuclein can be delivered intracellularly via viral vectors (intrabodies) and are able to reduce alpha-synuclein pathology in both *in vitro* and *in vivo* models of PD. To further establish the pre-clinical potential of our lead therapeutic, VH14-PEST, we aimed to optimize and select an appropriate AAV-based therapeutic paradigm to provide global CNS protection from alpha-synuclein toxicity. The engineered AAV9 capsid, AAV.PHP.eB, was designed and selected to increase viral penetration into the CNS through peripheral delivery. However, several studies have revealed inconsistencies with better CNS biodistribution of AAV.PHP.eB to be contingent on viral titer, delivery method, host strain, or transport receptor expression levels. Furthermore, high viral titers required for effective CNS transduction via intravenous delivery may cause peripheral tissue toxicity, immune responses, and impairments in neurogenesis. Vector delivery through circulating cerebrospinal fluid (CSF) presents a method to increase the CNS:peripheral tissue transduction ratio while distributing genetic material throughout the neuraxis at lower viral titers. However, it is unknown whether AAV.PHP.eB enhances CNS transduction compared to its parent serotype, AAV9, when delivered into the CSF of rats. To address this gap, we delivered equititer doses ($1x10^{13}$ vg/mL, 45 µL) of AAV.PHP.eB or AAV9 with green fluorescent protein (GFP) into the cisterna magna of aged Sprague-Dawley rats (n=5-6) and sacrificed animals 5-weeks post-injection. Using stereological estimations of GFP+ cells, we found intracisternal injections of AAV.PHP.eB significantly increased levels of transduced cells in the cerebellar cortex by five-fold when compared to AAV9-injected animals (AAV.PHP.eB=12974±3082, AAV9=2569±576, p=0.015). AAV.PHP.eB also demonstrated increased penetration in the substantia nigra, a primary target site for intrabody therapy (AAV.PHP.eB=6348±1312, AAV9=2890±330, p=0.046). Densitometric analysis of GFP+ cells and neurites revealed AAV.PHP.eB-mediated gene delivery also enhanced transduction in the motor cortex (+31.2%), somatosensory cortex (+48.4%) and thalamus (+30.0%) compared to AAV9. AAV.PHP.eB-injected animals also consistently demonstrated higher levels of GFP expression in white matter tracts throughout the brain. Parallel to our evaluation of CNS-targeting viral vectors, we have also generated a fully-human version of VH14-PEST (VH14-hPEST) to test with our global CNS therapeutic paradigm. VH14-hPEST demonstrated effective target engagement of alpha-synuclein in 3D-cerebral forebrain organoids derived from PD patient iPSCs. Here, we will present the complete comparison of CNS biodistribution of AAV.PHP.eB and AAV9, evaluate potential toxicities and side effects, and illustrate a path forward to validate disease-modifying potential of AAV-VH14-hPEST for the treatment synucleinopathies.

307. Anti-Epileptic Effects of AAV-AEG-1 in the Kainic Acid-Treated Animal Model of Temporal Lobe Epilepsy

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Although the expression of astrocyte elevated gene-1 (AEG-1) is well observed in neurons *in vivo*, the role of AEG-1 is rarely known in the adult brain. In recent, we have found that an intra-hippocampal injection of kainic acid (KA), which can induce temporal lobe epilepsy (TLE), upregulates AEG-1 expression in the dentate gyrus (DG) of mouse brain. To investigate the role of AEG-1 in the DG of KA-treated hippocampus, we induced AEG-1 upregulation by an injection of adeno-associated virus serotype 1 containing human AEG-1 (AAV-AEG-1) in the DG of mouse brain, and then treated KA in the hippocampus. Our results showed that AEG-1 transduction could significantly reduce seizure susceptibility and frequency of spontaneous recurrent seizures (SRS) caused by KA administration compared to KA alone. Moreover, its upregulation reduced granule cell dispersion (GCD), which is a morphological alteration characteristic in the DG with TLE, through the inhibition of KA administration-induced mammalian target of rapamycin complex 1 (mTORC1) activation. These results suggest that AEG-1 upregulation may have beneficial effects for preventing epileptic events in the hippocampus *in vivo*, and that the utilization of AAV-AEG-1 can be a beneficial strategy against TLE.**Acknowledgements** This work was supported by a grants from the National Research Foundation of Korea (NRF-2017R1A2B4002675 and 2017H1A2A1046044).

308. In Vitro and In Vivo Administration of a Sustained Release Nanocarrier-Based Formulation of an Inhibitor in Conjunctival Fibrosis

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Purpose: Nanotechnology enables the design of multifunctional targeted therapies, thereby increasing their therapeutic efficacy and reducing the side effects for patients. Sustained drug delivery is a large unmet clinical need in glaucoma. We hypothesised that a nanocarrier formulation of an MRTF/SRF inhibitor, CCG-222740, could be used as a sustained release antifibrotic therapy in glaucoma filtration surgery. **Methods:** Nanocarrier CCG-222740 was prepared by the thin film hydration technique using the DOTMA and DOPE liposomes in a 1:1:1 molar ratio (DOTMA: DOPE: CCG-222740). Nanocarrier size, zeta potential and morphology were determined by dynamic light scattering, laser Doppler anemometry and transmission electron microscopy, respectively. We tested the effects of nanocarrier CCG-222740 on *ACTA2* gene expression using real-time qPCR and on cell viability in human conjunctival fibroblasts. Drug release studies were performed using high performance liquid chromatography. We validated our results using a randomised, prospective, masked-observer study of 18 New Zealand white rabbits undergoing glaucoma filtration surgery. The animals received intraoperative 0.2 mg/ml mitomycin-C [N=6] or a postoperative subconjunctival injection of 68 µg nanocarrier CCG-222740 [N=6] or empty liposomes [N=6]. Bleb morphology was recorded over 30 days. Tissue sections on day 30 were immunohistochemically assessed. We analysed our results using the Kaplan-Meier curve Log-rank test and Student's t-test. **Results:** The vesicles were spherical particles of 137.0 ± 7.1 (SEM) nm, +63.0 ± 1.5 mV and 0.39 ± 0.02 polydispersity index. In vitro, nanocarrier CCG-222740 was not cytotoxic and decreased *ACTA2* gene expression by 61.0% (p=0.026) and 71.2% (p=0.033) at 5 and 10 µM, respectively, compared to empty liposomes. There was a sustained cumulative drug release of 32.1%, 45.1%, 63.7%, 79.8% and 93.1% at 1, 3, 6, 9 and 14 days. In vivo, nanocarrier CCG-222740 doubled bleb survival from

11.0 ± 0.6 days for empty liposomes to 22.0 ± 1.3 days (p=0.001) (Fig 1). Nanocarrier CCG-222740 significantly decreased conjunctival scarring compared to empty liposomes using H&E, picrosirius red, Gomori's trichrome and alpha smooth muscle actin staining. There were no adverse side effects and drug levels were undetectable in the aqueous and vitreous. **Conclusions:** A nanocarrier formulation of an MRTF/SRF inhibitor represents a safe and sustained release drug delivery system that could be used to prolong bleb survival and to prevent conjunctival fibrosis after glaucoma filtration surgery.

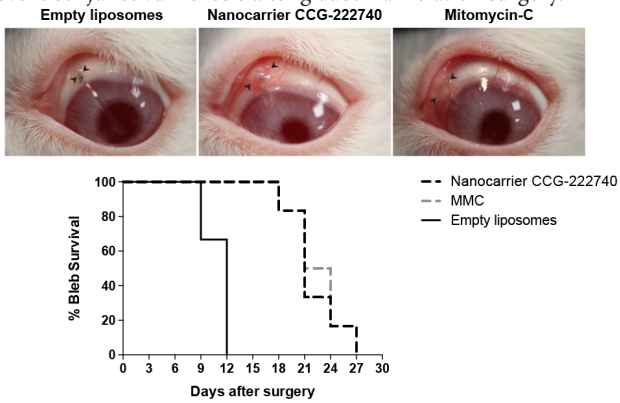

Fig 1. Nanocarrier CCG-222740 double bleb survival from 11.0 ± 0.6 days for empty liposomes to 22.0 ± 1.3 days (p = 0.001) in a pre-clinical rabbit model of glaucoma filtration surgery. Black arrows indicate bleb areas. Kaplan-Meier graph of bleb survival between nanocarrier CCG-222740 [N=6], empty liposomes [N=6] and mitomycin-C [N=6].

309. Transduction Efficiency of Synthetic and Conventional AAVs for Cochlear Lateral Wall

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Background: Adeno-associated viruses (AAVs) are popular vector choices for gene delivery in the inner ear due to their excellent biosafety profiles. While most AAV transduction studies in mammalian inner ear focus on the mechanosensory hair cells as well as supporting cells, many inner ear disease processes also affect the cells in the cochlear lateral wall. In this study, we examine the lateral wall transduction patterns of two conventional AAVs (AAV2/2 and AAV2/8) and three synthetic AAVs (AAV2.7m8, AAV8BP2, Anc80L65) in the neonatal mouse inner ear. Methods: Neonatal (P0-P5) CBA/J mice were used in this study. Five AAV-GFPs (AAV2/2, AAV2/8, AAV2.7m8, AAV8BP2, Anc80L65) were injected into mouse inner ear using the posterior semicircular canal approach. Immunohistochemistry was used to assess the infection efficiency. Results: AAV8BP2, AAV2/8, and Anc80L65 transduced various cell types in the cochlear lateral wall. AAV8BP2 transduced the marginal cells and intermediate cells in the stria vascularis with the highest efficiency. AAV2.7m8 and AAV2/2 transduced cells in the cochlear lateral wall at low levels. Conclusions: Our study showed AAV8BP2 transduced both the marginal cells and intermediate cells at high levels. AAV8BP2 is a useful viral vector for targeting the cochlear lateral wall.

310. Germline CRISPR/Cas9-Mediated Gene Editing Prevents Vision Loss in a Novel Mouse Model of Aniridia

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Aniridia is a rare eye disorder, which is caused by mutations in the paired box 6 (*PAX6*) gene, resulting in vision loss due to the lack of a long-term vision-saving therapy. One potential approach to treating aniridia is targeted CRISPR-based genome editing. For preclinical studies we chose a mouse model of aniridia, 'small eye' *Sey*, that carries the same loss-of-function *Pax6* mutation found in some patients. First, we endogenously FLAG tagged the *Sey* allele, thereby allowing for the differential detection of protein from the mutant allele, after correction by therapeutic approaches. Next, we optimized a CRISPR-based correction strategy *in vitro*, then tested it *in vivo* in the germline of our new mouse, also validating the causality of the *Sey* mutation. The genomic manipulations were analyzed by PCR, and Sanger and next generation sequencing. The mice were studied by slit lamp imaging, immunohistochemistry, and western blot analyses. We successfully achieved both *in vitro* and *in vivo* germline correction of the *Sey* mutation; with the former resulting in an average 34.8 ± 4.6% SD correction, and the latter in restoration of 3xFLAG-tagged PAX6 expression, and normal eyes. Hence, we have created a novel mouse model for aniridia, demonstrated that germline correction of the *Sey* mutation alone rescues the mutant phenotype, and developed an allele-distinguishing CRISPR-based strategy for aniridia.

311. Cellular Model for Spontaneous Initiation of alpha-Synuclein Aggregation

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Synucleinopathies are a group of neurodegenerative diseases with a shared hallmark, i.e. misfolding of the protein alpha-synuclein, which leads to the formation of insoluble cytoplasmic aggregates.Evidence supporting a causative role of alpha-synuclein in pathology comes from several lines of research, such as genetics, where mutations targeting the gene encoding for alpha-synuclein can lead to familial forms of the diseases. Studies also show that pathology can be induced in cellular and animal models by introducing pre-aggregated alpha-synuclein. This is thought to be a prion-like transmission, where aggregated alpha-synuclein acts as an infectious particle capable of spreading the pathology from cell-to-cell.With the hypothesis that alpha-synuclein aggregation is central to disease pathology, it is important to understand the events leading to initiation of aggregation and the events that happen as a consequence. We therefore set out to generate a cell model to investigate alpha-synuclein aggregation by expressing alpha-synuclein^{A53T}-GFP in Hek293T cells through lentiviral transduction. One of the generated monoclonal cell-lines

was observed to develop spontaneous GFP inclusions, rather than the expected diffuse GFP labeling. This occurred at the frequency of one out of 192 sorted single-cells. This phenotype was maintained upon subsequent divisions, as well as freeze-thawing. We therefore established these daughter cells as a monogenic cell line (AS001) with the main feature of spontaneously forming alpha-synuclein inclusions. Using this novel AS001-cell line we employed tagmentation and next generation sequencing and identified the lentiviral insertion point and subsequently the genetic ablation causing the shift in phenotype to the observed spontaneous aggregation. Further analysis by microscopy showed the aggregated alpha-synuclein to be phosphorylated, a feature of synuclein pathology. The aggregates were also found to co-localize with Rab8 and LAMP1, both markers associated with endo-lysosomes and autophagy, indicating the inclusions likely are targeted for degradation. We here present evidence that a single genetic perturbation is capable of causing alpha-synuclein to aggregate in cellular systems, although the induced aggregates are not immediately toxic to the cells as long as protein clearance is functional. That single-gene ablation can induce a strong aggregation phenotype, as demonstrated here, opens up for the possibility to screen for other genes that may have a similar effect on alpha-synuclein aggregation. The identification of such genes could lead to a better understanding of the molecular mechanisms of alpha-synuclein aggregation in synucleinopathies and the discovery of novel therapeutic targets.

312. Assessment of the Novel AAV-Based miQURE™ Gene Therapy in SCA3 Animal Models

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Spinocerebellar ataxia type 3 (SCA3) is a fatal neurodegenerative disorder characterized by brainstem and cerebellar atrophy. Clinical manifestations include progressive gait ataxia with involvement of cranial nerves. Expansion of a CAG trinucleotide repeat in the ataxin-3 gene (*ATXN3*) causes the accumulation of aberrant, toxic ataxin-3 protein in brain regions located in the posterior fossa. Lowering expression of the *ATXN3* gene is expected to alleviate mutant ataxin-3 toxicity. A non-allele-specific *ATXN3* silencing approach was investigated using the proprietary, next-generation miQURE™ technology. This strategy makes use of AAV5-delivered miRNA (AAV5-*miATXN3*) capable of inducing efficient *ATXN3* silencing. Following candidate selection in several cell-lines, including iPSC-derived neurons, a lead AAV5-*miATXN3* was selected for further *in vivo* assessment. The potency of AAV5-*miATXN3* was investigated in transgenic SCA3 mouse models by means of direct injection in cisterna magna or striatum. Following a one-time administration of AAV5-*miATXN3,* a potent reduction in ataxin-3 RNA and protein was observed in the brain of transgenic mice. Additionally, formation of insoluble ataxin-3 protein and aggregates was significantly prevented in the brain areas assessed. As these are disease-specific hallmarks that are closely related with mutant ataxin-3 toxicity, this suggests that AAV-*miATXN3* can result in alleviation of the cellular toxicity underlying SCA3. Indeed, darpp-32 lesion size was significantly smaller in brains of mice treated with AAV5-*miATXN3* compared to untreated littermates. In ongoing experiments, the potential of AAV5-*miATXN3* to improve behavioral deficits in transgenic SCA3 mice expressing the full human mutant ataxin-3 protein are being investigated. In conclusion, we report that administration of the miQURE™ based AAV5-*miATXN3* is able to significantly reduce expression of ataxin-3 and related toxicity. As such, this AAV-*miATXN3* is a promising treatment strategy for SCA3 and warrants further studies investigating potency and tolerability in larger animal models.

313. Cerebrospinal Fluid Chitotriosidase as a Surrogate Endpoint of the Efficacy of the PS System Gene Therapy in Neurodegenerative Lysosomal Diseases

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Background: There are 70 genetically distinct lysosomal diseases, the majority of which cause severe neurological deficits. Validated surrogate endpoints or biomarkers are critically important to accelerate approvals for gene therapy, by providing a more rapid and easier detection of efficacy than clinical outcomes. However, there are currently no surrogate endpoints or biomarkers that can predict long-term clinical benefit from gene therapy for lysosomal diseases. In an NIH-funded (U54NS065768) natural history study of two lysosomal diseases, GM1-gangliosidosis and GM2-gangliosidosis, chitotriosidase (chito) enzyme activity was one of the few analytes out of approximately 200 screened that appeared to relate to the most severe phenotypes of gangliosidoses. However, no clinical laboratories were positioned to pursue a more rigorous evaluation. Moreover, chito has never been evaluated as a biomarker of gene therapy efficacy. **Objectives:** To investigate CSF chito levels as a probable surrogate endpoint for clinical trials with gene therapy, this study aimed to (1) validate chito levels for important clinical outcomes in patients with lysosomal diseases and (2) assess chito's ability to detect effective gene therapy in murine models of lysosomal diseases. **Methods:** A novel method of quantifying 4-methylumbelliferyl-β-D-N,N',N"-triacetylchitotrioside in the CSF was developed under conditions comparable with concurrent measurements in serum. A total of 134 CSF and serum specimens were collected from 34 patients with the lysosomal diseases GM1-gangliosidosis (n=8), GM2-gangliosidosis (n=12), Gaucher disease (n=2), mucopolysaccharidoses (MPS, n=11), and multiple sulfatase deficiency (n=1). Gene therapies for three lysosomal diseases were studied in mice with GM1-gangliosidosis,

GM2-gangliosidosis, or MPS type I (MPS I). For each disease, CNS tissues were collected from heterozygotes, untreated mice, and treated mice. **Results:** Chito levels in the CSF were significantly higher in patients with gangliosidoses compared to MPS, suggesting distinctive neuroinflammation between the diseases: GM1-gangliodosis vs MPS ($p<0.0001$); GM2-gangliosidosis vs MPS ($p<0.0001$). CSF chito levels were higher in patients with the more severe phenotypes compared to milder phenotypes in GM1-gangliosidosis and GM2-gangliosidosis. Furthermore, higher CSF chito levels were significantly associated with higher neurological impairment in patients with GM1-gangliosidosis, GM2-gangliosidosis, and MPS ($p=1.12*10^{-5}$, $R^2=0.72$). In the CNS tissue for mice with GM1-gangliosidosis and MPS I (affected animals), high chito significantly correlated with low lysosomal enzyme activity ($R^2=0.86$). Moreover, MPS I mice treated with the CNS-effective, PS gene editing system had lower chito levels in the CNS compared to untreated mice ($p=0.0004$). **Conclusions:** These results motivate the use of CSF chito to measure the therapeutic effect and response to gene therapy in clinical trials for multiple lysosomal diseases. Chito is a probable surrogate endpoint that is supported by strong mechanistic rationale and a clinically validated biomarker. CSF chito may also be a valuable tool for clinical trial enrichment by objectively differentiating between the phenotypes of a lysosomal disease. This clinical laboratory (CLIA certification pending) is prepared to receive specimens to further assess the relationship between chito and response to gene therapies. **Funding:** Rare Disease Clinical Research Network. "Lysosomal Disease Network." (U54) NS065768.

314. Anti-Inflammatory Effects of Human Mesenchymal Stem Cells Treatment in a Lipopolysaccharide-Induced Animal Model of Cerebellar Ataxia

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Cerebellar ataxia(CA) is one of incurable motor neuron diseases that characterized with lack of voluntary movements and gait problems. Although there are reports that neruo-inflammation may be associated with CA, there is no suitable animal model to study inflammatory cerebellar ataxia (ICA). In this study, we evaluated the usability of intracerebellar injection of lipopolysaccharide (LPS) for establishment of ICA animal model, and investigated the therapeutic effects of human mesenchymal stem cell (hMSC) administration in the ICA model. Our results showed that the injection of LPS, inducing inflammatory responses such as symptoms. In addition, hMSC treatment significantly alleviated the abnormal behaviors by inhibition of LPS-induced inflammatory responses. These results suggest that LPS administration may be useful to study ICA, and hMSC treatment can be effective to control ICA. **Acknowledgements** This study was supported by grants from the National Research Foundation of Korea (NRF-2017R1A2B4002675), and from the Korea Health Technology R&D Project through the Korea Health Industry Develpment Institute (KHIDI; HI16C2210) by the Korean government

315. Addressing the Weakest Link in DMD Gene Therapy: Force Transmission Through the Rod Domain of Dystrophin

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Mutations in the dystrophin gene result in at least the three allelic diseases: Duchenne and Becker Muscular Dystrophy (DMD and BMD), and X-linked Dilated CardioMyopathy (XLDCM). Ambulation past age 13 in patients with BMD is generally attributed to preservation of an open reading frame, allowing synthesis of dystrophin of altered sized, but even patients with gene duplications can have severe phenotypes. Many BMD patients have detectable dystrophin subfragments at molecular weights by Western blot that implicate covalent bond cleavage of the protein at the site of internal deletion. These clinical findings raise unanswered questions about functional constraints on the structure of the rod domain of dystrophin, composed almost entirely of tandemly linked triple helices and comprising the vast majority of the protein's 427kd molecular weight. The cloning capacity of systemically deliverable vectors based on adeno-associated viruses (AAV) is limited to approximately 1/3rd the length of the dystrophin coding sequence, requiring removal of 80% of the rod's triple helices. Can this be done without creating recombinant proteins susceptible to cleavage at the protein's weakest link? To explore structure-function relationships for proteins that can safely and durably substitute for dystrophin, we have integrated multiple approaches as reported in other ASGCT abstracts and recent publications (e.g. Song et al, Nature Medicine 2019 25:1505, Greer et al, 2020). We used comparative phylogenomics to reconstruct the evolutionary history of all members of the dystrophin supergene family as well as their binding partners in the dystrophin-associated protein complex. We used several principles of molecular modeling to design candidate therapeutics, and with rigorous testing in DMD models. In the course of this research we made a startling discovery with immediate implications for both the mechanobiology of dystrophin and translational research in gene therapy. The findings suggest that dystrophin is mechanically loaded in such a way that "BMD dystrophins" have a common structural flaw: a weakest link at the deletion/duplication junction. We have tested this hypothesis by designing alternative transgenes guided by the structural biology of the crystallographically studied members of the supergene family. A key finding is that tertiary structural conservation of the core of the triple helix enables truncation by precisely placed internal deletion to eliminate the weakest link. This has critical implications for the durability of gene therapy for DMD.

316. AAV Mediated Gene Therapy Restores Partial Auditory Sensitivity in Mice Models of Autosomal Recessive Non Syndromic Deafness DFNB31 and Usher Syndrome Type IID

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The PDZ domain-containing protein whirlin (WHRN), encoded by the *WHRN* gene is essential for both hearing and vision. WHRN is

implicated in the stabilization, elongation and maintenance of the stereociliary bundles of the sensory hair cells in the inner ear. Two major WHRN splice variants have been shown to be expressed in auditory hair cells: the full-length isoform (*FL-WHRN*) of the protein is located at the tip and shaft of the stereocilia in inner hair cells and the ankle link complex in outer hair cells; the short isoform (*C-WHRN*) is restricted to the tips of the stereocilia in both inner and outer hair cell types (Mburu et al. 2003; Mathur et al. 2015; Ebrahim et al., 2016). Both isoforms have significant effects on hair bundle ultrastructure (Mathur et al. 2015; Ebrahim et al. 2016). Distinct disruptions of one or both of these isoforms lead to a variety of phenotypic configurations including Usher Syndrome type IID (USH2D) and autosomal recessive non-syndromic deafness 31 (DFNB31). To assess the efficacy of novel gene therapy approaches for USH2D and DFNB31, we used a synthetic viral vector, Anc80L65 (Landegger *et al.* 2017), encapsulating the wildtype cDNA encoding for the *FL-WHRN* and *C-WHRN* isoforms. Two mouse models were used for this study that closely recapitulate the human phenotype: DFNB31 (*Dfnb31*$^{wi/wi}$, Holmes et al. 2002; Mburu et al. 2003) have a c-terminus deletion resulting in the truncation of both *FL-WHRN* and *C-WHRN* isoforms and USH2D: (*USH2D*$^{neo/neo}$, Yang et al. 2010) which only lacks *FL-WHRN*. *USH2D*$^{neo/neo}$ mice received exogenous *FL-WHRN*, while *Dfnb31*$^{wi/wi}$ mice received either *FL-Whrn* only or a combination of *FL-WHRN* and *C-WHRN*. The vectors were injected to the murine inner ear at P1 to induce expression of the transgene *in vivo*. Efficacy of the treatments were assessed by comparing uninjected mice mutants versus injected mutants at the cellular, system and behavioral levels with analysis of mechanosensitive transduction currents, protein expression, stereocilia length quantification and evaluation of the behavioral phenotype via auditory brainstem responses, and balance assessments. Neonatal injection of sAAV.*FL-WHRN* in *Dfnb31*$^{wi/wi}$ mice yielded improved mechanotransduction in outer hair cells and inner hair cells. ABR recovery was moderate, down to 70 dB thresholds for the best performer, while balances tests revealed no significant recovery in performance compared to mutant controls. Impressively, dual sAAV.*FL-WHRN* and sAAV.*C-WHRN* injection in *Dfnb31*$^{wi/wi}$ mice lead to significant recovery in auditory thresholds and balance behavior. Injection of sAAV.*FL-WHRN* in *USH2D*$^{neo/neo}$ mice lead to improved transduction currents, bundle morphology and hair cell survival, but only a modest recovery in ABR thresholds. The extent of recovery observed in both single and double isoform delivery demonstrate that gene replacement strategy is viable for the treatment of USH2D and DFNB31.

317. MiniPromoters for rAAV Ophthalmic Gene Therapy: Novel PAX6 and Cone Promoters; Improved Müller Glia, Bipolar ON, and Brain Retinal Barrier Promoters; and Expanded Analysis of a Corneal Stroma Promoter

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Introduction: Ophthalmic gene therapy has been leading the field, with 42 "eye disease" clinical trials listed for rAAV-based therapies, and with over 50% using promoters that restrict expression based on cell type. Promoters that restrict expression have demonstrated increased efficacy, and can limit the therapeutic to the target cells thereby reducing unwanted off-target effects. Using bioinformatics and *in vivo* expression analysis, our team has previously developed numerous restricted human promoters (MiniPromoters). Here we increase our collection with new MiniPromoters for: PAX6-expressing cells and cones; as well improved promoters for the Müller glia, bipolar ON, and blood-retina-barrier (BRB); and expanded the analysis of a previously characterized promoter for the corneal stroma. PAX6 is best known as the essential transcription factor for panocular development, and mutations in *PAX6* produce the ocular disorder aniridia. Cones are responsible for colour vision, and dysfunction of the cones can lead to various cone dystrophies including Achromatopsia. Müller glia provide essential molecules to the neurons of the retina, and thus may be able to widely deliver a neuroprotective therapeutic. Bipolar ON cells transmit signals from the photoreceptors to the ganglion cells, and are an important target for stationary night blindness, melanoma associated retinopathy, and optogenetic strategies. Endothelial cells of the BRB are an important target for therapeutic accessibility to the retina. The corneal stroma is essential for the maintenance of clear vision and normal visual function, disorders of the cornea include keratoconus and macular corneal dystrophy, as well as infection and injury. **Methods:** MiniPromoters either were new bioinformatics-driven designs from genes with therapeutically interesting expression patterns, or were further reduced in size, strengthened, or combined, from our previous work. All promoters were cloned into a custom rAAV genome plasmid driving EmGFP (emerald GFP), and including an intron, WPRE (transcript stabilizer), and SV40 polyA, and then packaged into rAAV9. All viruses were tested by intravenous injection into newborn mice. A subset of MiniPromoters were further characterized by direct injection into the adult mouse eye, including subretinal, intravitreal, and intrastromal routes. Analysis included histological examination of MiniPromoter driven EmGFP immunofluorescence compared to the ubiquitous promoter smCBA. For the most promising promoters, co-staining with relevant cellular markers and quantification of epifluorescent signal was also undertaken. **Results:** Successful MiniPromoters include designs from: the *PAX6*

SIMO element with binding site modification (1,982 bp); a combination of previous *PAX6* MiniPromoters (2,148 bp); a novel design of *PDE6H* for the cones (2,005 bp); a re-design for reduced size and increased strength of *NR2E1* for the Müller glia (1,691 bp); re-designs for reduced size and increased specificity for *PCP2* for the Bipolar ON (986 bp) and *CLDN5* for the BRB (1,670 bp); and *PITX3* for the stroma (2,484 bp). **Conclusions:** Small-restricted MiniPromoters can be developed for clinically relevant cell types of the eye. All published Pleiades (Ple) MiniPromoters are available to the community through Addgene (www.addgene.org), and unpublished materials are available from Dr. Elizabeth M. Simpson (simpson@cmmt.ubc.ca).

Cancer - Immunotherapy, Cancer Vaccines

318. Non-Viral Generation of Chimeric Antigen Receptor T Cells Using CRISPR-Cas9

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Chimeric antigen receptor (CAR) T cells constitute an important immunotherapy targeted against cancer antigens that can provide complete remission for patients afflicted by cancer. Currently, there are over 500 clinical trials underway around the world with CAR T cell immunotherapies, and nearly all of them require the use of viral vectors to deliver the CAR transgene into T cells. Use of viral vectors for CAR T cell manufacturing constitutes a bottleneck in the supply chain and can be problematic due to batch-to-batch variability, use of xenogeneic components during manufacturing of viral vectors, and the high random integration of viral elements into the human genome. The poorly specified integration of the CAR transgene can lead to heterogeneous expression that can be readily silenced, in part by host cell recognition of viral genetic elements. Here we describe a novel method to generate CAR T cells using genome editing where the editing machinery consist only of proteins and nucleic acids without any viral vectors. We demonstrate CRISPR-Cas9 mediated genomic insertion of CAR transgenes into the T cell receptor alpha constant, *TRAC*, locus in primary human T cells collected from healthy donors. These cells, termed non-viral-CRISPR CAR T cells (NV-CRISPR CAR T cells), exhibit proper *TRAC*-specific integration of the CAR transgene, robust gene expression of the CAR mRNA, and translated CAR proteins on the T cell surface (Figure 1). The NV-CRISPR CAR T cells potently upregulate cytotoxic transcriptional programs and kill target-antigen-positive human cancer cells *in vitro* within co-culture assays. The NV-CAR T cells successfully cause GD2-positive tumor regression *in vivo* within human xenograft cancer models in mice at comparable efficiency to state-of-the-art, viral CAR T cells. NV-CRISPR CAR T cells can be manufactured in a xeno-free manner and have high potential to simplify and advance CAR T cell manufacturing by elimination of viral vectors.

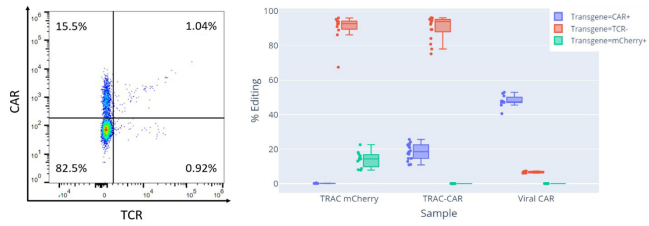

Figure 1. High efficiency CAR T cell generation using CRISPR-Cas9. Left) Representative flow cytometry diagram of NV-CRISPR *TRAC* CAR T cells. 16% of cells are CAR positive while 98% of cells are TCR negative. A second generation OX40 GD2 targeting CAR was inserted into the *TRAC* locus using CRISPR-Cas9 ribonucleoproteins and double stranded PCR donor templates. **Right)** Percent gene editing inferred from flow cytometry assays on T cells cultured for 10 days after nucleofection with non-viral CRISPR-Cas9 reagents. Boxplots for editing rates across three test groups: 1) NV-CRISPR *TRAC* mCherry, n=11 (i.e., T cells where the mCherry gene is knocked in at the *TRAC* locus using CRISPR-Cas9), 2) NV-CRISPR *TRAC* CAR, n=17 (i.e., T cells where the CAR gene is knocked into the *TRAC* locus using CRISPR-Cas9), 3) Viral CAR, n=11 (i.e., a retrovirus is used to generate CAR T cells).

319. Combination Therapy of GD2-Specific CAR-T Cells with Tyrosine Kinase Inhibitors for Neuroblastoma

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Background: Chimeric antigen receptor (CAR)-T cell therapy is a breakthrough therapy for patients with B cell malignancies, but its effectiveness against solid tumors is limited partly because of actively hostile tumor microenvironment and immune evasion due expression of immunosuppressive molecules such as PD-L1 on the surface of tumor cells. Thus, combinatorial therapies of CAR-T cells with immune checkpoint inhibitors and/or molecular targeted drugs are attractive therapeutic options for increasing the efficacy of CAR-T cells. As the expression of PD-L1 in tumor cells is partially regulated by the Raf/MEK and ALK pathways, we hypothesized that a molecular targeted drug disrupting these signaling pathways would enhance the function of CAR-T cells when used in combination therapy. In this study, we developed GD2-specific CAR (GD2-CAR)-T cells by *piggyBac* transposon (PB)-based gene transfer and evaluated whether combination therapy of GD2-CAR-T cells with tyrosine kinase inhibitors (TKIs), including a Raf/MEK inhibitor and an ALK inhibitor, would improve the killing activity of GD2-CAR-T cells against neuroblastoma (NB) cells. **Method:** We transfected unstimulated PBMCs with the GD2-CAR transgene and the PB transposase gene using electroporation and cultured them with defined T cell media

and feeder cells, supplemented with IL-7 and IL-15. The phenotypes of the GD2-CAR-T cells were assessed using flow cytometry. To evaluate cytotoxic activity, GD2-CAR-T cells were co-cultured with 3 NB cell lines (SK-N-AS, SH-SY5Y, and IMR32), and the numbers of live tumor cells were measured using real time cell analysis or flow cytometry. In addition, we also tested whether trametinib (MEK inhibitor) or alectinib (ALK inhibitor) would augment the antitumor activity of the GD2-CAR-T cells. **Results:** We successfully generated PB-based GD2-CAR-T cells, with a positivity rate of about 30-50% for GD2-CAR at day 14 after transfection. A dominant fraction of the GD2-CAR-T cells were CD45RA+/CCR7+, which is indicative of a naïve/memory stem cell phenotype. When co-cultured with GD2-positive NB cells, the GD2-CAR-T cells demonstrated sustained killing activity, even in multiple re-challenges. The combination of GD2-CAR-T cells with alectinib caused a synergistic increase in killing activity against NB cells. By contrast, addition of trametinib completely suppressed the killing activity of GD2-CAR-T cells, and production of IFN-γ in response to stimulation with tumor cells was inhibited. **Conclusion:** Alectinib synergistically enhances the cytotoxic activity of GD2-CAR-T cells by reducing the expression of PD-L1 in tumor cells, and may therefore be a promising therapeutic option for neuroblastoma when used in combination with GD2-CAR-T cells. Trametinib, on the other hand, completely inhibits the activity of GD2-CAR-T cells, which may make it useful as a safeguard for when serious adverse events such as off-target effects occur during CAR-T cell therapy.

320. Anti-NKp46 CAR T Cell to Target NK Cell Malignancies

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Introduction: Natural killer (NK)-cell neoplasms, including NK/T cell lymphoma and aggressive NK-cell leukemia, are rare malignancies (incidence of ~0.8 per 1,000,000 in the U.S., 3 to 10 times higher in South America and Asia) with high mortality (17-20-month overall median survival, but much worse for relapsed/refractory NK/T lymphomas and aggressive NK-cell leukemias). These malignancies are usually associated with Epstein-Barr Virus (EBV). To address this problem, we sought to develop CAR T cells to target NK malignancies. However, NK cells do not express a uniformly expressed cell surface receptor that is unique to NK cells. NKp46 (aka CD335) is an activating NK receptor that is expressed broadly by many NK cell subsets. Limited reports suggest NKp46 is expressed on approximately 90% of NK cell malignancies. Here we target NKp46 with CAR T cells. **Methods**: An anti-NKp46 antibody was synthesized as a scFv sequence and cloned into a lentiviral CAR backbone containing 4-1BB as the costimulatory domain and a downstream blue fluorescent protein (BFP) reporter. Primary PBMC were stimulated with CD3/CD28, transduced with anti-NKp46 CAR LV, and expanded. CAR T-cells were mixed with K562 cells transduced with NKp46 or autologous primary NK cells. Effector to target ratios of 2:1 were used unless otherwise specified. CAR T-cell function was evaluated by flow cytometry, wherein CAR-T cells were identified by BFP expression. CAR T-cell activation was evaluated by expression of cell surface CD137 and intracellular cytokine expression, and compared to anti-CD19 CAR T-cells. Killing of NKp46-expressing K562 cells was also assessed by flow cytometry. **Results**: Anti-NKp46 CAR T-cells were made from three different donors. The percentage of BFP+ CAR T-cells across three donors after 5-7 days of expansion was a median of 56% (range 53-65). The percentage of CAR T-(BFP+) cells expressing CD137 increased a median of 5-fold (range 4-6) when exposed to NKp46+ K562 cells compared to NKp46- K562 cells. Preliminary results also show anti-NKp46 CAR T-cell activation against autologous primary NK cells. The percentage of anti-NKp46 CAR T-cells with intracellular IL-2, TNF-alpha, and IFN-gamma expression increased a median of 10.5-(range 10-11), 11-(range 9-13), and 9-fold (range 7-11) when exposed to NKp46+ K562 cells compared to K562 cells. When tested in parallel, anti-CD19 CAR T-cells reacted similarly to CD19+ K562 cells compared to K562 cells, and showed no reaction to NKp46+ K562 cells. When mixed for 24 hours, anti-NKp46 CAR T-cells reduced the percentage of NKp46+ K562 cells by 83%, showing a dose-response with increasing E:T ratio. Anti-CD19 CAR T-cells did not deplete NKp46+ cells. **Conclusions**: We present a potent, scFv-based, second-generation anti-NKp46 CAR. The anti-NKp46 CAR T-cells are functional, show robust and specific activation, inflammatory cytokine release, and cell killing in response to target cells modified to express the NKp46 receptor. These results warrant further development and pre-clinical studies in small animal models. In vivo, this approach has the obvious potential toxicity of substantially depleting many NK cells. Short-term, this risk might be manageable with anti-viral prophylaxis, analogous to what is done during stem cell transplants. Long-term, this side effect would need to be anticipated and addressed, either with technology to regulate the anti-NKp46 CAR T-cells, or by consolidating therapy with a stem cell transplant.

321. An Engineered Allogeneic Artificial Antigen Presenting Red Cell Therapeutic, Rtx-321, for Hpv16+ Associated Cancers Promotes Antigen-Specific T Cell Activation & Expansion

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Recent successes in immuno-oncology have demonstrated the potential of modulating a patient's own immune system to combat cancer and drive tumor antigen specific T cell activation. This mechanism requires the T cell receptor (TCR) to bind to a cognate peptide/MHC complex, (signal 1), followed by a costimulatory signal (signal 2) and cytokine support (signal 3), and typically, these signals are delivered by antigen-presenting cells (APCs). Multiple therapeutics target APCs (e.g. cancer vaccines) or circumvent the necessity of APCs by engineering T cell therapies. The wider adoption of antigen-specific therapies has been limited by significant toxicity, limited effectiveness in solid tumors and difficulties in manufacturing at scale. We genetically engineered red cells to create an allogeneic artificial antigen-presenting cell (aAPC), RTX-321, that expresses HPV E7 peptide bound to MHC I, 4-1BBL, and IL-12 on the cell surface to mimic the human immunobiology of T cellAPC interactions. RTX-321 is manufactured using CD34+ hematopoietic progenitor cells from blood type O, Rh-negative, Kell-

negative donors. Cells are expanded and transduced with lentivirus, followed by further expansion and differentiation into enucleated red blood cells expressing exogenous proteins on the cell surface. RTX-321 is designed to sustain antigen presentation and circumvent the antigen processing mechanisms that limit the efficacy of vaccines. RTX-321 also promotes an active immune response, enhances both the quantity and quality of endogenous tumor-specific T cells, and eliminates the need for manufacturing patient-derived T cells. To assess the in vitro activity of RTX-321, primary CD8 T cells were engineered to express the HPV E7 TCR (E7 TCR-T). Presentation of HPV E7 peptide on the MHC I on RTX-321 initiated TCR signaling in transgenic TCRβ+ cells, as indicated by upregulation of early activation markers Nur77 and CD69. RTX-321 preferentially expanded E7 TCR-T cells over non-specific polyclonal CD8 T cells compared to all other permutations of signals 1, 2, and/or 3, supporting the rationale that display of all three signals potentiates a robust CTL expansion. Characterization of the E7-TCR T cells post RTX-321 treatment showed enrichment for effector memory phenotype cells (CD45RO+, CCR7-), an increase in activation markers 4-1BB, PD-1, and CD25, and effector function by IFNγ production. Additionally, a signal 1 control aAPC, engineered to present an irrelevant CMV peptide on MHC I, did not induce T-cell mediated responses suggesting RTX-321 effects are driven by HPV E7 peptide on MHC I. Overall, these expanded antigen specific CD8 T cells demonstrated elevated activation status, memory-like phenotype and cytotoxic markers supporting RTX-321's capability for improved responses toward tumor killing. Because human red cells are cleared within hours in mice, a mouse surrogate model mRBC-321 was generated to assess efficacy and toxicity. mRBC-321 presents MHC class I H-2K^b loaded with OVA 257-264 peptide, murine 4-1BBL, and murine IL-12. Studies in the EG7.OVA tumor model with adoptive cell transfer of OVA antigen-specific OT1 cells showed that mice treated with mRBC-321 had dramatic delays in tumor growth compared to mice dosed with mRBC only. Mice, previously treated and cured with mRBC-321, rejected re-challenge with EG7.OVA compared to EG7.OVA challenged age-matched naïve mice, indicative of a robust memory response driven by initial mRBC-321 treatment. These results support the potential of RTX-321 as an effective aAPC. We plan to file an Investigational New Drug application for RTX-321 for the treatment of patients with HPV 16 positive solid tumors by the end of 2020.

322. Modeling the Role of Tumor-Derived Extracellular Vesicle Mediated Resistance in Pediatric High Grade Gliomas

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Background: Pediatric high-grade gliomas (pHGGs) are the most common cause of cancer death in children (accounting for 26 percent). These tumors are mostly inaccessible to chemotherapy and surgical resection. The use of cancer immunotherapy, a means of harnessing the host immune system to attack tumors have proven to be effective against a growing number of cancers. However, pHGGs are one notable exception thus far. A pressing question is whether and how tumor-derived extracellular vesicles (tEVs) modulate immune responses to these tumors. Transcriptome profiles of gliomas has identified different molecular subtypes, two of which, proneural (PN) and mesenchymal (MES), predominate but with multiple glioma subtypes within the same tumor. Proneural to mesenchymal transition (PMT) has been associated with pronounced resistance to therapy and recurrence. ***Methods:*** We have developed a culture system to model PMT. Pediatric glioma stem-like cells are grown as neurospheres in serum-free neurobasal medium. When serum is added they differentiate into mature cells (glial cells, neurons, astrocytes). After several days, some cells detached from the monolayer and grow as floating cells (FC), especially under acidic or hypoxic conditions. When these FCs are put back into neurobasal medium, they resumed their spherical morphology and self-renewal properties. These FCs carry MES gene signatures, including increased CD44 expression and activation of NFkB transcription factor, and yield aggressive tumor growth and resistance to therapy. We then evaluated whether EVs collected from MES GSCs could induce a MES phenotype in recipient PN cells. We then evaluated whether tumor derived extracellular vesicles (tEVs) collected from MES pHGG cells could induce a MES phenotype in recipient PN pHGG cells. ***Results:*** This PMT model was reproducible in cultures from 6 pHGGS patients, in which tEVs modulate the transcription factor profile of other tumor cells through their ability to carry RNA and cytoplasmic/nuclear proteins from MES cells to PN cells. Using this system, we developed a multiplex high-throughput assay to screen for drugs that block PMT in pHGGs. ***Conclusions:*** We showed that tEVs are a critical component of PMT induced therapeutic resistance and it will be important to intercept this route of communication could inhibit migration/invasion, recurrence and therapeutic resistance.

323. Sialidase-CAR-T - A Next Generation Cellular Immunotherapy to Elicit Cooperative Killing From Unmodified Innate Immune Cells

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Clinical success has been demonstrated in recent years with the FDA approval of CAR-T cells targeting CD19 for a wide-range of hematological malignancies. Despite these achievements, CARs have lacked efficacy in the treatment of solid tumors due to a number of challenges, including overcoming the dense immunosuppressive tumor stroma and post-translational modifications on the tumor that favor survival. Our work aims to improve CAR-T cell therapy against solid tumors by targeting the tumor-specific glycoepitope Tn-MUC1, and enhancing cytotoxic effects of endogenous and unmodified immune cells by engineering sialidase function on CAR-T cells. Previous studies have demonstrated precedence for this approach showing that coupling current immunotherapies with glycoediting activity increases therapeutic efficacy. For example, CAR-T cells engineered to express heparanase showed an improved degradation of the extracellular matrix, which promoted T cell infiltration of the tumor and antitumor activity. Additionally, sialidase activity coupled to trastuzumab enhanced endogenous NK anti-cancer efficacy. Here, we present a cellular therapeutic approach where we designed a novel cell-surface human sialidase and expressed it on CAR-T cells targeting Tn-MUC1. Truncated, cancer-specific glycoforms, such as Tn-MUC1, are thought to play a role in decreasing cell-cell interactions and increasing the metastatic potential of cancer cells. We established that

Tn-MUC1-CAR-T cells induce minimal cytotoxic effects against the aggressive PC3 prostate cancer cell line, when co-cultured alone at equal effector:target ratios. When PC3 cells are treated with a *Clostridium perfringens*-derived sialidase, NK cells and Tn-MUC1-CAR-T cells show enhanced IFN-γ production and improved cytotoxic effects are observed compared with NK cells and Tn-MUC1-CAR-T cells alone. Similarly, these effector enhancements are observed in the presence of T cells presenting a human cell-surface sialidase. Taken together, these data suggest that glycoediting of tumor sialic acid presents a rational and unique opportunity to overcome barriers for adoptive immunotherapies in solid tumors, and engagement of the endogenous immune system by adoptively transferred cells is an effective strategy for improved anti-tumor activity.

324. Immunological Role of Altered Expression of T-Cell Immunoglobulin- and Mucin Domain-Containing (TIM)-3 Receptor on Natural Killer Cells in Glioblastoma

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Natural killer (NK) cells are part of the innate immune system, but are capable of participating in both innate and adaptive immune responses due to their wide range of cytolytic activities, from degranulation, secretion of cytokines to antibody-dependent cell-mediated cytotoxicity. These are possible due to the cells' ability to recognize self and non-self-entities via the net signal generated from their activating and inhibitory receptors upon engagement. One such receptor is Tim-3, which is expressed on various lymphocytes. In T-cells, Tim-3 is an exhaustion marker, but on NK cells, results are conflicting in regards to its function as the receptor exhibits both activating and inhibitory effects depending on disease type and activation status. We have found that Tim-3 is significantly downregulated on primary human NK cells, in both frequency and surface density, when exposed to solid tumor cells such as glioblastoma (both U87 and patient-derived GBM43 cells) and prostate cancer (PC3) cells at different E:T ratios under specific priming conditions. However, it is unknown why the downregulation occurs with solid tumors, and whether this change in expression affects NK killing capacity. Here, we report the role of TIM-3 on NK cell cytotoxicity against GBM43 cells in the context of killing percentage, degranulation and cytokine secretion. We also profile the expression of TIM-3 on clinical GBM samples to infer its presence in pathological settings. This information allows us to better understand the nuanced immunomodulatory role of Tim-3 on NK cell anti-tumor responses, and provide a basis for the development of immunotherapies targeting impaired NK cell function in solid tumors.

325. Evaluation of Tumor-Associated Antigen MUC1 Expression with the MACSima™ High-Content Imaging Platform

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We report the use of the MACSima™ imaging platform to perform high-content imaging for the pre-clinical validation of tumor target expression on tumor and healthy human tissues indicative for potential on-target/off-tumor toxicity in vivo. Major advances have been achieved in cancer therapy in the past decade. In particular, targeted-immunotherapy has progressed and clinical benefits for patients have been achieved. However, on-target/off-tumor toxicity is a potential threat which has been shown to be more pronounced in solid than in liquid tumors. These findings highlight the need for better pre-clinical assays to improve the safety profile of immunotherapies. On-target/off-tumor toxicity is mainly based on the expression of tumor-associated antigens (TAA) in healthy tissues under physiological conditions. Currently, most prediction methods for on-target/off-tumor expression are based on bulk mRNA expression data of healthy tissue. These prediction models, however, have limitations, mainly poor predictable relation between RNA and protein level. Moreover, it is frequently unclear which cell types are affected by on-target/off-tumor effects. We employ multi-parameter imaging to overcome these limitations to analyze the expression of TAAs directly at the protein level. Additionally, we gain spatial information about tissue and cellular distribution of TAAs. Notably, our novel high-content imaging technology potentially allows for the analysis of hundreds of markers in a single experiment, paving the way for high-dimensional characterization of cells within complex solid tissues. High-content imaging with the MACSim™ platform was performed to validate the expression of known TAAs across tumor and healthy tissue samples. Subsequently, we employed unbiased cluster analysis revealing correlation patterns within the datasets to identify cell types at risk. In detail, we analyzed several high-grade serous ovarian carcinoma and pancreatic ductal adenocarcinoma for the expression of known TAAs, described tumor markers, and tissue lineage markers. Additionally, we evaluated the expression of these TAAs across several healthy human tissues. Next we performed pixel- and object-based data analysis for unbiased cluster analysis. Thereby we identified cell clusters that express TAAs in ovarian and pancreatic cancers, as well as in healthy tissues. We found that primarily epithelial cells express the analyzed TAAs in different tissues and that TAA expression shows inter- and intra-tumor heterogeneity. In conclusion, we established a novel workflow for multi-parameter characterization of tissues employing the MACSima™ imaging platform. Our work enables efficient identification of potential tissues for on-target/off-tumor toxicity, paving the way for improved pre-clinical evaluation of TAAs as new targets.

326. Bicistronic CAR Constructs for Dual CD19 and CD20 Targeting

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Chimeric antigen receptors (CARs) are fusion proteins with antigen-recognition domains and T-cell signaling domains. Two anti-CD19 CAR T therapies have been approved by the U.S. Food and Drug Administration for treatment of B-cell lymphoma and acute lymphoid leukemia (ALL). However, 30-70% of initial responders with ALL will eventually relapse with CD19-negative disease (Orlando, E. Nature Med. 2018). Loss of CD19 highlights the importance of targeting other antigens in addition to CD19 by CAR T cells. Loss of CD19 expression by lymphomas after anti-CD19 CAR T-cell therapy has also been observed (Brudno J.N. ASCO abstract 2018). CD20 is a phosphoprotein expressed on all stages of B cell development except for early pro-B cells and plasma cells. Cell surface density of CD20 is higher than CD19 in normal B cells and in in B-cell leukemias including CLL and B prolymphocytic leukemia (B-PLL). In diffuse large B-cell lymphoma, one study reported that CD20 expression was well-preserved in nineteen post-relapse tumor samples with loss of CD19 after anti-CD19 CAR T therapy. Immunohistochemistry analysis demonstrated that CD20 was coexpressed with CD19 in nearly all of 96 pretreatment tumor samples despite all patients having previously relapsed after receiving rituximab-based regimens (Neelapu, S.S. ASH abstract 2019). Taken together, the loss of CD19 expression in lymphoma and leukemia and the consistent expression of CD20 on a variety of lymphoma and leukemia encouraged us to design bicistronic CAR constructs targeting both CD19 and CD20 simultaneously. We designed bicistronic CAR constructs including both the previously-reported fully-human anti-CD19 CAR Hu19-CD828Z and one of a series of novel anti-CD20 CARs. The constructs were encoded by a gamma-retroviral vector which allowed simultaneous expression of both an anti-CD19 CAR and an anti-CD20 CAR. The construct design was the anti-CD19 CAR, a 2A self-cleavage peptide, and the human anti-CD20 CAR. Both the anti-CD19 and anti-CD20 CAR had CD8α hinge and transmembrane domains. The anti-CD19 CAR had a CD28 costimulatory domain and the anti-CD20 CARs had 4-1BB costimulatory domains. T cells expressing the bicistronic CAR constructs exhibited strong antigen-specific biological activities *in vitro* including cytokine release, degranulation, cytotoxicity, and proliferation. Because of the presence of rituximab in the serum of patients after rituximab treatment, we tested rituximab interference on the function of anti-CD20 CAR T cells *in vitro* and *in vivo*. We found no blocking of anti-CD20 CAR T-cell recognition towards cell lines with higher levels of CD20. In cell lines with modest CD20 expression, approximately 50% of CD20-specific IFNγ release was retained when clinically-relevant concentrations of rituximab were added to CAR T cell plus target cell co-cultures. T cells expressing bicistronic CARs did not recognize fifteen CD20-negative primary cell lines and tumor lines without CD19/CD20 expression. Anti-CD19/CD20 bicistronic CAR T cells eradicated pre-established tumors of 3 different lymphoma cell lines in immunocompromised nod-scid common gamma-chain knockout (NSG) mice. Anti-CD19/CD20 CAR T cells eradicated CD19-negative lymphoma tumors in mice. In addition, anti-CD19/CD20 bicistronic CAR T cells completely eradicated pre-established tumors in the presence of rituximab. We conclude that redirecting T cells with two independent CARs via a bicistronic construct is a promising approach to prevent CD19 antigen loss-induced clinical relapse, and we have initiated a clinical trial of T cells expressing a bicistronic anti-CD19/CD20 CAR construct.

327. Preclinical Development of Synthetic DNA Delivery Technology for the *In Vivo* Launch of a Bispecific T Cell-Engager Targeting HER2

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Bispecific antibody constructs targeting the T cell and tumor antigens have been successfully employed to mediate T cell killing of tumor cells. However, the widespread application of such therapies has been hindered by the difficulties associated with the manufacturing of such constructs, their instability and short *in vivo* half-lives. A simplified way to produce and deliver these therapies could advance their widespread usage and increase patient uptake. We have designed and engineered synthetic DNA plasmids which are optimized for *in vivo* expression to encode functional bispecific T cell-engagers. This technological advancement bypasses the requirement for the complex manufacturing and delivery procedures associated with recombinant constructs, and permits durable *in vivo* expression of a functional construct after a single administration. Here we describe the proof of concept and scalability of the technology to achieve durable *in vivo* production of tunable levels of a bispecific T cell-engager targeting the HER2 tumor antigen. In preclinical models we demonstrated that expression of this bispecific T cell engager mediates the killing of HER2-expressing tumors. In side by side studies we illustrate the increased durability of the *in vivo* expressed construct compared to its recombinant counterpart. In large preclinical animal models we confirm the scalability of the approach after a single administration of the synthetic DNA plasmid to muscle or skin tissue. In summary, these studies highlight the potential of a novel *in vivo* gene delivery approach to harness the power of bispecific T cell engagers while addressing many of the current limitations associated with recombinant constructs. The synthetic DNA-based HER2-targetting construct may represent a highly effective immunotherapy in the treatment of ovarian and other cancers.

328. Preclinical Development of Chimeric Antigen Receptor T-Cell Therapies Targeting CD30+ Lymphomas

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CD30 is a cell-surface protein expressed on Hodgkin lymphomas as well as some T-cell and B-cell lymphomas. CD30 is not expressed on normal organs, so CD30 has a favorable expression pattern for a chimeric antigen receptor (CAR) target. Drawbacks of targeting CD30 include expression on activated T cells. We designed and constructed 3 anti-CD30 CARs that each incorporated one of 3 single-chain variable fragments. When evaluated with in vitro cytokine-release and degranulation assays, the fully-human 5F11 scFv was associated with the best CD30-specific T-cell activation of the 3 scFvs. We compared 3 different CAR designs incorporating the 5F11 scFv; 5F11-28Z had hinge, transmembrane, and costimulatory domains from CD28 and a CD3-zeta activation domain; 5F11-CD828Z had hinge and transmembrane domains from CD8-alpha, a CD28 costimulatory domain, and CD3-zeta; 5F11-CD8BBZ was identical to 5F11-CD828Z except for replacement of the CD28 moiety with a 4-1BB moiety. Compared with T cells expressing CARs containing CD28, T cells expressing 5F11-CD8BBZ had lower levels of CD30-specific degranulation and cytokine release. T cells expressing 5F11-CD8BBZ also had relatively high non-specific interferon gamma release when stimulated with CD30-negative target cells. We established tumors of the CD30+ human HH lymphoma cell line in nod-scid common gamma-chain knockout (NSG) mice and treated the tumors with T cells expressing different CARs. We found a hierarchy of effectiveness at eradicating tumors. T cells expressing 5F11-28Z were most effective. T cells expressing 5F11-CD828Z had intermediate effectiveness, and T cells expressing 5F11-CD8BBZ were least effective. One dose of $2x10^6$ 5F11-28Z CAR T cells could eradicate HH tumors, and 5F11-28Z T cells also eradicated tumors of the L428 CD30+ lymphoma cell line. Hodgkin lymphoma is made up of a small number of malignant Hodgkin Reed-Sternberg (HRS) cells with much more numerous non-malignant leukocytes. HRS cells originate from B cells as shown by the presence of clonal B-cell receptor gene rearrangements in HRS cells; 12-20% of Hodgkin lymphoma cases have HRS cells that express B-cell antigens such as CD20. Clonal populations of blood CD19+ B cells with B-cell receptor sequences matching those of the patient's HRS cells have been detected. We hypothesized that treating Hodgkin lymphoma with T cells targeting both CD30 and CD19 might be able to eliminate CD19+ HRS stem cell as well as CD30+ HRS cells. In addition, some non-Hodgkin lymphomas express both CD30 and CD19, providing another rationale for dual targeting of CD30 and CD19. Three strategies for targeting CD30 and CD19 simultaneously are: tandem CARs that include 2 antigen-binding domains fused together in the same CAR, bicistronic CAR constructs expressing 2 separate CARs from the same gene-therapy vector, and co-transduction of the same T-cell population with 2 different gene-therapy vectors each encoding a different CAR. Co-transduction yields a mixed population of T cells expressing either of the 2 CARs or both CARs. We designed tandem CARs and bicistronic CAR constructs targeting both CD30 and CD19. We have also tested the co-transduction approach by transducing T cells simultaneously with one vector encoding an anti-CD30 CAR and one vector encoding an anti-CD19 CAR. In vitro, we demonstrated CD30 and CD19-specific cytokine-release and degranulation with all 3 of the simultaneous targeting strategies. We have demonstrated anti-tumor activity against both CD30-negative, CD19+ tumors and CD30+, CD19-negative tumors in mice with the tandem CAR strategy. Studies to determine the optimal strategy to target both CD30 and CD19 are continuing.

329. CD123-CAR NK Cells are Activated by and Kill Acute Myeloid Leukemia

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Purpose: NK cell adoptive transfer is a promising anti-cancer immunotherapeutic tool. The degree of NK cell mediated cytotoxicity is determined by a constellation of competing inhibitory and activating signals. Genetic modification of NK cells to favor activation when bound to tumor target is one strategy that can overcome tumor evasion mechanisms and improve specific cytotoxicity. We aimed to elicit a specific and enhanced antitumor response by engineering NK cells with Chimeric Antigen Receptors (CARs) .We designed 9 different CARs, all targeted to the AML-associated surface antigen CD123 (IL3Ra), utilizing combinations of DAP10, 2B4, 4-1BB, TCRζ, and FcεRIγ hinge, transmembrane, and intracellular domains. **Methods**: CARs were synthesized with standard cloning techniques. To generate CAR-NK cells, healthy donor PBMCs were depleted of CD3+ cells, then stimulated with lethally irradiated K562.mbIL15.41BBL feeder cells. Cells were maintained in IL-2 and transduced with transiently produced γ-retrovirus on D4 of culture. CAR expression was measured on D8 using FACS. Co-culture assays were performed using Raji (CD123-) and MV-4-11 (CD123+) cancer cell lines to assess NK cell activation and cytotoxicity. Supernatant following 24-hour co-culture was used for quantification of IFN-γ by ELISA. Cytotoxicity was evaluated by FACS following 72hr co-culture. **Results:** CAR-NKs containing transmembrane 2B4 or CD8α demonstrated transduction efficiencies ranging between 70-98% as well as high CAR surface density. DAP10 and FcεRIγ containing CARs had lower CAR-surface expression and percent transduced cells (25-83%). All CAR-NKs showed good proliferative capacity with fold expansion ranging from 61-197X calculated on day +18. CAR-NKs demonstrated CAR-specific activation with increased IFNγ production following co-culture with CD123+ MV-4-11 as compared to unmodified NK cells. There was no increase in IFN-γ secretion, again compared to unmodified NK cells, when CAR-NK were in co-culture with CD123-negative Raji cells. Analysis of CAR-NK cytotoxicity revealed 2B4.ζ and 4-1BB.ζ as powerful stimulants of NK cytotoxicity against MV4-11 in both 1:1 (2B4.z: 98±1.4%; 41BB.z: 97±1.6%) and 1:5 E:T ratio co-cultures (91±7.4%; 96±0.1% respectively). There was no observed difference in killing of Raji between unmodified and CAR-NK cells. **Conclusion:** Higher transduction efficiency and CAR surface expression in NK cells were evident if CARs had transmembrane domains derived from 2B4 or CD8α. All CAR-NKS demonstrated higher IFNγ production in co-cultures with MV-4-11 cells compared to unmodified cells, indicating CAR-stimulated production of cytokine. The presence of a costimulatory molecule (4-1BB or 2B4) plus an activation domain (CD3ζ) was associated with higher IFN-γ production. 2B4.ζ and

41BB.ζ CARs demonstrated potent anti-AML cytotoxicity. All of the NK cells, including unmodified NK cells, equivalently killed CD123-negative Raji cells, suggesting antigen and CAR-enhanced specificity of killing. With further optimization, CD123-CAR NK cells may offer a therapeutic option for AML treatment.

330. Base Editor-Mediated CD33 Engineering in Hematopoietic Stem and Progenitor Cells to Improve CD33-Targeted Cancer Therapies

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CD33 is a sialic acid binding receptor expressed on normal myeloid cells as well as in some malignancies, including acute myeloid leukemia (AML). For this reason, CD33 immunotherapies have been used as treatment but these therapies are limited by on-target/off-leukemia toxicity on healthy hematopoietic cells, which may lead to life-threatening cytopenia in treated patients. Recent studies by our group and others demonstrated that CRISPR/Cas9-editing of CD33 in hematopoietic stem and progenitor cells (HSPCs) efficiently generate CD33-null hematopoiesis without compromising cell engraftment and multilineage differentiation. Importantly, edited cells were protected in vivo from CD33-directed drugs whereas non-edited cells were efficiently eliminated. While promising, this CRISPR-based strategy suffers from off-target activity due to cleavage of a nearby CD33 homolog pseudogene and from activation of endogenous TP53-mediated DNA damage responses. To address these limitations, we have explored the use of base editors (BE) that introduce precise genetic alterations without the need for double-stranded DNA breaks. We first validated several targets using cytidine BEs to engineer CD33 non-sense and splicing mutations in human CD34+ HSPCs. Normal engraftment and differentiation of these edited cells was established in the mouse xenotransplant model, resulting in hematopoiesis deficient for CD33 expression. Next-generation sequencing analysis of peripheral blood nucleated cells confirmed the in vivo persistence and specificity of BE-induced mutations. Edited cells were protected from treatment with the CD33 antibody drug conjugate gemtuzumab ozogamicin (GO). This was evidenced by a drop in CD14+ blood monocytes in GO treated animals, which correlated with in vivo editing levels. Together, these results validate the use of BE for the generation of CD33-engineered hematopoiesis to improve safety and efficacy of CD33-targeted therapies.

331. Preclinical Development of Novel Synthetic DNA Encoded Multi-Neoantigen Targeting Vaccine

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Neoantigens are tumor specific antigens that arise because of somatic mutations in DNA of tumor cells. These epitopes can arise because of single point mutations, frameshift mutations or gene fusions. Neoantigens represent an ideal target for cancer vaccines which have traditionally focused on Tumor Associated Antigens (TAAs). Neoantigens are highly specific to the tumor and hence there is minimal risk of adverse events resulting from 'On target Off tumor activity' that are associated with targeting TAAs. Additionally, neoantigens are foreign antigens and have the potential to be highly immunogenic since they are not subject to central immune tolerance. Multiple approaches are being investigated for targeting tumor neoantigens, such as vaccines delivered as DNA, RNA, dendritic cells and peptides; or adoptive transfer of neoantigen targeted T cells. Initial studies using peptides and mRNA based vaccines targeting neoantigens revealed primarily $CD4^+$ T cell driven immune response in pre-clinical models as well as in the clinic. In addition, these platforms have complex manufacturing processes which puts an enormous economic burden on the healthcare system. Synthetic DNA vaccines have recently shown strong $CD8^+$ and $CD4^+$ T cell responses in several clinical trials. Here we report development of synthetic DNA encoded vaccine targeting 40 neoantigens in a MC38 mouse model of colon carcinoma. Vaccinating C57BL/6 mice with MC38vax led to strong immune responses as measured by IFN-γ ELISPOTS. Flow cytometric analysis revealed that this response was almost entirely MHC class I $CD8^+$ T cell mediated. In addition, there was increased expression of degranulation marker CD107a on $CD8^+$ T cells, indicative of increased cytolytic function. MC38vax was able to prolong mouse survival in absence of immune checkpoint blockade molecules. Experiments are ongoing to test synergy of this approach with immune checkpoint molecules.

332. PD-L1-Blocking scFv Secreting, Genetically Engineered NK Cells Induce Potent Anti-Tumor Immunity in Glioblastoma

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Background: Despite aggressive treatments and care, the median life expectancy for GBM patients is still only around 15 months, highlighting the need for new therapeutic approaches. NK cells, innate cytotoxic effectors, are showing potential for cancer immunotherapy including GBM. Even though GBM tumors are infiltrated by NK cells [1]. However, their activities are impaired by the immunosuppressive TME *via* various mechanisms, including adenosine-mediated downregulation of NKG2D [2]. Therefore, it is critical to understand more about how the TME in GBM modulates the NK cell-mediated immunity so that we can develop novel NK cell-based therapies specifically for GBM. **Methods:** We isolated peripheral blood NK cells from healthy volunteer donors (**Fig. 1A**). K562, U87MG and GBM43 cells were used as targets. To investigate PD-L1 expression on NK cells, we co-cultured the NK cells with above three cancer target cells for 24 h at an E:T ratio of 10:1. We have generated gene-modified NK cells expressing NKG2D CAR directed against its ligands alongside secreting anti-PD-L1 scFv targeting PD-L1. These cells are being evaluated for their efficacy against GBM *in vitro* and in orthotopic intracranial mouse xenografts. **Results:** We have found that that both U87MG and GBM43 as well as K562 cells can significantly upregulate PD-L1 expression on NK cells from the different donors. Interestingly, compared with K562 cells, the effects induced by GBM43 and U87MG cells are both lower,

while, the increase in PD-L1 expression induced by U87MG cells is higher than that due to GBM43 cells (**Fig. 1B**). When we looked at the expression of major histocompatibility complex (MHC) class I molecules, namely HLA-A, B, C antigen on these three cancer cell lines, we found that K562 cells have the lowest expression, while GBM43 cells have the highest (**Fig. 1C**). In addition, we also examined the expression of PD-L1 on these target cell lines and found that U87MG cells have the highest expression, while GBM43 cells have the lowest expression (**Fig. 1D**). Based on these results, we have designed and synthesized a multifunctional CAR construct that enables NK cells to express a secretable PD-L1 blocking scFv and a CAR redirected against ligands for NKG2D (**Fig. 1E**). **Conclusions:** We have for the first time shown that GBM cells can induce PD-L1 expression on NK cells. Additionally, based on the HLA-A, B, C expression, this enhanced expression capacity may be associated with target susceptibility to NK cell cytotoxicity. At the same time, the increase in expression of PD-L1 appears to bear no relationship with the stimulating cells' own levels of PD-L1 expression. Stimulated by these results, we have been generating multifunctional NK cells that can, at once, secrete the PD-L1-blocking scFv to target PD-L1 both expressed on GBM cells and NK cells, and express the NKG2D CAR to specifically target its ligands on GBM cells. Based on these results, we are currently testing the *in vitro* and *in vivo* therapeutic efficacy of these engineered NK cells. And we believe our findings will provide an advanced NK cell-based immunotherapy for GBM.

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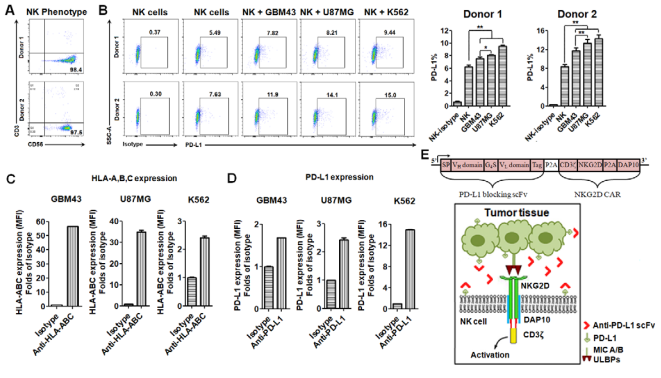

Fig. 1. (**A**) Phenotype of isolated human primary NK cells (CD56^{+}CD3^{-}); (**B**) PD-L1 expression on human primary NK cells after co-incubation with different types of cancer target cells, K562 (human leukemia cell line), U87MG (human glioblastoma cell line) and GBM43 (a human patient derived glioblastoma cell) at a E/T ratio of 10 for 24 h, respectively; (**C**) HLA-A,B,C expression on different cancer target cells, including K562, U87MG and GBM43 cells; (**D**) PD-L1 expression on different cancer target cells, including K562, U87MG and GBM43 cells; (**E**) Multifunctional genetically engineered NK cells for GBM immunotherapy. Data are presented as the mean ± SEM. *P < 0.05 and **P < 0.01.

333. Screening the Membrane Proteome to Determine Antibody Specificity and De-Risk CAR-T Cell Development

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Off-target reactivity is the single largest cause of failed preclinical drug programs, accounting for 62% of all failures. We developed the Membrane Proteome Array (MPA) platform to de-risk lead selection by testing biotherapeutics for specificity and off-target binding. The MPA enables safety analysis by screening antibodies against an expression array of 6,000 membrane proteins. Each membrane protein is presented in live cells in its structurally intact and native conformation with human post-translational modifications. Binding interactions are assayed by high-throughput flow cytometry allowing for high sensitivity detection and rapid analysis. In the process of testing hundreds of antibodies, we found that, despite their presumed specificity, up to 20% of monoclonal antibodies (MAbs) exhibit detectable off-target binding. In many cases, off-target interactions occurred with unrelated proteins and could not be predicted by protein sequence homology. We present a case study where a MAb targeting the SLC2A4 transporter showed cross-reactivity to Notch1, a protein with no structural or functional similarity. The unexpected cross-reactivity was explained by a shared epitope contained in a six amino acid loop that was mapped on SLC2A4. A second case study shows the ramification of low-level cross-reactivity of a Claudin 6 (CLDN6) MAb with the highly homologous Claudin 3 (CLND3) protein as revealed by the MPA. CAR-T cells formatted with this MAb as an scFv CAR were robustly activated by both CLDN6 and CLND3 antigen, suggesting that even minimal off-target reactivity is sufficient to activate the killing mechanisms of CAR-T cells, with possible serious consequences. In a third case study, we show that the MPA is capable of screening for non-specific activation of CAR-T cells in addition to binding interactions. In summary, our findings indicate that detailed specificity profiling is necessary to better predict off-target binding and de-risk preclinical biotherapeutics prior to entering first-in-human studies.

334. Adaptive Immune Responses Against Solid Tumors Mediated by Macrophage Checkpoint Blockade

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Introduction: The macrophage checkpoint CD47-SIRPα is emerging as a clinical target against hematologic and potentially solid tumors. CD47 is considered to be a marker of 'self' on healthy cells and tumor cells, inhibiting phagocytosis when it binds its macrophage receptor, SIRPα. When combined with a tumor-opsonizing antibody such as rituximab against lymphoma, blockade of CD47-SIRPα with anti-CD47 has shown a fraction of patients respond in small, early phase clinical trials. However, major safety and efficacy challenges remain. The ubiquitous expression of CD47 results in significant off-target toxicities by phagocytosis of healthy cells, and efficacy against solid tumors and metastasis remains uncertain. Here, we validate a solid tumor animal model of melanoma that re-capitulates some key human patient responses in that anti-CD47 on its own shows no efficacy and neither does the tumor-opsonizing antibody. We demonstrate an ability of macrophages to initiate systemic adaptive immunity and apply these insights to the development

of a safer, efficacious macrophage-based preclinical cell therapy. **Methods:** The B16F10 syngeneic melanoma model in immunocompetent C57BL6/J mice is known to be minimally immunogenic and is widely used to challenge pre-clinical immunotherapies against solid tumors. CRISPR-Cas9 editing enables complete ablation of CD47 signaling in B16F10 tumors; importantly, these cells grow normally in vivo relative to wild-type (WT) CD47-expressing control tumors, matching clinical observations that anti-CD47 monotherapy is never effective. Anti-Tyrp1 is a monoclonal antibody that binds the melanoma cell surface, providing an opsonizing, pro-phagocytic signal via macrophage Fc-receptors. Primary bone-marrow derived-macrophages are harvested and differentiated with M-CSF to assess in vitro phagocytosis of intact and blocked CD47. Engineered marrow cells are generated by harvesting fresh donor C57 marrow, pre-loading Fc-receptors with anti-Tyrp1 to prime eating, and blocking CD47-SIRPα with anti-SIRPα. These marrow cells are quickly re-infused into tumor-bearing mice. **Results**: Phagocytosis of B16F10s in vitro occurs at low levels when only CD47 is blocked or anti-Tyrp1 opsonizes Fc-receptors but increases 2-3-fold in the presence of both blockade and opsonization. Off-target eating of CD47-blocked red blood cells in vitro also abrogates on-target eating of B16F10s. CD47 knockout B16F10 tumors in combination with anti-Tyrp1 produces some durable, reproducible cures in primary subcutaneous tumors and clearly suppresses tumor growth in lung metastases. Cured mice resist tumor growth when re-challenged with the same cells but without additional anti-Tyrp1, implicating a persistent adaptive immune response. In contrast, NSG mice that lack any adaptive immunity only show delayed growth of CD47 knockout tumors in some treated mice without any cures. Serum from treated mice exhibit increased levels of anti-B16 IgG and a clear broadening of epitopes beyond Tyrp1. WT CD47 tumors treated with engineered marrow show similarly delayed growth and curative potential, and importantly, no significant anemia is observed unlike anti-CD47 infused in both mouse and human. **Conclusion:** CD47-SIRPα checkpoint therapies can shrink hard-to-treat tumors that are widely used in immunotherapy development but only when combined with an opsonizing antibody. Cures associate with robust adaptive immune responses. Macrophage-based adoptive cell therapy offers potential to overcome challenges with anti-CD47 based checkpoint blockade for treatment of solid tumors.

335. A Novel CAR Containing JAK-STAT Signaling Domain Demonstrates Superior Antitumor Effects Without Inducing Non-Specific Activation

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Adoptive cancer immunotherapy using chimeric antigen receptor (CAR) gene modified T cells have shown impressive clinical results. Despite recent successes, considerable clinical challenges remain to be addressed. To improve T cell persistence and function, which are critical for clinical responses, various efforts have been made to optimize the CAR design such as the inclusion of a costimulatory domain(s). It is known that non-specific activation of CAR-T cells is greatly influenced by the CAR design. Indeed, excessive T cell activation leads exhaustion and depletion of naïve/memory subsets important for durable clinical responses. Thus, the CAR construct needs to be optimized so that transduced T cells persist and induce potent antigen-specific response with minimal non-specific activation, which results in maximal efficacy and minimal toxicity. Three signals, which are TCR signal (signal 1), co-stimulatory signal (signal 2), and cytokine signal (signal 3), are essential for optimal T cell activation and proliferation. Second generation CARs contain CD3ζ and a co-stimulatory domain such as an intracellular domain of CD28 and 4-1BB but not a cytokine signaling domain. Recently, we have developed a new generation JAK-STAT CAR composed of a truncated cytoplasmic domain of the IL-2 receptor β chain and STAT3/5 binding motifs, CD28 co-stimulatory domain, and CD3ζ domain. The novel anti-CD19 JAK-STAT CAR-T cells showed antigen-specific activation of the JAK-STAT signaling pathway, enhanced proliferation, and limited terminal differentiation *in vitro* compared to second generation CAR-transduced T cells. Furthermore, the anti-CD19 JAK-STAT CAR-T cells demonstrated superior *in vivo* persistence and antitumor effect in multiple mouse models (Kagoya et al., Nat Med. 2018). In addition, we previously showed that a hinge region and the composition of a single chain variable fragment (scFv) such as the order of VH and VL regions critically influence not only antigen-dependent activation but also undesired antigen-independent activation known as tonic signaling. In this study, we have explored the optimization of 28ζ CAR and JAK-STAT CAR constructs. We have found that CAR constructs encoding scFvs in a VL-VH orientation consistently showed superior antigen-specific activation and reduced tonic signaling compared to CAR with scFvs in a VH-VL orientation for all the targets tested (CD19, CD20, Mesothelin, and GD2). Moreover, JAK-STAT CAR-T cells showed superior antigen-specific proliferation with less differentiated status, whereas 28ζ CAR-T cells showed antigen-independent proliferation and displayed higher exhaustion marker expression after repetitive stimulations. These results suggest that our JAK-STAT-CARs with the optimized scFv design possess enhanced antigen-specific response with reduced tonic signaling, potentially representing a new generation CAR construct with improved efficacy. Clinical translation of our novel JAK-STAT CARs targeting various antigens is warranted.

336. TurboCAR™ T Cells: CAR T Cells with Constitutive, Programmable Cytokine Signaling Outputs

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CAR T cell therapy has attained unprecedented success in the treatment of hematological malignancies. However, clinical benefit in solid tumor indications has been limited, potentially in part due to suppressive solid tumor microenvironments (TME) that inhibit T cell effector function and persistence. While the provision of cytokine support can help CAR

T cells overcome suppressive TME, combining CAR T therapy with systemically-administered cytokines/cytokine mimetics can result in toxicities and adverse events. Even cell therapies locally secreting cytokines can still activate neighboring cells and cause pleiotropic effects. To gain the benefit of cytokine stimuli without adverse effects, we designed TurboCAR™ T cells, which are CAR T cells co-expressing an inducible chimeric cytokine receptor or Constitutively Active Chimeric Cytokine Receptor (CACCR). CACCRs are homodimeric receptor chimeras that mimic cytokine signaling in a constitutive, CAR T cell-intrinsic fashion. For example, CACCRs may comprise (i) a membrane-tethered dimerization and JAK-binding domain derived from the thrombopoietin receptor (TpoR), fused to (ii) one or more intracellular signaling domains derived from the cytokine receptor(s) of interest. Mutations in the TpoR transmembrane domain result in constitutive receptor dimerization and activation of JAK2, which in turn phosphorylates the cytokine receptor signaling domains and cause STAT signaling. Each of these domains were individually optimized, and then combinatorially fused to generate CACCRs that mimicked downstream STAT signaling of cytokine receptors of interest. CACCRs mimicking signaling from multiple cytokines were generated. An EGFRvIII tool CAR was utilized to screen and select CACCRs based on TurboCAR™ T cell manufacturability and in vitro serial killing activity. Functional benefits conferred by CACCRs were further validated in the context of a CAR directed towards BCMA in a range of functional assays, demonstrating improved activity compared to the parental CAR. In conclusion, CACCRs are novel homodimeric cytokine receptor chimeras that can be tailored through fusion of dimerization and signaling domains to increase TurboCAR™ T cell activity, while circumventing safety risks associated with cytokine combination therapy.

337. Abstract Withdrawn

338. Abstract Withdrawn

339. GD2-Redirected T Cells Encapsulated in Hydrogel Control Retinoblastoma and Protect Mouse Vision

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Retinoblastoma is a localized tumor of the eye that may benefit from conservative and localized treatment. We found that GD2 antigen is overexpressed in human Y79 retinoblastoma (Rb) cells and Rb tissues but not on the normal human retina. Thus GD2 can be used as a target in Rb immunotherapy. We developed a GD2-specific chimeric antigen receptor (CAR) studied and analyzed the antitumor effects of GD2.CAR T cells in vitro and an in-situ grafting murine model of retinoblastoma. GD2.CAR T cells effectively eliminated tumor cell lines in vitro but have limited effects on an engrafted animal model likely due to short persistence. When combined with interleukin 15 that promotes T cell proliferation, GD2.CAR T cells significantly controlled tumor growth, but tumor recurrence occurred in some animals. To further increase the local persistence of CAR R cells we then developed a biomaterial to encapsulate CAR T cells. We found that the chitosan-polyethylene glycol thermoresponsive hydrogels serves as a sustained delivery vehicle and extends the lifespan of enclosed lymphocytes. CAR T cells encapsulated in hydrogel eliminated the tumor and reduced tumor recurrence upon subretinal injection, with the absence of any adverse immune response and toxicity to healthy tissues. Compared to T cells suspended in PBS, encapsulation in hydrogel significantly improved the lifespan T cells and vision of mouse from ERG. This method is the first attempt to treat retinoblastoma using immunotherapy, and localized CAR T delivery using injectable hydrogel may be used for other localized solid tumors.

Cancer - Oncolytic Viruses

340. Mathematical Modeling for Systemically Administered Oncolytic Adenovirus Loaded with Mesenchymal Stem Cells

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To define both the growth dynamics of tumor xenografts model and the effects of the immune response during the mesenchymal stem cells (MSC)s-based oncolytic virotherapy, here we developed an integrated mathematical-experimental model. The model was utilized to explore the conceptual clinical feasibility, particularly, in evaluating the relative significance of potential immune promotive/suppressive mechanisms induced by MSCs loaded with oncolytic viruses. As a resultant, we found conditions which may significantly contribute to the success or failure of MSC-based virotherapy. Notably, one of the most impactful outcomes shown by this study was the initially counter-intuitive, since tumor-promoting MSCs as carriers is not only helpful but necessary in achieving efficient tumor control. Considering the fact that it is still currently a controversial debate whether MSCs exert a pro- or anti-tumor action, mathematical models such as this one help to quantitatively predict the consequences of using MSCs for delivering oncolytic viruses *in vivo*. Taken together, our results show that MSC-mediated systemic delivery of oncolytic viruses is a promising strategy for achieving synergistic anti-tumor efficacy with improved safety profiles

341. Triple Combination Immunotherapy Using Oncolytic Herpes Simplex Virus, G47Δ, Prolongs the Survival of Transgenic Mice with Pancreatic Ductal Adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related death and notable for its dense tumor stroma which is a major contributor to resistance to systemic chemotherapies and immune checkpoint inhibitors (ICIs). G47Δ is a triple-mutated, third generation oncolytic herpes simplex virus type 1 (HSV-1) that can selectively replicate in and kill tumor cells, inducing antitumor immune responses. Focal adhesion kinase inhibitor (FAKi) is a non-receptor tyrosine kinase inhibitor that depletes the stroma and suppresses the tumor growth. In this study, we examined the combination therapy of G47Δ, FAKi and ICIs, in a transgenic mice model of invasive PDAC. Methods: To mimic the tumor microenvironment, PKF (*Ptf1a*$^{cre/+}$;*LSL-Kras*$^{G12D/+}$;*Tgfbr2*$^{fl/fl}$) mice were used and treated with a single intratumoral injection with G47Δ (1×10^7 pfu), intraperitoneal injections with ICIs (anti-PD-L1 antibody 200μg/body and anti-CTLA4 antibody 250μg/body) every 4-5 days and daily oral FAKi administration (50mg/kg). For cytotoxicity assay, a PDAC cell line (#146) derived from *Pdx1*$^{cre/+}$;*LSL-Kras*$^{G12D/+}$;*Trp53*$^{fl/+}$ was used. Subcutaneous tumors were generated by implanting #146 cells to the flank of C57BL/6 mice and treated with G47Δ on days 4 and 7, and daily FAKi administration. Flowcytometry (FCM) analysis on tumor-infiltrating cells was performed on day 9 and the numbers of CD8(+) T cell, CD4(+) T cell, NK cell, regulatory T cell (Treg) and myeloid-derived suppressor cell (MDSC) in CD45(+) lymphocyte, as well as tumor-associated macrophage (TAM) and PD-L1 status, were analyzed. Histological analyses were performed with anti-CD8 antibody and anti-alpha smooth muscle Actin antibody (SMA). Results: *In vitro,* G47Δ and FAKi showed a synergistic cytotoxic effect. In #146 subcutaneous tumor models, the G47Δ and FAKi combination treatment significantly inhibited the tumor growth compared with FAKi alone ($p < 0.01$). The FCM analysis showed that the combination therapy significantly increased the infiltration of CD8(+)/CD45(+) cells within the tumor ($p = 0.01$) and decreased the MDSC/CD45(+) cells compared with each monotherapy ($p < 0.05$). The treatment with G47Δ significantly increased the proportion of CD45(-)PDL1(+)/CD45(-) cells (Mock vs. G47Δ $p < 0.001$; FAKi vs. G47Δ + FAKi $p < 0.001$). The TAM1/TAM2 ratio significantly increased in the combination therapy compared with G47Δ alone ($p < 0.05$). Immunohistochemistry showed an increase in CD8(+) cells in the combination therapy, and a decrease in alpha SMA(+) cells by FAKi indicating a decrease in tumor stroma. Whereas PKF mice had no additional survival benefit with G47Δ with or without ICIs, PKF mice treated with G47Δ, FAKi and ICIs survived longer than those treated with FAKi alone ($p < 0.01$). Conclusion: The results suggest that the combination therapy using G47Δ, FAKi and ICIs may cause enhanced induction of systemic antitumor immunity and could be useful for treating PDAC.

342. Abstract Withdrawn

343. Cellular Innate Immune Mechanisms Mediating Long-Term Rejection of Disseminated Lung Tumors after Systemic Therapy with Oncolytic Adenovirus

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Currently, some of the most advanced and clinically effective solid cancer treatment approaches are based on counteracting immune checkpoint inhibitory pathways (ICI) to activate cytotoxic activity of tumor antigen-specific CD8+ T cells. Unfortunately, the use of ICI targeting drugs provides clinical benefits only to a minority of cancer patients. Although major efforts are put forth to improve understanding of CD8+ T cell-centric adaptive immune mechanisms to increase the proportion of cancer patients responding to this therapy, the effector functions of innate immunity in mediating tumor rejection remain poorly understood. Using a CD8+ T cell-deficient mouse model and a disseminated human lung cancer model, allowing for a productive replication of human oncolytic adenovirus, AVID-317, in this study we analyzed the recruitment and activation states of hematopoietic and stromal cells that enter the lungs of tumor-bearing mice after systemic oncolytic virus therapy and their correlation with the efficacy of anti-tumor response. Intravenous administration of AVID-317 resulted in a significant suppression of tumor growth and extension of survival of mice with disseminated tumors in the lungs. However, the longitudinal analyses showed that two distinct cohorts of mice could be identified within 21 days after the beginning of the therapy - one where mice responded by a complete rejection of disseminated lung tumors (complete responders) and the other, where mice exhibited unimpeded tumor progression (non-responders). Multi-dimensional flow cytometry analysis revealed that after oncolytic virus treatment, there were quantitative and qualitative differences in tumor-infiltrating cells recovered from the lungs of responder and non-responder mice. After oncolytic virotherapy, the tumor infiltrating cells in non-responder mice were significantly enriched for inflammatory myeloid Ly-6G-, Ly6C-, and CD11b-expressing cells, which also expressed high levels of CD80/CD86 and MHC class II proteins, indicating their highly activated state. In contrast, in responder mice, the tumor infiltrating cells were significantly enriched for B cells, NKT cells, and Ly-6C- and Sca-1-positive stromal cells. The influx of B cells, NK, and NKT cells in the lungs after systemic AVID-317 therapy significantly correlated with tumor burden reduction. Although the relative abundance of NK cells was not different between responder and non-responder mice, the single cell transcriptomics analysis showed that NK cells in responder mice expressed higher levels of KLRG1 and KLRC1 terminal effector markers and perforin. Taken together, our data provides evidence that a systemic therapy with oncolytic adenovirus promotes significant alteration of the tumor microenvironment, leading to the recruitment of lymphocytes, namely B cells, NK cells,

and NKT cells, with effector functions, which mediate long-lasting rejection of disseminated lung tumors via innate immune mechanisms. Disclosure: D.M.S is a founder and shareholder of AdCure Bio, which develops adenovirus technologies for clinical use.

344. Natural Killer Cells Induce Potent Therapeutic Efficacy in Pancreatic Cancer Model via Efficient Tumor Homing and Cytolysis

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In this study, we have isolated allogeneic human natural killer (NK) cells from healthy donors and expanded them *in vitro* to generate cryopreserved products. These NK cells were thawed to evaluate their therapeutic efficacy against desmoplastic pancreatic tumors, ultimately aiming to develop a readily accessible, mass-producible, and off-the-shelf NK cell therapeutic. The cultured NK cells post-thawing retained highly pure populations of activated NK cells that expressed various activating receptors and a chemokine receptor. Furthermore, systemic administration of NK cells induced greater *in vivo* tumor growth suppression than chemotherapeutic used as standard of care for the treatment of pancreatic cancer patients in clinic. The potent antitumor effect of NK cells was mediated by efficient tumor-homing ability and infiltration into desmoplastic tumor tissues. Moreover, the infiltration of NK cells led to strong induction of apoptosis, elevated expression of interferon-γ, and inhibited expression of immunosuppressive transforming growth factor-β in tumor tissues. Collectively, our study demonstrates that *ex vivo* expanded and cryopreserved allogenic NK cells are strong candidates for future cell-mediated systemic immunotherapy against pancreatic cancer.

345. The Role of Polycytidine Tract Length in Mengovirus Oncolytic Activity

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An attenuated miRNA-targeted oncolytic Mengovirus (MC_{24}) restricted by miRNAs enriched in nervous and cardiac tissues (MC_{24}-NC) demonstrated significant oncolytic activity in the absence of toxicity against a multiple myeloma mouse model, but was not curative. Partial (MC_{24}) or complete (MC_0) deletion of a 55 nt long polycytidine (polyC) tract in the 5' non coding region (NCR) of Mengovirus causes significant *in vivo* attenuation, but the underlying mechanism remains unknown. With the goal of improving therapeutic efficacy, we investigated the oncolytic potential of Mengoviruses carrying variations of the polyC tract length and the mechanism behind polyC attenuation. Mengovirus variants with polyC tract lengths of $C_{44}UC_{10}$ (Mwt), $C_{13}UC_{10}$ (MC_{24}), or no polyC (MC_0) show equally robust *in vitro* oncolytic activity against a panel of human and mouse tumor cell lines. However, the length of the polyC tract significantly impacts Mengovirus replication kinetics in macrophages and dendritic cells. This cell type-specific difference in replication also holds true for miRNA-targeted Mengovirus polyC variants. The viral attenuation induced in macrophages by polyC truncation cannot be explained by variations in type I IFN activation, as all polyC variants are capable of suppressing IFN production and all demonstrate similar susceptibility to IFN response modulation. Similarly, a deleted or truncated polyC tract did not attenuate the oncolytic activity of Mengovirus in IFN responsive nor IFN-non-responsive tumor cell lines *in vitro*. The presence of a shorter polyC tract in the 5'NCR does not hamper Mengovirus IRES-dependent translation, but rather reduces the synthesis of new viral genomes during Mengovirus replication in macrophages. These preliminary data suggest that the polyC tract evolved as a specific mechanism to overcome a replication block present in cells of the hematopoietic cell lineage and grant a successful infection in the target tissues. Unlocking the molecular mechanism behind polyC tract-dependent attenuation of Mengovirus in immune cells will be pivotal in exploring new avenues for augmenting its safety and therapeutic efficacy as an oncolytic agent, particularly with regards to immunotherapy combinations.

346. Improving Oncolitic Virus Immunotherapy for Glioblastoma with Cancer-Specific Targeting of Hypoxia

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Glioblastomas are not only difficult to treat but also hard to study and model. One of the reasons is their heterogeneity, which means that any individual tumor consists of cancer cells that are unlike each other. The heterogeneity dampens cytotoxic and immune therapies as the response depends on the cellular composition of the tumor and its adaptation to the microenvironmental niche. To understand these obstacles, we developed heterogeneous patient-derived intracranial xenograft (PDIX) that was used for the analysis of cell transcriptome, both *ex-* and *in vivo*. Our data revealed that tumor microenvironment worsened patient outcomes by driving tumor cell adaptation to hypoxic stress and immune escape. So, we tested if the immunotherapy approach using oncolytic viruses (OV) will benefit once combined with the inhibition of hypoxia response. But, key factors of hypoxia response (HIF1A and EPAS1) also coordinate metabolism, proliferation, differentiation and development of immune T cells. Because of that, and the lack of cell specificity to target hypoxia response in cancer cells, no selective HIF1A/EPAS1 inhibitors are currently clinically approved. So, we used the strategy of targeting long-Non-Coding RNA HIF1A-AS2, the antisense gene to HIF1A that is upregulated by hypoxia in glioblastoma in a cell-type dependent manner. HIF1A-AS2 knockdown diminished the cancer cell response to hypoxia and was associated with increased OV tumor penetration and prolonged survival of tumor-bearing animals treated with OV. As the considerable obstacle contributing toward the resistance to immunotherapy is enhanced by immune evasion in the hypoxic tumor environment, our data demonstrated that the successful treatment of glioblastoma requires closer inspection of hypoxic regions within a tumor that constitute an immunosuppressive environment.

347. Development of Sequence-Optimized Nitroreductase for Retroviral Replicating Vector (RRV)-Mediated Prodrug Activator Gene Therapy in Human Glioma Models

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Significantly enhanced survival benefit can be achieved when tumor-selective retroviral replicating vectors (RRV) are employed to deliver genes encoding prodrug-activating enzymes in a variety of cancer models. Early-phase clinical trials of RRV-mediated prodrug activator gene therapy using yeast cytosine deaminase (RRV-CD, Toca511) delivered by local tumor site injection in recurrent high-grade glioma have shown highly promising evidence of therapeutic benefit and increased survival, leading to registrational Phase 3 trial completed in late 2019. Despite the failure of the Phase 3 trial to meet primary and secondary endpoints, promising results were observed within predetermined subgroups, and combined with the highly favorable safety profile of this approach, this makes it worthwhile to consider alternative prodrug-activating enzymes in order to increase therapeutic efficacy. In this study, we employed RRV to deliver modified versions of another prodrug activator gene, *E.coli* nitroreductase (NTR), which activates the prodrug CB1954 to generate a potent bifunctional alkylating agent. We constructed RRV encoding two different isoforms of wild-type *E.coli* NTR genes (NfsA, NfsB) as well as variants of both isoforms extensively modified to optimize human codon usage and vector stability. Further efforts to improve the potency of these prodrug activator enzymes included the construction of additional new vectors incorporating point mutations at the active site which have previously been demonstrated to improve prodrug conversion efficiency.NTR transgene insertion did not affect RRV replication, which resulted in increasing NTR expression over time in U87 human glioma cultures for all vectors, with sequence optimization resulting in increased genomic stability of the newly developed viruses upon serial passage in U87 human glioma cells. *In vitro* cytotoxicity was examined by MTS assay after CB1954 treatment of fully transduced U87 and U87-EGFRviii cells. Cytotoxicity upon prodrug treatment was much more rapid and potent than RRV-CD, with viability of RRV-NTR-transduced glioma cells reduced by >80% within 48 hrs with the use of sequence-optimized vectors, as confirmed in multiple cell lines. In orthotopic U87-EGFRviii intracerebral tumor models, stereotactic intratumoral injection of sequence-optimized RRV-NTR and repeated cycles of prodrug treatment resulted in rapid and significant reduction of tumor burden as monitored by bioluminescence imaging, leading to prolonged survival benefit. These data indicate that improved therapeutic efficacy can be achieved with optimized NTR prodrug activator gene therapy delivered by RRV in experimental glioma models.

348. Mathematical Modeling of Heterogeneity in Viral Spread and Tumor Dynamics

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The major challenge in oncolytic virus mediated cancer therapy is to select which virus, out of a burgeoning number of oncolytic viruses and engineered derivatives, can maximize both viral spread and therapeutic efficacy. To this end, an in-depth understanding of the virus-tumor interaction is crucial. Here, we develop a novel integro-differential system with distributed delays embodying the dynamics of an oncolytic adenovirus with a fixed population of tumor cells *in vitro*, allowing for heterogeneity to exist in the virus and cell populations. The parameters of the model are optimized in a hierarchical manner, the purpose of which is not to obtain a perfect representation of the data. Instead, we place our parameter values in the correct region of the parameter space. Due to the sparse nature of the data it is not possible to obtain the parameter values with any certainty, but rather we demonstrate the suitability of the model. Using our model, we quantify how modifications to the viral genome alter the viral characteristics, specifically how the attenuation of the E1B 19 kDa and E1B 55 kDa gene of adenovirus affect the system performance, and identify the dominant processes altered by the mutations. We conclude that the deletion of the E1B 55 kDa gene significantly reduces the replication rate of the virus in comparison to the deletion of the E1B 19 kDa gene. We also found that the deletion of both the E1B 19 kDa and E1B 55 kDa genes resulted in a long delay in the average replication start time of the virus. This leads us to propose the use of E1B 19 kDa gene-attenuated adenovirus for cancer therapy, as opposed to E1B 55 kDa gene-attenuated adenoviruses.

349. A Novel Method to Generate High-Sequence Diversity Libraries of Plasmids and Recombinant Adenovirus Based Oncolytic Viruses for Targeted Therapy

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Targeted therapies against a disease like cancer are more effective when available, but hard to design against a specific marker of interest. Conventional strategies of targeted therapy based on Antibodies (Ab) and Small molecule inhibitors (SMI) are effective only if a targeted marker is specific and critical for tumorigenesis/progression. However, Oncolytic viruses (OV) can potentially target any tumor-specific marker irrespective of its functional relevance to tumor biology. Specificity of OVs is only to gain access to tumor cells and they subsequently kill them by infecting and/or delivering other oncolytic agents. Therefore, markers which were thought to be undruggable by Abs and SMIs can be targeted with OVs. One of the major challenges in designing rAdVs is to achieve high specificity to targets. Adenoviruses (AdV) have a natural affinity towards CAR protein and is determined by AB-loop region of viral protein 'Fiber'. Since there is no easy way to identify a sequence which can alter the specificity of AdV towards a marker of interest, one has to generate a large library with sequences diversity in AB-loop region and screen for binding ability. Again, major hurdle in this field is to have a method to generate libraries with large sequence

diversity. There are several related methods published before and some of them claim to generate over ~40billions sequences. However, we found no substantial experimental evidence to support such diversity in their reports and they also had highly biased distribution. They relied only on statistical extrapolations of small scale data. Therefore, we intend to challenge such claims and overcome decades old limitations and provide a solution by developing novel methods. Here, we demonstrate, our novel method can generate libraries of plasmids and recombinant viruses with unprecedented sequence diversity and uniform distribution. We have highly stringent NGS based validation of our libraries. Our novel library generation methods are not only limited to rAdVs but also can be applied to libraries of phages, single-chain antibodies, Nanobodies, CRISPRs, shRNAs, Phage display, DARPins, Ribosome display, Peptides, other types of recombinant viruses including lentiviruses, AAVs etc. They will be highly valuable tools for research, drug discovery and novel therapeutic strategies.

350. Abstract Withdrawn

351. Modification of Extracellular Matrix Enhances Oncolytic Adenovirus Immunotherapy in Glioblastoma

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Complex interplay between heterogeneous cells and extracellular matrix (ECM) in the tumor microenvironment drives the pathogenesis of glioblastoma (GBM). Glycosaminoglycan hyaluronic acid (HA) is a major component of GBM ECM, and associated with tumor growth, invasion, and treatment resistance. Delta-24-RGD oncolytic adenovirus is currently under clinical evaluation for GBM. We hypothesized that degradation of HA would enhance oncolytic adenovirus immunotherapy of GBM by overcoming the immunosuppressive functions of GBM ECM. Intratumoral injections of the oncolytic adenovirus ICOVIR17 which carries hyaluronidase PH20 cDNA increased animal survival, compared with its parental virus ICOVIR15, in an orthotopic GBM model generated with murine stem-like GBM cells in immunocompetent mice. ICOVIR17 promoted the degradation of HA, induced an increase in tumor infiltrating CD8+ T cells and macrophages, upregulated immune checkpoint PD-L1 on GBM cells and macrophages, and downregulated immune checkpoints TIM3 and CTLA-4 on CD8+ cells. Combination therapy of ICOVIR17 with systemic administration of anti-PD-1 antibody significantly prolonged survival of GBM bearing mice compared to no treatment and monotherapy controls, and achieved long-term remission in 21% of treated animals. Antibody and clodronate-mediated in vivo depletion of immune subsets revealed that any depletion of CD4+, CD8+ T cells or macrophages abrogated efficacy of combination therapy. Thus, ICOVIR17-mediated degradation of HA within GBM dramatically alters the immune landscape of the tumor microenvironment, and offers a mechanistically rational combination immunotherapy with PD-L1/PD-1 blockade.

352. Exploring the Natural Adenovirus Diversity to Improve Oncolytic/Cancer Gene Therapy Vectors for HPV Associated Cancers

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Human Papillomaviruses (HPV) cause cervical cancer, and are cofactors in the development of head and neck cancer as well as non-melanoma skin cancer. The oncogenic potential of HPVs is based on the HPV-oncoprotein mediated suppression of the retinoblastoma protein pRB an degradation of the tumour suppressor gene p53 causing malignant progression by driving cell cycle progression and inhibiting apoptosis. Previous studies showed that CRISPR/Cas9 mediated HPV-oncogene disruption induces apoptosis and cell cycle arrest in HPV positive cancer cells. We translated these findings towards potential in vivo delivery approach using high capacity adenoviral vectors (HCAdV) expressing HPV oncogene specific CRISPR/Cas9 as cancer gene therapy vectors. Transduction of HPV positive cervical cancer cells with these vectors specifically reduces tumour cell vitality and proliferation and induces apoptosis. To improve tumour gene therapeutic, or oncolytic approaches using adenoviral vectors, we investigate a series of 21 human Adenovirus (AdV) serotypes, covering the natural AdV diversity, for their efficiency to transduce and replicate in different HPV related cancer cell lines. With this study we aim to identify possible vector candidates that could serve as improved cancer gene therapeutic vector or oncolytic AdV for virotherapies of HPV related cancers. Monolayer and 3D spheroid cultures of HPV positive cervical carcinoma cells lines were transduced with 21 different GFP-luciferase reporter gene expressing AdVs. Transduction efficiency was determined by quantifying luciferase activity q-PCR based detection of internalized vector genomes. To investigate antitumoral effects of the different AdVs, we assayed cell viability of transduced cells and monitored spheroid growth and integrity using microscopy, assisted by automated size measurements using the FIJI software. The ability of different AdVs to replicate within transduced tumour cells, was investigated by a q-PCR based quantification of AdV genome copy numbers 3h and 48h and 96 h post transduction, fluorescent microscopic imaging over a time, as well as reinfection experiments using progeny viruses derived from transduced spheroids. In all cell lines AdV3, 5 and 35 showed highly efficient tumour cell transduction in monolayer and 3D cultures. Even though AdV4 and 14 showed less pronounced initial transduction, they exhibited strong virus replication. Interestingly lytic replication of AdV4 and 35 disturbed tumour spheroid integrity. For HPV related tumours AdV3, 5 and 35 are promising candidates for the development of improved tumour gene therapy vectors. AdV4, 14 may be useful alternatives for oncolytic virotherapy of HPV related cancers. Future work will focus on converting these AdV serotypes into AdV vectors and establishing suitable vector production platform enabling the exploitation of these alternative AdV serotypes for future gene therapeutic approaches.

353. Mesenchymal Stem Cells Enable Delivery of an Oncolytic Adenovirus Specifically to the Tumor without Posing Any Risk Associated with Systemic Administration of Naked Virions to the Host

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Systemic delivery oncolytic virus to tumors remains a major challenge due to its poor tumor tropism and immunogenicity. In this regard, mesenchymal stem cells (MSC) with low immunogenicity and tumor-homing property could serve as a promising systemic delivery tool for oncolytic viruses. We showed that MSCs could be effectively infected by oncolytic adenovirus (oAd) and the virus replicated efficiently in the MSC carrier. Importantly, systemically administered oAd loaded in MSCs (oAd/MSC), which were initially infected with a low viral dose, led to significantly and preferentially elevated viral accumulation in tumor tissues, while attenuating virus detection in normal organs in respect to systemically administered naked oAd. Efficient retargeting of oAd to tumor tissues prevented the induction of oAd-associated hepatotoxicity that arise due to native hepatic tropism of systemically administered oAd. Further, pharmacokinetic profiling of oAd/MSC revealed that cell carrier improved and prolonged oAd retention in blood circulation compared with naked Ad. Importantly, these attributes enabled oAd/MSC to elicit potent antitumor effect, while attenuating systemic toxicity. Collectively, these results demonstrate that MSC-mediated systemic delivery of oAd is a promising strategy for achieving synergistic antitumor efficacy with improved safety profiles.

354. Oncolytic Herpes Simplex Virus Armed with an Immunomodulatory Cytokine Alters the Tumor Microenvironment of Syngeneic Glioblastoma and Enhances Survival Outcomes

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Glioblastoma multiforme (GBM) is the most common form of human brain cancer. Despite a well-established standard of care, the 5-year mortality rate of GBM patients is 95%, highlighting the need for novel therapeutic interventions. A variety of oncolytic viruses, including those derived from herpes simplex virus (oHSV), have been designed for GBM therapy, but early-phase clinical trials have reported few complete responses without evidence of long-term anti-tumor immunity believed to be essential for prevention of almost inevitable recurrence. Recently, our lab and others have used syngeneic animal models to demonstrate that viral-mediated oncolysis alone is insufficient to generate an effective anti-tumor immune response. Potential reasons for the lack of efficacy are limited vector potency (i.e., virulence) and the presence of a highly immunosuppressive tumor microenvironment (iTME). We are attempting to address these obstacles by comparing the anti-tumor activity of different oHSV designs and by arming these oHSVs with immunomodulatory payloads designed to encourage the development of anti-tumor immunity. We first compared the activity of two oHSV vectors, (i) an HSV-1 KOS strain derivative designated KG4:T124 or colloquially, 2A5B, and (ii) an F strain derivative designated rQNestin34.5v.1 (a similar oHSV, rQNestin34.5v.2 is currently in a phase I clinical trial for GBM). We demonstrate that these viruses have similar replication capacity *in vitro* in two murine GBM cell lines, CT2A and GL261. In syngeneic mice, rQNestin34.5v.1 reduced orthotopic GL261 tumor burden compared to 2A5B, but neither virus conveyed superior survival in either tumor model. Regarding the second impediment to effective therapy, GBM is regarded as an immunologically cold tumor with few activated lymphocytes and large numbers of suppressive myeloid cells (macrophages, myeloid derived suppressor cells [MDSCs], microglia) that produce an abundance of immunosuppressive cytokines (IL-10, VEGF, MIF, etc.). Evaluation of the CT2A TME revealed that both viruses induced a high influx of MDSCs, some macrophages, and a transient population of neutrophils and minimal numbers of NK, T cells and dendritic cells. As a strategy to enhance oHSV efficacy, we sought to differentiate the highly immunosuppressive MDSC population into pro-inflammatory macrophages using oHSV armed with IL-12, a cytokine that has been shown to provide this activity. We show that oHSV:IL-12 induced a significant increase in tumor-associated macrophages and increased the survival of animals bearing either tumor type. Although we observed a low percentage of complete responses with the presence of anti-tumor CTLs demonstrated by tumor challenge experiments, IL-12 alone was not sufficient to significantly improve overall long-term animal survival, suggesting that additional arming genes should be considered in combination with IL-12.

355. Exploiting the Mechanisms by Which Tumors Escape Oncolytic Viroimmunotherapy to Treat Tumor Recurrence

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Tumor escape following frontline therapy represents a major cause of treatment failure. We have shown previously that murine tumors which escape virotherapy, adoptive T cell therapy or chemotherapy acquire very specific novel properties which allow them to escape immune surveillance and clearance (*Nat. Med.* 19: 1625, 2013; *Cancer Immunol. Res.* 5: 1029, 2017). Using RNAseq analysis we now show that selective pressures applied through these frontline treatments induce mutational mechanisms which generate a pool of tumor cells from

which escape variants can be selected to evade the frontline treatment. In particular, we show that oncolytic virotherapy leads to the induction of Type-I IFN-mediated induction of APOBEC3 expression which mutates both the incoming virus and the target tumor cell genome. Our data show that APOBEC3 induction leads to a mutation based loss of fitness of the virus which, in this context, decreases its efficacy as an oncolytic agent and reduces treatment efficacy. Simultaneously, APOBEC3 also generates a range of cellular mutations, some of which also permit escape from viral replication and oncolysis. By screening tumors which escape VSV-IFN-ß oncolytic immunovirotherapy, a virus that we are currently testing in clinical trials, we identified one such gene, *CSDE1*, which becomes highly selectively mutated in cells which escape oncolysis. Thus, tumor cells infected at low MOI by VSV-IFN-ß, which are not killed by the virus exhibit mutation in CSDE1 at very high clonality. We show that replication and oncolysis by VSV-IFN-ß are significantly dependent upon expression of (wild type) CSDE1, a protein not previously associated with VSV replication. Thus, a point mutation in *CSDE1* in VSV-IFN-ß-resistant cells reduces viral titers and cytotoxicity by one-two logs. However, we also show that VSV repeatedly passaged through CSDE1 mutated tumor cells can itself adapt to growth in these cells by equally highly selective point mutations in specific parts of the viral genome. In this respect, we were able to use a highly specific, mutant CSDE1-adapted VSV to treat tumors which escaped oncolysis by the original wild type virus. Therefore, we show here that it is possible to treat recurrence, therapy-resistant tumors with modified viruses selected for their ability to overcome tumor escape.

356. Spectrum-Wide Exploration of Human Adenovirus for the Treatment of Breast Cancer

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Breast cancer (BC) is the most commonly diagnosed cancer and the leading cause of cancer deaths in women worldwide. Current treatment for BC is surgery-based and often combined with chemotherapy or radiation therapy. Exploration of multidisciplinary approaches and alternative therapeutic strategies will benefit the BC patients' life quality and overall survival rates. Oncolytic virotherapy is one of the most promising novel treatment concepts for cancer and has been actively studied in clinical trials. Since the efficacy of virotherapy relies first on efficient transduction of targeted tumors, initial screening of a broad range of viral agents to identify the most effective vehicles is essential. As one of the most used vectors in gene therapy clinical trials, adenoviral vector (AdV) is also popular candidate for oncolytic virotherapy. AdVs are non-enveloped viruses with double-stranded, linear DNA genome of approximately 36 kb. More than 80 Human adenoviruses (HAds) were identified, which utilize varying types of receptors in the process of cell infection. Therefore, adenoviral types also display distinctive cell tropisms. AdVs are especially interesting in the scope of tumor therapy as they are able to infect malignant cells and to lysate them in the course of their replication cycle. Additionally, it is possible to genetically modify AdVs to achieve tumor-selective virus replication. The aim of this research project was to elucidate the transduction efficiencies and oncolytic potency of reporter gene tagged (GFP and Luciferase) AdVs on breast cancer cell lines with distinctive characteristics (i.e. hormone sensitive, HER2/neu, triple negative) and breast epithelial cells. For this project 20 different reporter gene tagged adenovirus types were screened with respect to their transduction rates measured by the expression of the luciferase enzyme and GFP. Adenovirus types with high transduction rates were furthermore, included in experiments characterizing their respective infection efficiency, replication patterns and oncolytic potency. The adenoviral serotypes 3, 5, 35, 37 and 52 displayed high transduction rates measured by luciferase and GFP expression levels. Additionally, adenoviral serotypes 3, 35 and 37 featured high rates of initial (3 hours post infection) virus particle uptake in a qPCR-based assays. In the longitudinal analysis of replication patterns serotypes 3 and 5 showed the highest replication rates 48 hours post infection. Regarding the oncolytic potency adenovirus types 3, 5, 35 and 52 displayed the strongest lytic activity. The adenoviral types 35, 37 and especially 52 should therefore, be the subject of further research as they represent interesting candidates for the construction of novel viral vectors in the treatment of breast cancer.

357. Bacteriophage-Mediated Systemic Gene Therapy of Diffuse Intrinsic Pontine Glioma Through the Blood-Brain Barrier

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Diffuse intrinsic pontine glioma (DIPG) is an aggressive and invasive brain tumour in children which is almost invariably fatal. Consequently, there is an unmet need to develop novel and effective therapies to save children affected by these devastating tumors. A critical challenge in devising effective treatment is the frequent presence of an intact blood-brain barrier (BBB), which limits the delivery of systemically administered conventional and biological therapies. In this study, we investigated the ability of a novel and safe intravenous tumour-targeted delivery system to cross the BBB and target the delivery of therapeutic genes to human DIPG in a pre-clinical setting. We selected the bacteriophage vector with a natural ability to cross the BBB and modified it to achieve selective tumour homing by capsid display of the cyclic RGD4C ligand which binds to the αvβ3 integrin receptor, commonly overexpressed on tumor cells and tumor angiogenic vasculature. We found high expression of this integrin on human primary DIPG cells while little receptor expression was detected in normal glial cells, astrocytes. Next, we designed a soluble form of the tumour necrosis factor-related apoptosis-inducing ligand (sTRAIL) using IL-2 signal peptide, followed by insertion into our bacteriophage vector. The targeted phage vector carrying sTRAIL destroyed human primary DIPG cells both in 2D cultures and in spheroids settings without harming the normal astrocytes. Consistently, we detected high expression of the TRAIL death receptor 5 in DIPG cells with lower level of expression in astrocytes, which may explain why normal astrocytes are protected against the sTRAIL. Preliminary studies of the effect of this vector in an orthotopic DIPG mouse model show strong tumor accumulation of the targeted phage particles throughout the tumors, which means that the vector was able to cross the BBB in DIPG after

intravenous administration. Based on these findings, we establish that the receptor targeted by our delivery system is a valuable cell surface target receptor to use in guiding therapeutic delivery to DIPG and this will provide a comprehensive *in vivo* foundation for the application of phage in children with cancer.

358. Sustained Delivery of Oncolytic Adenovirus Using Injectable Hydrogels Potentiates Antitumor Effect in Non-Small Cell Lung Cancer Xenografts

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Development of implantable depot to control the release of oncolytic viruses could be a promising strategy to prolong viral persistence of oncolytic agent in tumor tissues. In this study, we propose an *in situ* forming injectable hydrogel depot for the controlled delivery of potent oncolytic adenovirus. The injectable hydrogels were developed by the physical cross-linking of pH- and temperature-responsive sulfamethazine containing polyurethane copolymers. The free-flowing copolymer solutions at pH 9.0 and room temperature allowed the easy loading of oncolytic adenoviruses and formed hydrogel depot after subcutaneous implantation into the warm-blooded animal model. The biocompatible hydrogel depot controlled the initial burst and sustained delivery of oncolytic adenoviruses over 10 days. The soft texture and pliable nature of injectable hydrogels provided protective environment for oncolytic adenovirus without loss of bioactivity. From the *in vitro* cytotoxicity test, it has been found that oncolytic adenovirus delivered by the injectable hydrogels exhibited strong cytotoxicity to cancer cells than naked adenovirus. Oncolytic adenoviruses delivered near the tumors in a sustained manner were effectively infiltrated into the thick solid tumors and provide a durable and enhanced anti-tumor response in the H1975 non-small cell lung cancer xenograft model *in vivo*. These results suggest that sulfamethazine containing polyurethane copolymer-based injectable hydrogels could be a potential medical intervention for the treatment of cancers.

359. Novel Anti-Tumor Combination of Virotherapy & Phytotherapy Against Cancer Cells that Enhance P53 Expression

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Background, the majority of malignant tumors remains an incurable disease in the most cases, for that reason we need novel treatment strategies such as oncolytic virotherapy using viruses that replicate selectively in tumor cells only. Newcastle Disease Virus (NDV) AMHA1 oncolytic Iraqi strain is a promising anti-tumor agent. However, oncolytic virotherapy still not very successful to eliminate tumors completely. Combination therapy seems the best option to target the cancer cells by many different mechanisms. Simply by combining an oncolytic virus with the existing standard radiation or chemotherapeutics, or other novel traditional modalities such as Phytotherapy. The long-term goal of such combination therapy must be to have a synergistic enhancement to the tumor growth inhibition effect. Phytotherapy, is the use of plant-derived medications in the treatment and prevention of disease. Convolvulus arvensis, family Convolvulaceae, is perennial weed plant. It widely spread in north of Iraq. The aerial parts have many medical properties. The extracts of C. arvensis showed interesting anti-tumor activity and it is very interesting to combine such two novel modalities (virotherapy using NDV and phytotheray using c. arvensis extracts) to target cancer cells through different novel mechanisms and study the involvement of p53 pathway in the proposed synergistic anti-tumor activity.**Method**, the present study aims to determine the tumor cytotoxic effect of *Convolvulus arvensis* crud roots extract and Newcastle Disease Virus combination therapy on several cancer and normal cell lines in vitro using MTT assay. and studied this combination using Chou-Talalay analysis. The cell lines were: Rhabdomyosarcoma (RD), Glioblastoma-Multiform primary culture (AMGM5), mouse mammary adenocarcinoma (AMN3) and Rat Embryo Fibroblast (REF). P53 concentration was measured to evaluate the mechanism of this proposed synergism using a p53 quantitative enzyme-linked immunosorbent assay (ELISA) kit.**Results**, the results showed a clear cytotoxic activity of all concentrations of *C. arvensis* roots extractions alone on all (RD, AMGM and AMN3) cancer cell lines tested, the IC50 of plant extract were: (24, 15.7 and 15.7) ug/ml respectively. The growth inhibitory rates were: (68, 74 and 61) % respectively. There are no decreases in cellular viability of normal REF cell line treatment with the all concentrations of plant extract alone and the IC_{50} value was 7475 ug/ml. NDV was effective and the IC_{50} were: 7.3 HAU (hemagglutination units), 82.4 HAU and 23.8 HAU respectively. The growth inhibitory rates were: 68, 82 and 70 % respectively. The IC50 value of NDV alone on normal REF cell line was 1163 HAU. Chou-Talalay analysis of combination therapy of NDV and *C. arvensis* root extracts showed synergistic effect as shown by the dose oriented isobologram technique in RD and AMGM cells with no synergism against AMN3 cells. The level of P53 in AMGM cell line treated with combination therapy was three-fold higher than the level in the untreated cells.**Conclusions,** the present study proposes a novel combination modality of phytotherapy and NDV virotherapy as a promising treatment for cancer which mediated by p53 elevation that need further *in vivo* evaluation.

360. Next Generation ONCOS Double Transgene Oncolytic Adenovirus Exhibits Enhanced Anticancer Effect in Melanoma

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Oncolytic virotherapy is a promising and fast emerging anti-cancer strategy. To date, herpes virus: Talimogene laherparepvec is the only FDA approved oncolytic virus (OV), however, many other types of OVs are showing promising results in clinical studies. One example

is chimeric oncolytic adenovirus ONCOS-102, expressing GM-CSF as a transgene, which recently reported 33% ORR in advanced anti-PD1 refractory melanoma phase I study (NCT03003676).Genetically modified OVs can be armed with different co-stimulatory molecules in order to boost the anti-tumour immune responses. Based on the ONCOS-102 backbone, we have engineered next generation ONCOS virus, ONCOS-214, expressing novel double transgenes designed to enhance apoptosis of the cancer cells, leading to increase the release of tumour antigens and thereby enhance cross presentation of tumour antigens and priming of cancer specific T cells.The oncolytic properties of ONCOS-214 were confirmed in 4 melanoma cell lines *in vitro*, demonstrating robust cell lysis and anti-cancer properties. Anti-cancer effects of the viruses were also assessed in i) immunodeficient xenograft and ii) humanized xenograft mouse models to further understand the anti-cancer and immune stimulatory potency of the constructs. Tumor growth analysis showed that the double transgene virus ONCOS-214 had the most profound antitumor properties with a significant tumor growth reduction of the A2058 tumour compared to other treated groups in the immunodeficient mouse model. Moreover, the treatment with ONCOS-214 at different doses kept most of the tumors volume under 600 mm^3 after the second round of treatment. Synergistic anti-tumor effect of ONCOS-214 was observed. Interestingly the treatment with ONCOS-214 resulted in highest infiltration of CD4+ T cells.

361. Combination Therapies to Reverse Tumor Antiviral Immunity Enhances Vesicular Stomatitis Virus Oncolytic Efficacy

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Prevention of the type 1 interferon (IFN) induced antiviral state is an emerging strategy to enhance the replication, spread, and efficacy of therapeutic viral vectors. The induction of the antiviral state may explain the discrepancy observed between *in vitro* viral cytoxicity and *in vivo* efficacy for many oncolytic viruses. Vesicular Stomatitis virus (VSV) is an RNA oncolytic virus in preclinical and clinical development, that is sensitive to the IFN mediated antiviral response. Here we demonstrate that while sensitive to viral oncolysis *in vitro*, treatment of CT26WT, a murine syngeneic colon carcinoma model, with VSV had minimal therapeutic efficacy *in vivo*. RNA sequencing analysis revealed that CT26WT upregulated antiviral gene networks in response to subcutaneous implantation in mouse flanks. This data was corroborated by a reduction in viral cytotoxicity and lowering viral titers by a factor of 105 in CT26WT cells pretreated with IFN *in vitro*. To investigate the impact of this response on efficacy, targeted pharmacologic blockade of antiviral signaling and effector functions, with ruxolitinib or sunitinib, was used in our CT26WT *in vitro* and *in vivo* models. While both drugs could restore viral titers and cytoxicity in IFN pretreated cells *in vitro*, only sunitinib increased VSV replication and enhanced survival *in vivo.* Considering the pharmacological limitations of these drugs, we hypothesized that coinfection of VSV with Vaccinia Virus-Lister (VV), which is known to encode numerous antiviral signaling and effector antagonists, would control the antiviral state more efficiently. VV was capable of preventing IFN induction, enhancing viral cytotoxicity, and completely restoring VSV titers in IFN pretreated CT-26WT cells *in vitro*. VV infection of mice bearing CT26WT flank tumors sensitized the tumors to oncolytic viral therapy, significantly increasing the intratumoral replication of VSV and enhancing survival in combination treated animals. VV more consistently enhanced VSV replication *in vivo*, as 60% of animals treated with VV and VSV had intratumoral titers of VSV > 10^4 $TCID_{50}$/g of tumor, compared to only 20% in the sunitinib combination group. Our results demonstrate that antiviral immunity can be suppressed and even reversed with infection of VV or the systemic use of sunitinib, and coinfection of CT26WT tumors with VV is capable of overcoming innate immune resistance to VSV oncolysis.

362. Oncolytic Coxsackievirus Therapy as an Immunostimulator

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Oncolytic virotherapy is as a promising treatment platform for cancer therapy. Previously, we found that coxsackievirus B3 (CVB3) possessed specific oncolytic activity against many cancer cell types in vitro and in vivo. Therein, we also demonstrated that CVB3 triggered immunogenic cell death (ICD)-like features in its infected cells. However, the contribution of CVB3-triggered ICD to antitumor activity and overall therapeutic efficacy remains largely unknown. In current study, we first established mouse fibrosarcoma MCA205 cells transduced with human coxsackie and adenovirus receptor (hCAR) (hCAR-MCA205). The established cells were also engineered with transduction of shRNA against one of the major ICD determinants calreticulin (CRT) (shCRT-hCAR-MCA205). We investigated whether (i) CVB3 infection could evoke antitumor activity without its direct oncolysis, thereby inducing ICD, (ii) CRT played a pivotal role in ICD-mediated therapeutic efficacy following CVB3 infection. Flow cytometric analysis showed significant increase of the cell surface exposure of CRT on CVB3-infected live hCAR-MCA205 cells, and the maximum exposure of surface CRT was observed 16 hr after CVB3 infection. Next, in vivo data demonstrated that in contrast to mice vaccinated with cisplatin (non-ICD inducer) treated hCAR-MCA205 cells, those vaccinated with either CVB3 infected or dinaciclib (ICD inducer) treated hCAR-MCA205 cells showed stronger tumor rejection to challenged live MCA205 cells which couldn't be infected with CVB3. Of note, splenocytes from mice vaccinated with CVB3-infected hCAR-MCA205 cells secreted higher amount of granzyme-B. Furthermore, these antitumor effects were significantly diminished in mice vaccinated with CVB3-infected shCRT-hCAR-MCA205 cells. In conclusion, our results indicated that besides its direct oncolysis surface exposure of CRT on the infected cells was important process to exhibit antitumor effects in oncolytic CVB3 therapy.

363. S-1 Facilitates C-REV Induced Anti-Tumor Efficacy in Triple Negative Breast Cancer Model

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AbstractBreast cancer is the most common cancer in women in Japan. Breast cancer with distant metastasis is difficult to treat, especially the triple negative breast cancer (TNBC), whereas an effective treatment for triple negative breast cancer is desired. S-1 is currently used as a key chemotherapeutic agent for metastatic recurrent breast cancer. Canerpaturev (C-REV) is a highly attenuated, replication-competent mutant strain of oncolytic herpes simplex virus type 1. C-REV showed antitumor effect by its oncolytic infectious ability, followed by the systemic anti-tumor immune responses in various tumor models. In this study, we evaluated the antitumor effect of combination of C-REV with S-1 for TNBC. C-REV inhibited tumor growth and showed the additive effect in combination with S-1 with statistic difference. Furthermore, C-REV+S-1 decreased MDSC (myeloid-derived suppressor cell) in spleen and also in non-injected tumor. Considering that MDSC is known as the cells to suppress both innate and adaptive immune responses, the combination could induce a systemic antitumor immune response. From our result, S-1 is expected to be a promising agent in combination with C-REV in TNBC.

364. Designing an Enhanced EGFR-Retargeted Oncolytic Herpes Simplex Virus

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During the past decade, oncolytic viral therapy has come of age as a viable treatment option for multiple cancer types. Oncolytic viruses (OV) have been created from a variety of viruses and have been tested in human clinical trials for an array of solid tumors with varying success. The HSV-based oncolytic vector (oHSV) Imlygic has received FDA approval to treat unresectable melanoma and has quickly become part of the standard of care for melanoma patients. Considerable effort has gone into devising strategies to achieve safety and efficacy for oHSV. Vector delivery has largely relied on intratumoral inoculation with off-target replication minimized by engineered mutations that reduce virulence. An attractive alternative approach is vector retargeting, whereby virus infection is restricted to cells expressing tumor-specific cell surface receptors. Strategies for full retargeting of HSV infection require virus detargeting from its cognate receptors (HVEM and nectin1), recognized by the virus attachment/entry component glycoprotein D (gD), and introduction of a new ligand into gD that allows entry through interaction with its cognate cellular receptor. Our laboratory has previously shown that an EGFR/EGFRvIII retargeted virus, KNE, can effectively treat an orthotopic human glioblastoma model in nude mice. In this EGFR-retargeted gD design, a single-chain antibody (scFv) specific to EGFR/EGFRvIII was introduced in place of the HVEM-binding region of gD, and nectin-1-detargeting was solidified by a single residue deletion to ensure stable, uniquely EGFR-dependent virus infection. However, EGFR-dependent infection by this retargeted virus was not as efficient as nectin-1-dependent infection by wild type gD virus and spread to neighboring uninfected cells was mildly impaired. Here, in an attempt to enhance the efficiency of EGFR-specific oHSV, we explored the impact of the nature of the targeting ligand, its site of insertion into gD, and the mutations used to eliminate nectin-1 binding, on retargeted gD function. We evaluated three nanobodies, i.e. heavy chain variable region-only (VHH) antibodies, as EGFR-targeting ligands in gD. The nanobodies and original scFv were inserted at different positions within detargeted gD, the recombinants and wild type gD were recombined into a gD-null HSV backbone, and the viruses were compared for EGFR-dependent entry, spread and tumor cell killing. Two nanobody-retargeted gD constructs in particular were found to mediate enhanced EGFR- and EGFRvIII-specific virus entry and tumor cell killing compared to scFv-retargeted gD. These viruses also demonstrated increased incorporation of gD into purified virions, which may be a contributing factor in their enhanced activity. Analysis of specific point mutations in gD identified substitution mutations that ablate gD entry through nectin-1 as tightly as the previous single-residue deletion, while providing increased EGFR-retargeted virus entry. Our improvements in EGFR-retargeted gD promise enhanced viral anti-tumor activity and provide a valuable model for the design of oHSV targeted to other tumor-associated receptors.

365. Development of Systemically Applicable Cancer-Targeted Oncolytic Adenovirus in Pancreatic Cancer

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Pancreatic cancer is the third leading cause of cancer-related deaths in the US, and is predicted to become second around 2020. Oncolytic Adenovirus (OAd) has high potential for systemic cancer therapy. However, its systemic application efficacy has been limited because most of the virus goes to the liver. The improvement of cancer selective transduction and vector distribution to avoid liver sequestration would overcome the obstacles for systemic delivery to enable efficient systemic treatment of cancer with OAd. To improve the cancer-selective transduction, we have generated the pancreatic cancer-targeted OAd by high-throughput screening system with Adenovirus library. The pancreatic cancer-targeted OAd binds to Mesothelin (MSLN) protein, which is overexpressed on the surface of pancreatic cancer. MSLN-targeted OAd showed selective and powerful cytocidal effects against MSLN-positive cancer cell line in *in vitro*. Next, in order to assess the potential of MSLN-targeted OAd for systemic application, therapeutic effect of MSLN-targeted OAd was analyzed in a subcutaneous tumor xenograft model of AsPC-1 cells. When we compared the tumor volume of MSLN-targeted OAd injection group and untargeted OAd (Ad5 WT virus) injection group with the low-dose single intravenous (i.v.) injection (10^9 vp/mouse), MSLN-targeted OAd exhibited stronger antitumor effect compared to its equivalent Ad5 WT virus. In addition, the antitumor

effect of MSLN-targeted OAd was also seen in patient-derived xenograft (PDX) model. After intravenous administration, only the MSLN-targeted OAd showed significant antitumor effect compared to the untreated group or Ad5 WT virus injected group ($p<0.05$). Importantly, when we assessed viral distribution after i.v. injection, the liver sequestration of MSLN-targeted OAd was lower than Ad5 WT virus. By day 7, the viral copy number of MSLN-targeted OAd in the tumor was significantly higher than Ad5 WT virus. In this study, systemic injection of MSLN-targeted OAd showed remarkable anti-tumor effect in both pancreatic cancer cell line-derived xenograft tumor model and PDX model at low dose. Interestingly, systemically injected MSLN-targeted OAd showed significantly lower liver sequestration and better tumor accumulation. Our results indicated that tumor targeted-OAd can embody efficient systemic treatment for pancreatic cancer which are mostly found with spread or metastatic lesions.

366. Systemic Administration of Polymer-Coated Oncolytic Adenovirus Loaded into Human Mesenchymal Stromal Cells Induces Efficient Tumor Homing and Infiltration to Elicit Potent Antitumor Effect

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Oncolytic adenovirus (oAd)-mediated gene therapy is a promising approach for cancer treatment because of its cancer cell-restricted replication and therapeutic gene expression. However, oAd cannot be utilized to treat disseminated metastases due to its immunogenic nature and poor tumor homing ability. In this study, human bone marrow-derived mesenchymal stromal cell (hMSCs) was used as a viral replication-permissive carrier for oAd with an aim to improve the systemic delivery of the virus to tumor tissues. To overcome the poor delivery of oAd into hMSCs, oncolytic Ad (oAd) was complexed with biodegradable polymer (poly(ethyleneimine)-conjugated poly(CBA-DAH); PCDP), generating oAd/PCDP complex. oAd/PCDP complex enhanced the internalization and endosomal escaping ability of oAd into and in hMSC, thus resulting in superior viral production and transgene expression. Furthermore, systemic administration of oAd/PCDP-treated hMSCs elicited more potent antitumor effect compared to naked oAd or naked oAd-treated hMSC in xenograft tumor model. In conclusion, these results demonstrate that hMSCs are effective carriers for the systemic delivery of oAd to tumor sites and treatment of cancer.

367. Engineered Vaccinia Virus for Cholangiocarcinoma

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While oncolytic vaccinia virus-based therapy has shown promising results for uncured patients with cancer, its effects on cholangiocarcinoma (CCA) remain unclear. Here, we evaluated the anti-cancer activity of the cancer-favoring oncolytic vaccinia virus (CVV), which was recognized as a promising therapy for stem cell-like colon cancer cells (SCCs) and metastatic hepatocellular carcinoma (HCC) in previous studies. CCA presents major challenges, such as clinical complexity, stem cell cancer characteristics, a high refractory rate, resistance to conventional therapy, and a dismal prognosis. In the present study, we confirmed the oncolytic activity of the CVV in CCA with a slightly alkaline microenvironment (pH 7-8), in which the CVV was stable and highly effective at infecting CCA. Taken together, our findings suggest that CVV-based therapy is highly suitable for the treatment of CCA.

Hematologic and Immunologic Diseases

368. Ancestral Sequence Reconstruction of Coagulation Factor IX Identifies Variants with Superior Activity Independent to and Additive with Human Factor IX Padua

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Tremendous efforts have been directed at optimizing tropism, transduction efficiency and antigenicity of adeno-associated viral (AAV) vectors. Significantly less effort has been directed toward optimizing transgene sequences. Liver-directed AAV vectors have been used successfully in clinical trials to transfer factor IX (fIX) sequences, improve fIX activity levels and prevent bleeding episodes in subjects with hemophilia B. Although capsid choice and AAV genome cassette design are critical, arguably the greatest impact on clinical effectiveness has been incorporation of the Padua mutation (R338L) into fIX coding sequence, where 7 - 8-fold benefit to human fIX activity levels was observed. Based on these findings, transgene sequences that encode for products with enhanced activity and/or expression advantages clearly provide benefit to clinical gene therapy strategies. Based on our previous success developing high activity/expression coagulation factor VIII variants through Ancestral Sequence Reconstruction (ASR), we applied the same discovery platform to fIX. ASR facilitates the prediction and resurrection of ancient protein sequences from extant sequences enabling high resolution mapping through comparison of sequential phylogenetic branches. Of the 8 resurrected ancestral (An) fIX in our initial screen, two variants (An96 and An97; 90 and 91% identical to human fIX, respectively) common to the primate lineage and predicted to have existed 80 - 90 million years ago, demonstrated 11-fold greater fIX activity production than human fIX in recombinant expression systems despite the absence of other reported 'high activity' fIX substitutions, e.g. R338 Padua. An96 activity was superior to the human fIX Padua variant and, impressively, addition of the Padua mutation (R338L) to An96 further increased the activity to levels 4-fold greater than human fIX Padua. Highly purified AN96 Padua displayed a specific activity of 6,450 IU/mg, which was approximately 32x higher than commercial recombinant fIX. To explore the potential for An96 in gene therapy applications, AAV2/8-An96-Padua and AAV2/8-

hfIX-Padua vectors were generated and administered to hemophilia B mice. Over 8-fold greater plasma fIX activity was observed over the course of 12 weeks in mice administered AAV2/8-An96-Padua as compared to mice administered AAV2/8-hfIX-Padua at $5x10^{11}$ vg/kg with both vectors containing identical liver-directed promoters and similarly designed liver-enhanced codon optimized transgene sequences. Mice that received AAV2/8-An96-Padua demonstrated supraphysiological levels of plasma fIX (> 1 IU/mL). In a subsequent log10 dose finding study, a clear dose response was observed down to a minimal dose of $5x10^{09}$ vg/kg AAV2/8-An96-Padua, which conferred sustained fIX levels of ~0.2 IU/mL. Phenotypic correction was observed in mice subjected to the saphenous vein bleed challenge, where the average time to hemostasis and total number of hemostasis mirrored that of wild type (wt) mice. Further validation was obtained using thromboelastrography of whole blood obtained from AAV2/8-An96-Padua treated mice. Through ongoing mapping studies, it is clear that a significant number of non-native amino acids in An96 can be removed to minimize immunogenicity concerns without loss of activity. Furthermore, no fIX inhibtors were identified in 25 AAV2/8-An96-Padua treated animals. Collectively, these findings support the clinical development of a novel hemophilia B gene therapy candidate and further validate ASR as a powerful discovery platform facilitating rapid exploration of evolutionary design space to identify enabling biopharmaceutical design elements.

369. A Novel Human Factor VIII Variant with Increased Secretion Confers Improved Expression After AAV-hFVIII Administration to Hemophilia A Dogs

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Hemophilia A (HA) is a bleeding disorder caused by a deficiency in coagulation factor VIII (FVIII). Ongoing efforts to develop adeno-associated viral (AAV) vector approaches focus on decreasing the vector dose to reduce the potential for the immune response to the AAV capsid and AAV integration as well as demands on AAV production. Several strategies to reduce the vector dose have been tested including codon-optimization (CO) of the transgene and using enhanced promoter elements. Here we demonstrate the use of novel FVIII variants in conjunction with these strategies to yield an additive effect to increase the human FVIII (hFVIII) expression level and allow us to maintain efficacy at a lower vector dose. First, we characterized hFVIII variants of the 2 major proteolytic cleavage sites, where hFVIII is intracellularly processed to yield the secreted heterodimer form. In HA mice we compared 3 variants: (1) Δ3, deletion of the first three out of four amino acids of the furin cleavage site (1645-RHQR-1648); (2) SP/DE, the modification of a secondary cleavage site in the acidic region 3 (S1657P/D1658E); and (3) Δ3-SP/DE, a variant that combines both modifications. These variants were initially introduced into an AAV8 non-CO B-domain deleted (BDD) hFVIII cassette with an enhanced promoter (TTRm) and administered into HA-CD4KO mice. At the lower dose ($4x10^{12}$vg/kg), BDD, Δ3 and SP/DE have similar hFVIII expression, while Δ3-SP/DE exhibits a 2-fold increase. In the higher dose cohort ($1.2x10^{13}$vg/kg), Δ3 and Δ3-SP/DE have a 2-fold and 5-fold increase, respectively, in circulating hFVIII protein over BDD. Since the combined Δ3-SP/DE variant shows significant improvement over BDD, we incorporated it into a CO expression cassette with the same promoter and carried out preclinical studies in HA-CD4KO mice and HA dogs tolerant to hFVIII. After AAV8-hFVIII-BDD-CO and AAV8-hFVIII-BDD-CO-Δ3-SP/DE delivery ($2x10^{12}$vg/kg and $4x10^{12}$vg/kg) in the HA mice, the hFVIII expression of the Δ3-SP/DE variant was 2-fold higher than hFVIII-BDD demonstrating that the variant combined with the CO sequence resulted in an additive improvement in hFVIII expression. To evaluate these hFVIII variants in a large animal model, HA dogs (n=4) were tolerized to hFVIII by delivering a retroviral vector (RV) expressing hFVIII-BDD on day 2 of life. This results in a sustained stabilized low level expression of hFVIII of ~0.5-2%. Challenge with hFVIII protein (Xyntha; 25 IU/kg per week x 6 weeks, I.V.) demonstrated that these dogs were tolerant to hFVIII in contrast to naïve HA dogs that develop an immune response after the same challenge regimen. Two of these tolerized dogs were treated with AAV8-TTRm-hFVIII-CO-BDD (S29) or AAV8-TTRm-hFVIII-Δ3-SP/DE (S28) ($2x10^{12}$vg/kg). S29 expressed 3-4% of hFVIII while S28 treated with the combined variant had a 4-fold increase in circulating protein (12%) at the same vector dose. No IgG1, IgG2 or total anti-hFVIII antibodies were observed in either dog post-AAV delivery. These studies show that this novel hFVIII variant, Δ3-SP/DE, can be combined with other strategies to yield a significant improvement in FVIII expression, permitting the use of a lower vector dose to achieve therapeutic hFVIII expression levels.

Table 1: hFVIII Expression After AAV Administration of hFVIII Variants

Variant	HA-CD4KO mice		HA Dogs
	$2x10^{12}$ vg/kg	$4x10^{12}$ vg/kg	$2x10^{12}$ vg/kg
AAV8-TTRm-CO-BDD	161% ± 7	176% ± 10	4%
AAV8-TTRm-CO-Δ3-SP/DE	267% ± 24	352% ± 80	12%

370. The CCR5 Gene Editing of Hematopoietic Stem and Progenitor Cells for the Treatment of HIV Infection

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HIV infection, a major public health problem worldwide having claimed more than 32 million lives in the past few decades, has no cure so far. Transplantation of hematopoietic stem and progenitor cells (HSPCs) with CCR5 Δ32 genotype is emerging as a promising cure. To circumvent the availability of CCR5 null HSPCs in the Indian population, we used the CRISPR-Cas9 system to edit the CCR5 gene in the HSPCs of the healthy donor. Our optimized gene editing conditions resulted in greater than 80 percent of gene-edited cells. The CFU analysis confirmed the editing frequency and the presence of bi-allelic CCR5 gene-edited colonies. The suspension culture differentiation of HSPCs into macrophages retained the gene-editing

pattern of the HSPCs and showed a reduction in the CCR5 expression. The loss of CCR5 expression did not hamper the functional activity of the macrophages. The transplantation experiments in the NSG mice showed the engraftment of CCR5 edited HSPCs in the bone marrow 16 weeks post-transplantation. The pattern of lineage differentiation remained similar to that of control edited cells. We will also discuss the on-going functional characterization experiments with the gene-edited HSPCs.

371. Gene Therapy Approach for Severe Congenital Neutropenia by CRISPR/Cas9 Gene-Editing of Hematopoietic Stem Cells and iPSCs

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Severe congenital neutropenia (CN) is a pre-leukemia bone marrow failure syndrome presented with profound neutropenia early after birth due to markedly diminished granulocytic differentiation of bone marrow hematopoietic stem and progenitor cells (HSPCs). CN patients are chronically treated with daily s.c. injections of recombinant human granulocyte-colony stimulating factor (rhG-CSF). This lead to a significant decrease in the numbers and severity of infections. The curative therapy for Cn patients is hematopoietic stem cell transplantation with its associated side effects and risks. A clinical need for gene therapy for CN is imminent. CN is caused by inherited autosomal dominant mutations in *ELANE*, or autosomal recessive *HAX1* mutations. Other gene mutations have been also detected. We recently described the CRISPR/Cas9 mediated *ELANE* knockout as a possible gene therapy approach for CN patients with *ELANE* mutations (*ELANE*-CN) (Nasri et al. 2019). We report here the establishment of different strategies for the efficient CRISPR/Cas9-mediated correction of CN-associated gene mutations in *ELANE* or *HAX1* in patients primary HSPCs or patient-derived iPSCs (as a proof-of-principle). Our platform uses CRISPR/Cas9-sgRNA RNP complexes (for allele-specific knockout or correction of mutated *ELANE* in HSPCs or iPSCs), or a combination of CRISPR/Cas9 RNP with rAAV6-based delivery of DNA templates for the homology-directed repair of the *ELANE*- or *HAX1* mutations in HSPCs.We reached high gene correction efficiency in primary HSPCs from three CN patients harbouring selected *ELANE* mutations and in three CN patients with *HAX1* mutations. Additionally, iPSC-based gene correction platform established in ou rlaboratory yields very high gene correction efficiency with fast turn-around time required for generation of gene-corrected single-cell derived iPSC lines.The effect of *ELANE*- or *HAX1* mutations correction on the neutrophilic differentiation of CN HSPCs, was evaluated *in vitro* using CFU and liquid culture differentiation assays. We compared the gene mutation-corrected cells with the cells from the same patients that were edited in the AAVS1 safe harbor, or electroporated with intact Cas9 protein, as isogenic control. We observed marked improvement of granulocytic differentiation in *ELANE*- or *HAX1* mutations-corrected cells, as compared to the respective isogenic controls.We also performed live-cell imaging of neutrophil extracellular traps (NET) formation after PMA stimulation, chemotaxis and phagocytosis *in vivo* in zebrafish embryos, as described by us in Nasri et al. 2019. We detected qualitatively improved o rcomparable functions of gene-corrected cells, as compared to control edited samples.Taken together, we establsihed a protocol for efficient correction of CN-associated genen mutations in primary HSPCs and iPSCs using CRISPR/Cas9 gene-editing. We reached sufficient gene-editing to correct pathological effects of CN-associated gene mutations, as documented by markedly improved neutrophilic differentiation *in vitro* and *in vivo*. This approach is enticing to be investigated further for clinical translation.

372. Impact of High Affinity scFv on FVIII Specific CAR Treg Function

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Hemophilia A (HA), an X-linked genetic disorder, is caused by mutations in the factor VIII (FVIII) gene. The degree of FVIII mutations manifest as mild, moderate or severe versions of the disorder in patients. The administration of plasma derived/recombinant FVIII to restore coagulation is the most common available therapy, which, however, can be complicated by the formation of inhibitory antibodies (inhibitors) in up to 30% of severe patients. There is, therefore, a dire need to establish tolerance to FVIII treatment, which conventional immune tolerance induction (ITI) does not adequately address. Regulatory T cells (Tregs) have been shown to play an important role in peripheral immune suppression to FVIII, although the frequency of FVIII specific Tregs can be limited, particularly in severe patients with little to no endogenous FVIII expression. Here, we evaluated the use of chimeric antigen receptor (CAR) expressing Tregs to specifically suppress inhibitor formation to FVIII protein therapy. A FVIII specific 2nd generation CAR construct expressing a high affinity single chain fragment variable (scFv) to the C2 domain of FVIII and murine CD28 and CD3ζ signaling domains was synthesized. Soluble FVIII stimulated CAR Tregs upregulated several Treg associated activation markers (LAP, GITR, FOXP3, Ki67, PD1, CD44, CD28) and proliferated robustly both *in vitro* and *in vivo*. Assessment of polyfunctionality by transcription factor and cytokine analysis revealed heterogeneity in the expression of pro and anti-inflammatory immune mediators. Interestingly, the same cell did not co-express IFNγ, IL-10 and IL-4, indicating the presence of divergent subpopulations with contrary effector functions. When adoptively transferred into FVIII deficient BALB/c *F8*e16$^{-/-}$mice in an experimental model of prophylactic FVIII administration, recipient mice developed very high titer inhibitors indicating a loss of regulatory function of the infused FVIII specific CAR Tregs. Using a combination of western blot and phospho flow cytometric analysis, we tested signaling differences between the CAR molecule and the endogenous Treg cell receptor (TCR). CAR signaling induced a rapid and increased phosphorylation of key signaling intermediates (Zap70, AKT, ERK, S6) in a dose dependent manner, as compared to TCR signaling. With this in mind, we modified the FVIII CAR design with site specific mutations allowing us to analyze

the contribution of proximal and distal CD3ζ ITAMs, as well as CD28 motifs on CAR mediated signaling. A total of 8 different constructs were generated and their suppressive function were tested *in vivo*. This study provides a deeper insight into CAR mediated signaling outcomes, which is dependent on scFv affinity and antigen dose/density, emphasizing the need for tailoring the magnitude of CAR signaling activity in Tregs.

373. In Vivo HSC Gene Therapy in Mouse Models for Thalassemia and Sickle Cell Anemia: Phenotypic Characterization

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We have developed a new and simple *in vivo* HSC gene therapy approach that does not require myeloablation and HSC transplantation. It involves subcutaneous injections of G-CSF/AMD3100 to mobilize HSCs from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating helper-dependent adenovirus (HD-Ad5/35++) vector system. These vectors possess CD46-affinity enhanced fibers that allow for efficient transduction of primitive HSCs while avoiding infection of non-hematopoietic tissues after i.v. injection. We demonstrated in adequate mouse models that HSCs transduced in the periphery return to the bone marrow where they persist and stably express the transgene long-term. Recently, using an HDAd5/35++ vector expressing the human gamma globin gene under the control of different versions of the beta-globin LCR, we demonstrated the safety and efficacy of our approach in a mouse model of thalassemia intermedia. More recently, we also used HDAd5/35++ vectors expressing base editors or CRISPR/Cas9 to reactivate fetal gamma globin in Townes mice, a model for Sickle Cell Anemia. We present hematological data, reticulocyte percentages, and red cell morphology before and after treatment for both models.

374. Preclinical Development of ASC-618, an Advanced Human Factor VIII Gene Therapy Vector for the Treatment of Hemophilia A: Results from FRG-KO Humanized Liver Mice, C57Bl/6 Mice and Cynomolgus Monkeys

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Although several AAV-based gene therapies for hemophilia A are currently under evaluation in clinical trials, there is still an unmet medical need for different AAV serotypes, more efficient transgene vectors and reduced AAV doses to achieve high and sustained factor VIII expression with milder immunosuppressive treatments. ASC-618 is an advanced recombinant AAV2/8 vector with the shortest vector genome compared to other gene therapy constructs that have been tested in the clinic. It encodes a liver specific codon optimized (LCO) bioengineered B-domain deleted hFVIII (ET3) under a synthetic Hepatic Combinatorial Bundle (HCB) promoter (HCB-ET3-LCO). The HCB-ET3-LCO construct was previously characterized by Expression Therapeutics/Emory University in hemophilia A murine model and licensed in for further therapeutic development at ASC Therapeutics. Compared to the standard hFVIII transgene (HSQ), used in most hemophilia A gene therapies, ET3 demonstrates 10-100 fold enhancement in biosynthesis, and thus significantly better therapeutic potential. Here we present the results of safety and efficacy experiments comparing ET3 with HSQ in three species, namely C57Bl/6 murine model, cynomolgus monkeys and humanized liver mouse model (FRG-KO). In all 3 species, higher levels of circulating therapeutic product FVIII were seen in animals treated with AAV2/8 HCB-ET3-LCO, compared to AAV2/8 HCB-HSQ-LCO vector. In the C57Bl/6 murine pilot study, stable human FVIII expression was detected by human FVIII specific ELISA, with highest mean ET3 FVIII levels reaching around 50% (0.5 IU/mL), 300% (3 IU/mL) and 350% (3.5 IU/mL) of normal levels at the 5E10, 5E11, and 5E12 vg/kg dose, respectively. The highest mean HSQ FVIII expression levels were 7-fold and 3-fold lower at the 5E11 and 5E12 vg/kg dose, respectively, while at 5E10 vg/kg dose no HSQ expression was detected. The same trend of higher expression levels of ET3 was observed in the cynomolgus monkey study, with ~ 30% (0.3 IU/mL) of normal levels at the 5E11 vg/kg dose of AAV2/8 HCB-ET3-LCO. Importantly, when both vectors were tested in the humanized liver FRG-KO model at the 3E12 vg/kg dose level, the mean human FVIII levels reached around 480% (4.8 IU/mL) of normal levels after ET3 treatment while after HSQ treatment they only reached around 30%. Moreover, RNAscope analysis of liver tissue in FRG-KO model confirmed that ASC-618 drove high ET3 mRNA level expression in human hepatocytes. Safety studies conducted in all three models, including clinical observations, food consumption monitoring, body weight and temperature, liver enzyme and gross pathology evaluation, showed no toxicity effects. Together, these results demonstrate that ASC-618 is well-tolerated in animal models and has the potential of providing therapeutic benefit to patients at reduced vector doses.

375. Development of a Lentiviral Gene Therapy Vector for Treatment of CARD9 Deficiency

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Human CARD9 deficiency is an autosomal recessive disorder resulting in a primary immunodeficiency. Patients with CARD9 deficiency are highly susceptible to fungal infections in particular, which are often persistent, severe, and invasive, primarily affecting the skin,

subcutaneous tissue, mucosal surfaces, and central nervous system. C-type leptin receptors (CLRs), including Dectin-1, Dectin-2, and Mincle, bind carbohydrates on fungal cell walls and initiate signaling cascades necessary for antifungal responses. CARD9 is an essential adaptor protein to several CLRs. Binding of zymosan to Dectin-1 leads to Syk kinase activation and subsequent formation of the CARD9-BCL10-MALT1 complex. This complex activates ERK and NF-kB signaling, triggering downstream release of pro-inflammatory cytokines including TNF-a, IL-6, and IL-1b. Absence of CARD9 hinders signaling through the CLR pathway leading to impaired fungal killing in CARD9-deficient patients. However, different *CARD9* mutations affect a range of responses to pathogenic fungi, which complicates the use of cytokine replacement therapies. While allogeneic hematopoietic stem cell transplantation is effective across *CARD9* mutations, it is high risk in these chronically infected patients. Autologous transplantation of genetically corrected hematopoietic stem/progenitor cells could be curative while bypassing the need for HLA-matched donors, and minimizing or avoiding use of immune suppressants, and most importantly, the risk of graft-versus-host disease. We developed a CARD9-lentiviral vector (LV) for the treatment of CARD9 deficiency. To assess this vector in vitro, we utilized a CARD9-deficient cell model involving monocyte-like THP-1 cells containing an NF-kB luciferase reporter, in which endogenous CARD9 was knocked out by Cas9. Following transduction, approximately half of the treated cells expressed CARD9 (41% versus 88% for unmodified THP-1 normal control cells). We confirmed functional improvement by CARD9-LV by measuring TNF-a production and luciferase reporter activity of transduced cells, which demonstrated increased luciferase activity in response to zymosan stimulation (4.058 vs. 12.540 RFU, p=0.0010), while non-transduced cells did not (2.391 vs. 1.741 RFU, p=0.6954). Additionally, transduced CARD9$^{-/-}$ cells and normal control cells produced similar levels of TNF-a in response to stimulation (265.3 vs. 192.1 pg/mL, p=0.0849), higher than that of non-transduced cells (p<0.0006). Combined, our data shows that CARD9-deficient cells treated with CARD9-LV express functional CARD9 protein that responds to stimulation, with increased NF-kB signaling and TNF-a production. These findings suggest that a CARD9 lentiviral vector could improve antifungal immune responses in CARD9-deficient patients. We plan to test CARD9-LV in CARD9-deficient patient hematopoietic stem cells and assess ERK signaling, pro-inflammatory cytokine production, and fungal killing following stimulation in myeloid-differentiated cells. Additionally, we will determine the efficacy and safety of the lentivirus in vivo by challenging CARD9-LV treated CARD9$^{-/-}$ mice with *Candida albicans* and assessing infection clearance and pathology. In summary, these preliminary results show promise for the development of a CARD9-LV gene therapy approach for CARD9-deficient patients.

376. Clinical Outcomes from a Phase I/II Gene Therapy Trial for Patientsaffected by Severe Transfusion Dependent Beta-Thalassemia: Two Years Follow Up

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Transfusion-dependent beta-thalassemia (TDT) is a genetic disorder due to mutations in the gene encoding the beta-globin chain causing a reduced or absent production of haemoglobin A leading to severe anemia and lifelong transfusion dependence. Gene therapy has been recently accepted as possible alternative to the only curative treatment represented by allogeneic bone marrow (BM) transplantation, available for a minority of patients and associated with risk of complication and mortality. In September 2015 we started a phase I/II hematopoietic stem cell (HSC) gene therapy clinical trial. Investigational medicinal product consists of autologous G-CSF and Plerixafor mobilized HSC engineered by GLOBE lentiviral vector, expressing a transcriptionally regulated human beta-globin gene, administered following a myeloablative treosulfan and thiothepa conditioning. Nine patients with severe TDT (3 adults followed by 6 pediatric subject with different genotype have been treated with a median cell dose of 19.5x10^6 CD34+ cells/kg, with a transduction efficiency from 38 to 77% (median 60%) and a median vector copy number (VCN)/genome in bulk CD34+ cells of 0.9 (range 0.7-1.5). No product-related adverse events were observed and the procedure was well tolerated. At latest follow-up (range 24 months-48 months) we observed a robust and persistent engraftment of gene corrected cells in 7 out of 9 patients with a marking of BM progenitors that, in engrafted patients, ranged between 25.3 and 79.8% (median 41.7%) and with a median VCN in CD34$^+$ cells of 0.77 (range 0.3 - 2.2). As a relevant target for transgene expression, BM CD36$^+$ and GlycophorinA$^+$ erythroid cells were stably marked (VCN range 0.3 - 2.5). In the 2 patients with a lower *in vivo* marking the percentage of LV^{+ve} CD34$^+$ cells was around 7.0% with a VCN/cell of 0.1. The three adult patients had a reduction of transfusion requirement improving their quality of life. Among the pediatric patients, 4 out of 6 have discontinued transfusion shortly after gene therapy and are transfusion independent at the last follow-up. Polyclonal vector integrations profiles

with no evidence of clonal dominance have been detected in all patients with the expected genomic distribution for lentiviral vectors. As of November 2019, all patients had reached at least 2 years follow up and completed the trial and enrolled in a long-term follow-up study that will provide additional information on clinical efficacy and safety of this treatment.

377. Intein-Mediated Protein *Trans*-Splicing in Hemophilia A

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Hemophilia A is the most common X-linked bleeding disorder (1:5'000 males), caused by a deficiency of clotting factor VIII (F8). About 50% of cases are severe having circulating F8 levels less than 1%. The current treatment is frequent prophylactic F8 administrations that can be burdensome and predispose to neutralizing antibodies development. Thus, gene therapy holds great promise for a single-administration life-long cure. Previous delivery of the full-length F8 gene, using adeno-associated viral (AAV) vectors, has been challenging due to the gene's large size of 7 kb exceeding their cargo capacity. To overcome this limitation, a protein *trans*-splicing (PTS) strategy with two AAVs, each encoding half of the F8 gene flanked by split inteins, was designed. PTS can seamlessly reconstitute large proteins from shorter precursors which include split inteins at their extremities. The occurrence of intein-mediated PTS was verified *in vitro* by chromogenic F8 activity assay on cell supernatants of transfected HEK293 cells as well as by Western blot detection of the excised intein. Next, AAV2/8 F8 inteins were produced and injected into adult hemophilic mice that are currently being analyzed. However, full-length F8 is known to be less expressed and secreted than its B-domain deleted SQ-N6 variant, which contains a portion of the original B domain rich in asparagine-linked oligosaccharides that significantly improves its secretion. To achieve higher levels of secretion, we also designed split inteins of this variant. When transfected into HEK293 cells, the F8 SQ-N6 inteins are able to reconstitute the full-length protein detectable by Western blot. More importantly, they reach secretion levels similar or higher compared to the singly expressed plasmid. The *in vivo* evaluation of these F8 SQ-N6 inteins is currently ongoing. The ability of split inteins to reconstitute the large wild type F8 as well as the highly secreted F8 SQ-N6 variant could provide an alternative AAV gene transfer strategy to the current therapies under evaluation in the clinic.

378. Abstract Withdrawn

379. Correction of β-Thalassemia Phenotype by Editing the Human α Globin Locus to Modulate α and β Globin Expression

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Adult hemoglobin consists of two pairs of globin subunits (α;2β;2), whose production is strictly regulated to ensure their balanced expression in erythroid cells. β-thalassemias (β-thal) are a group of blood disorders caused by mutations in the β-globin gene (*HBB*) cluster, which alter α/β-globin chain balance leading to accumulation and precipitation of free α-globin (HBA) chains. These toxic aggregates damage cell membranes causing hemolysis and ineffective erythropoiesis. Several studies have shown that coinheritance of the α-thalassemia trait, the deletion of one or more of the 4 *HBA* genes (αα/αα), significantly ameliorates severity of β-thal patients. To reduce β-thal associated globin imbalance, most clinical approaches aim to increase β and β-like globin chains. Alternatively, globin balance can be restored by reducing free α-globin excess by RNAi or genome editing of regulatory elements, as shown by *ex vivo* studies in β^{+}-thal cells. Here, using CRISPR/Cas9 we aim to treat both β^{+}- and β^{0}-thal by combining these two approaches: α-globin reduction by recreating the α-thal trait (-3.7kb deletion, -α/-α), and concomitant targeted integration and expression of a *HBB* transgene under the control of the endogenous HBA promoter. Notably, we use a single CRISPR/gRNA to perform both genomic deletion and targeted integration, thus minimizing technical hurdles and possible off-targets. In HUDEP2 β^{0}-thal, deletion of 1 or 2 *HBA* genes resulted in reduction of α-globin precipitates (by HPLC) and amelioration of the α/β-globin balance (α/β-like mRNA ratio, from 6.4±2.4 to 2.1±0.6 for -α/αα, to 1.0 ±0.5 for -α/-α). Combination of HBA deletion and knock-in (KI) of β^{AS3}-globin transgene under the control of the HBA promoter achieved wild type level of HBB protein and restored adult hemoglobin production (~40% of normal level). Efficient HBA deletion (~1 deletion/cell) and β^{AS3} integration (~60% of cells) were observed also in primary human hematopoietic stem progenitor cells (HSPCs). By transplanting modified HSPC in immunodeficient mice, we demonstrated that edited cells retain their long-term engraftment ability and give rise to multiple hematopoietic lineages. We also tested our strategy in therapeutic conditions using β^{+}- and β^{0}-thal patients' HSPCs. We achieved sufficient levels of *HBA* deletion and β^{AS3} KI to ameliorate α/β globin imbalance, without affecting erythroid differentiation or multilineage potential of edited HSPC. Finally, to address the safety of this approach, we verified that the selected CRISPR/gRNA is specific, with undetectable off-target events (1% InDels threshold) as assessed by PCR analyses of *in silico* predicted off-targets and unbiased genome wide screening (IDLV capture). To reduce the DNA damage response associated with the generation of double-strand breaks, we investigated the use of Cas9 nickase (Cas9D10A). Interestingly, although less efficient than Cas9 (17.9±7.3% vs 53.4±17.0% of KI in HSPCs), Cas9D10A did not leave any scar in the genome (no InDel, only seamless α deletion in 96 HSPC

colonies analyzed) thus representing an interesting alternative strategy for HSPC editing. Overall, our data show that the combination of HBA deletion and HBB integration is a promising editing strategy for the correction of all forms of β-thal, regardless of the underlying mutations.

380. Base Editing Approaches for the Treatment of β-hemoglobinopathies

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β-hemoglobinopathies are caused by mutations affecting the synthesis or the structure of the adult hemoglobin (Hb). Transplantation of autologous, genetically modified hematopoietic stem cells (HSCs) is an attractive therapeutic option for patients lacking a suitable HSC donor. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing fetal γ-globin expression in adult life - a condition termed hereditary persistence of fetal Hb (HPFH). To reactivate γ-globin expression, genome editing approaches based on site-specific nucleases have been explored. Site-specific nucleases, however, generate double-strand breaks (DSBs) in the genome and raise safety concerns for clinical applications, particularly when used in DSB-sensitive HSCs. Base editing (BE) is a CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations (C>T by cytidine deaminase or CBE and A>G by adenine deaminase or ABE) in the DNA without generating DSBs. HPFH mutations in the 2 *HBG* γ-globin promoters either generate BS for fetal Hb (HbF) activators (e.g. KLF1, TAL1 and GATA1) or disrupt the binding sites (BS) of HbF repressors (e.g. LRF and BCL11A). In this study, we used BE and sgRNAs to target specific DNA sequences in the *HBG* promoters and mimic the effect of HPFH mutations. Firstly, we performed a sgRNA screening in an erythroid cell line (K562) and identified the most efficient sgRNAs for each target. We succeeded in generating the BS for KLF1 and TAL1 with ABE and disrupting the BCL11A BS by creating different types of HPFH and/or HPFH-like mutations, either with the CBE or the ABE. Of note, editing of the BCL11A BS by ABE, led simultaneously to disruption of this BS and creation of a *de novo* GATA1 motif. The absence of the canonical *Spy*Cas9 NGG PAM close to the LRF BS, prompted us to the generate BE containing non-NGG Cas9 variants that allowed the editing of 6 out of 8 cytosines of the LRF BS. These C>T conversions include not only known HPFH mutations but also mutations that can further impair LRF binding. The overall BE efficiency reached up to 60%. In the majority of cases, we detected no insertions or deletions in base-edited samples, as compared to nuclease-edited samples. The 4.9-kb deletion that can be generated upon cleavage of the two identical *HBG* promoters by the Cas9 nuclease was barely detected in base-edited samples. Then, we tested our base editing strategy in human adult erythroid progenitor cells expressing mainly β-globin. BE efficiency was similar or higher than in K562 cells. Efficient generation of the TAL1 and KLF1 activator BS by ABE led to a high frequency of HbF^+ cells and up to ~50% of HbF, as measured by flow cytometry and HPLC. Simultaneous disruption of the BCL11A BS and generation of the GATA1 activator BS by ABE caused a substantial γ-globin reactivation. Finally, editing of the BCL11A BS by CBE or disruption of the LRF BS by non-NGG enzymes also led to HbF de-repression. Importantly, erythroid differentiation was not affected upon BE of the *HBG* promoters. In conclusion, we developed an efficient BE strategy to disrupt repressor BS or create activator BS in the *HBG* promoters that led to therapeutically relevant HbF levels. It is worth noting that our DSB-free approach allows the simultaneous editing of different targets (i.e. activators and/or repressors BS; TAL1-KLF1, BCL11A-LRF, BCL11A-TAL1, BCL11A-KLF1) and the consequent synergistic HbF reactivation. The above-described results are currently being validated in patient HSCs, *in vitro* and *in vivo*, to provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSCs for the therapy of β-hemoglobinopathies.

381. Pediatric Sickle Cell Disease: A Role for Gender as a Risk Factor?

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Pediatric Sickle Cell Disease: A role for gender as a risk factor? Background: Sickle cell disease (SCD) is one of the most common monogenic disease worldwide. The only curative treatment for this disease is allogeneic hematopoietic stem cell transplantation, however, new strategies such as gene therapy and gene editing are currently being explored. The incidence of sickle cell disease is not gender related, since it is transmitted as an autosomal recessive disorder. However, there have been reports of sex-related differences in SCD mortality and morbidity in adult patients. No studies are currently available about gender heterogeneity in the pediatric population. Aim: We retrospectively analyzed the clinical records of 39 pediatric patients with a diagnosis of SCD (hemoglobin SS genotype), focusing on gender differences analyzing various aspects of the disease including acute symptoms and late complications. Methods and Results: We found various gender-related differences in our pediatric population. Pain crisis frequency per year was significantly increased in the male population with a mean number of crisis per year of 1.6 versus 0.6 in the female population ($p=0.04$). In addition, severe complications were mostly found in the male population; in fact, of the 4 cases of osteomyelitis, 3 occurred in male patients and 1 in female patient. Moreover, we observed one case of transient ischemic attack and one of portal vein thrombosis, both of them occurring in male subjects. To evaluate cardiac complications of the disease, we analyzed the echocardiographic findings, available in 31 patients (17 males and 14 females). SCD-related cardiac complications were observed mainly in the male population: among 13 patients with echocardiographic alterations, 10 of them were boys (77%) and 3 were girls (23%) ($p=0.04$). The number of blood transfusions per year did not differ between males and females. The age at diagnosis and was lower in males (median age of 2 years) than in girls (median age of 4 years), without reaching statistical significance ($p=0.08$). Conclusions: Our data support the hypothesis that gender could play a role in determining the clinical course of SCD. The higher morbidity in males is a well-known feature of SCD in adults but this is, to the best of our knowledge, the first study that confirms this finding in a pediatric population. These differences have, in adults, been attributed to hormonal variations

found in the two sexes after puberty. In a pediatric population, other factors must be held responsible for these differences that needs to be addressed in further studies. These findings suggest that gender could be a risk factor for these patients at diagnosis, and possibly guide therapeutic decisions. In the prospect of the emergent gene therapy strategies that are currently being developed in this field, it could be possible that gender, having a role in determining the morbidity of this disease, might be taken into account when considering the eligibility of a patient for such therapies.

382. A Prevalent CD34^{dim} Population Present in SCD BM-Derived CD34+ Cells Has an Engraftment Deficiency in the NSG Mouse Model

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For *ex vivo* lentiviral gene therapy, the marker CD34 is often thought of as a surrogate for long-term reconstituting hematopoietic stem cells (LT-HSCs), although CD34+ cells are a heterogeneous mix of hematopoietic stem and progenitor cells (HSPCs). Until recently, the primary source of CD34+ cells from patients with sickle cell disease (SCD) for *ex vivo* gene therapy had been bone marrow (BM) rather than mobilized peripheral blood (mPB). CD34+ cells collected from the BM have been shown to be phenotypically distinct from CD34+ cells isolated from mPB of healthy donors (HD) (Bender et al, Blood 1991). These observed differences can be exacerbated in SCD compared to HD. For example, the CD34 expression by flow cytometry for BM-derived HSPCs is bimodal, with clear CD34^{bright} (high CD34 levels) and CD34^{dim} (low CD34 levels) populations, whereas HSPCs from mPB are predominantly CD34^{bright}. Internal observations and literature suggest that up to 25% of HD BM CD34+ cells are CD34^{dim} and in SCD BM, the contribution of CD34^{dim} population is higher and can be up to 50% of the CD34+ cell product. In addition to differential expression of CD34, it has been internally observed that a subset of SCD BM CD34^{dim} cells have aberrant expression of markers of differentiation, such as CD19 and CD15, suggesting that these cells are neither true HSCs nor early progenitors with engraftment potential. We were, therefore, interested in characterizing the BM CD34^{dim} population and its role in *ex vivo* gene therapy products by measuring: frequency of colony forming cells, transducibility with a lentiviral vector (LVV), and engraftment potential of LT-HSCs by NSG xenotransplant. We have previously shown that within a population of transduced HSPCs, there is a non-uniform distribution of LVV insertions, with the differentiated progenitor and precursor cells being more highly transduced than quiescent HSC populations. CD34+ cells from HD BM were sorted based on CD34 expression (CD34^{dim} versus CD34^{bright}), transduced with an LVV, and assessed for both transducibility and colony formation. CD34^{dim} cells from two HDs demonstrated enhanced transducibility (1.25- and 1.98-fold higher) and diminished colony formation (2.88- and 6.61-fold lower) than donor-matched CD34^{bright} cells. We further characterized the CD34^{dim} and CD34^{bright} populations using LVV-transduced BM-derived CD34+ cells from two SCD donors. We confirmed that these cells had diminished colony formation (19.32- and 140.50-fold less) and a higher vector copy number (VCN) (1.25- and 2.01-fold higher). For one SCD donor, these sorted cells were transplanted into NSG mice; there was a 33.32-fold lower engraftment potential ($p < 0.0001$) in the CD34^{dim} compartment compared to the matched CD34^{bright} population. These data suggest that the BM CD34^{dim} population contains few repopulating HSCs or early progenitor HSPCs, and that LVV may disproportionately transduce these cells. Though this finding most acutely impacts *ex vivo* LVV gene therapy for SCD using BM as a starting material, it may also play a role in any gene therapy cell product that contains a similar CD34^{dim} population. These observations highlight the utility of deeper phenotypic characterization of HSPC populations for *ex vivo* gene therapy.

383. Optimizing Munc13-4 Expression from Lentiviral Vector Transduced Human T Cells

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Hemophagocytic Lymphohistiocytosis (HLH) is a hyperinflammatory disorder typified by fever, cytopenias, neurological symptoms, organ failure, and eventual death if left untreated. Familial variants of the disease are caused by mutations to genes whose protein products are necessary for the exocytosis of cytolytic granules from cytotoxic cells. Of the eleven genes that when mutated can lead to familial HLH, and its associated disorders, we are most interested in developing a gene therapy-based treatment for mutations in the *UNC13D* gene, which results in mutated Munc13-4 protein. Specifically, we are focused on transducing human T cells with lentiviral vectors encoding a *UNC13D* transgene. As such, we created several codon-optimized sequences with varying promoters driving expression of a 3,270 bp construct. Expression levels from two strong promoters, MND and the long form of EF1α, were similar. Subsequent studies utilized the EF1α promoter. We then compared several laboratory-based methods for producing and purifying lentiviral vectors containing this large transgene sequence. For example, we optimized plasmid ratios using multiple plasmid systems and identified an optimal ratio using a four plasmid production platform. In addition, we compared various purification methods, including ultracentrifugation, sucrose ultracentrifugation, and commercially available column purification. Overnight concentration followed by sucrose ultracentrifugation provided a reproducibly high-quality vector with little toxicity to target cells, in contrast to using only overnight centrifugation. We then compared the use of conventional transduction protocols to a less traditional method that employed the use of microfluidics devices. As anticipated, transduction in microfluidic devices provided a more robust transduction efficiency at lower volumes of vector. In addition, to further optimize the transduction process, we compared the use of commercially available transduction enhancers, such as Lentiboost, Cyclosporin H, and retronectin. To date, these have not provided a significant benefit, but we are continuing to optimize their use. Furthermore, we characterize these different transduction protocols on their ability to transduce different subsets of T cells and the ability of these cells to generate long-term memory T cells. Finally, we demonstrate the refinement of the transduced cell population using

a standard CD271 (delta LNGF) cell selection method. These studies provide the establishment of a transduction platform to genetically engineer T cells from Munc13-4 deficient patients to express Munc13-4 for the purpose of resolving hyperinflammation prior to a curative bone marrow transplant.

384. CB 2679d-GT - A Novel Human Factor IX Variant Shows Enhanced Activity After Delivery Into Hemophilic Mice Using an AAV Capsid with High Liver Transduction

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Catalyst Biosciences has developed a next generation subcutaneously delivered engineered coagulation Factor IX, dalcinonacog alfa, with increased catalytic activity, resistance to antithrombin inhibition and improved affinity for activated FVIII. The triplet substitutions (R318Y, T343R and R338E) provide this FIX variant with a 22-fold enhanced potency over wild-type (wt) FIX. We previously presented the superiority of this molecule by AAV delivery (CB 2679d-GT) over the R338L Padua FIX variant in a preclinical proof of concept study in hemophilia B mice using a self-complementary rAAV-DJ8 FIX expression construct (Blouse et al., Hemophilia, 2019, vol. 25: S1, P124). The FIX Padua variant was originally discovered in a patient with juvenile thrombophilia having an 8- to 9-fold enhanced FIX specific activity compared to wildtype and has since garnered usage as the leading FIX construct in haemophilia B gene therapy trials. In the present study we tested delivery of different FIX rAAV genes using the novel chimeric KP1 AAV capsid, which had previously been shown to exhibit high transduction rates in murine and human hepatocytes *in vivo* (Pekrun et al., JCI Insight, 2019, vol. 4, 22). Codon optimized wildtype (wt), CB 2679d-GT, and Padua FIX sequences containing a truncated version of the first FIX intron were cloned downstream of a robust hepatocyte-specific ApoE/SerpinA promoter and packaged into AAV-KP1. The *in vivo* performance of the constructs was assessed in FIX-deficient hemophilia B mice injected with 2 $x10^{10}$ vg/mouse, 2 $x10^{9}$ vg/mouse, and 2 $x10^{8}$ vg/mouse and followed over several months. FIX antigen and activity levels were assessed by ELISA and aPTT assays, respectively. As expected, FIX activity levels were significantly increased in plasma from mice receiving the CB 2679d-GT and Padua FIX rAAV constructs as compared to those that had received the wt FIX rAAV. The FIX antigen and thus activity levels using the KP1 capsid and the new single-stranded vector construct were between 5- and 10-fold higher compared to the previous study that employed the DJ8 capsid and a self-complementary vector construct. Specific FIX activity in plasma from mice injected with CB 2679d-GT was 2- to 3- fold higher than in plasma from Padua rAAV injected mice, similar to the expected improvement observed in the previous study. This study demonstrates that combining a next generation AAV vector with the potency enhanced FIX variant CB 2679d-GT has the potential to improve transgene expression and effectively lower the viral dose to one tenth or even one hundredth of the dose currently needed to achieve therapeutically relevant FIX activity levels. This would not only substantially reduce the cost of rAAV based FIX gene therapy but would importantly reduce the risk of adverse immune responses that even now limit this therapeutic approach.

385. Development of Coagulation Factor VIII Transgenes That Confer Greater Potency to Gene Therapies for Hemophilia A Through Ancestral Sequence Reconstruction

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Clinical adeno-associated viral (AAV) vector gene therapy candidates for hemophilia A currently require vector doses >10X higher than is required for similar hemophilia B gene therapy despite the 25-fold lower requirements for coagulation factor VIII (fVIII) concentration. This highlights a >250-fold difference in expression. Additionally, clinical hemophilia B gene therapy benefits from incorporating amino acid substitution(s) that increase factor IX (fIX) activity. It is anticipated that fVIII bioengineering would provide comparable advantages. Our group has pioneered efforts to capture the inherent advantageous biological properties of certain animal fVIII orthologs. More recently, we utilized ancestral sequence reconstruction (ASR) as a platform to study the evolutionary variation in coagulation factors V, VII, VIII, IX, X and von Willebrand factor and discovered additional functional diversity that can be captured to engineer next-generation gene therapy and protein pharmaceuticals for bleeding disorders. For these studies, phylogenetic trees were constructed from extant sequences. Other than a single inconclusive variation within the inferred factor VII phylogeny, all reconstructions were identical, further supporting the robustness of ASR. Here, cDNAs corresponding to 9 distinct ancestral B domain deleted (BDD) fVIII proteins were synthesized de novo. These inferred ancient fVIII variants share 87-98% sequence identity with BDD human fVIII (HSQ). Using transient expression from HEK293T/17 cells, the ancient fVIII variants showed 2 - 27-fold higher fVIII activity than HSQ ($n \geq 3$, $p<0.0001$). The top 3 ancestral (An) fVIII candidates, termed An63, An84 and An70, all had comparable fVIII activity at 27-, 24- and 22-fold greater than HSQ, respectively, as determined by one-stage coagulation assay ($n \geq 10$, $p<0.0001$). Furthermore, codon optimized An63 and An70 in a liver-directed AAV cassette exhibited significantly greater fVIII production than HSQ when transfected into Huh7 cells ($p<0.05$, 0.01, respectively), suggesting high-expressing ancestral FVIII proteins can be efficiently expressed from liver cells. Upon purification of the candidate molecules, An63 was notably secreted in single chain form, as opposed to the processed heavy-chain/light-chain heterodimer. The specific activities of An63, An84 and An70 were: 20500, 16000 and 20000 units/mg, respectively, roughly 2 - 4-fold higher than HSQ. In an effort to 'humanize' the lead candidate molecules, the C1 and C2 domains of HSQ were swapped with their cognate ancestral C1 and C2 domains. The resulting ancestral hybrids were non-inferior to their fully ancestral counterparts; however, the

An70/HSQ hybrid had significantly diminished activity compared to An70 (n=5, $p < 0.0001$). These findings suggest that the C domains of An70 are required for high activity, while An63 and An84 C domains are not. This demonstrates the first observation of substitutions in the C domains of fVIII influencing the activity of a fVIII variant. Further studies are in progress to identify the mechanistic role for the C domain(s) in AnfVIII biosynthesis and/or activity. Collectively, these findings validate the ASR approach as an enabling platform for the development of more potent gene therapy product candidates for hemophilia A, as has been achieved in hemophilia B with factor IX Padua.

386. Highly Efficient CCR5 Gene Knockout in Primary Human Hematopoietic Stem Cells with CCR5-Uco-TALEN

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Background. The HIV epidemic remains a global healthcare problem, with no curative method for this infection available to date. Functional HIV cure has been accomplished in patients after allogeneic hematopoietic stem cell (HSC) transplantation from donors homozygotic for the CCR5 Δ32 mutation and thus lacking this essential for HIV entry coreceptor. Based thereon, targeted CCR5 gene knockout has been suggested as a potentially curative HIV therapy. We previously introduced a protocol for CCR5-Uco-TALEN mediated CCR5 gene editing in primary human HSC for subsequent transplantation and eventual generation of HIV-resistant immune system. To achieve this goal, high rates of CCR5 gene disruption are needed. Therefore, the aim of this study was to maximize the CCR5 on-target efficiency with minimal off-target effect. **Methods and results.** Protocol modifications currently suggested in the literature include optimization of electroporation regimen, nuclease mRNA concentration, application of codon-optimized mRNA, the introduction of chemical modifications of mRNA, usage of small molecules for HSC activation, different pre-activation step duration and hypoxic conditions. Mentioned protocol modifications and their combinations were tested in a set of experiments. MACS-selected CD34+ primary cells from healthy donors were activated using cytokine cocktail supplemented with small molecules. We have tested different concentrations of codon-optimized, custom-synthesized mRNA (Trilink) of CCR5-Uco-TALEN with or without 5-methoxyuridine (5-moU) chemical modification. CCR5 gene editing efficiency and off-target activity at CCR2 homolog (the most frequent off-target locus; Mock et al., 2015) were estimated by previously described digital droplet PCR (ddPCR) assay. We have demonstrated that the pre-activation conditions were the key factor defining the efficiency CCR5 gene editing. With modification of cytokine/small molecule cocktail composition and HSC pre-activation step duration we were able to achieve up to 90-99% of CCR5 knockout rates, as well as to control on/off-target ratio, defined by CCR5/CCR2 knockout assessment based on a highly sensitive and robust ddPCR method, by adjusting concentrations of codon-optimized mRNA (25 μg - 100 μg per ml) during the electroporation step. Next, we tested the protocol toxicity and its influence on biological properties of CCR5-edited cells using flow cytometry and colony-forming unit (CFU) assay and did not observe any negative impact on granulocyte-macrophage as well as an erythroid colony-forming unit (CFU-GM & BFU-E) capacity for mRNA concentrations up to 50 μg/ml. Finally, we found that the introduction of 5-moU in the CCR5-Uco-TALEN mRNA structure had no significant effect on the efficiency and toxicity of the HSC gene-editing procedure. **Conclusion.** In summary, we have developed a reproducible GMP-compliant protocol for CCR5 knockout in primary human hematopoietic stem cells. Using this protocol we achieved the highest CCR5 gene editing efficiency reported to date (DiGiusto et al., 2016 ; Romito et al., 2018; Xu et al., 2016), which opens up a perspective for further development of the cell product. Deep off-target analysis using unbiased approaches and *in-vivo* tumorigenicity studies with optimal CCR5-Uco-TALEN mRNA concentration will be performed before the initiation of a clinical trial.

387. Lentiviral Gene Therapy for Hemophilia B: Exploration of Endothelial Promoters in Mesenchymal Stem Cells

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Hemophilia B (HB) is an X-linked recessive disorder caused by a mutation in the FIX genewhich leads to the absence of activated coagulation factor IX (F9). In coagulation cascade, F9 plays a role in intrinsic coagulation pathway and converts the prothrombin to thrombin. Current protein replacement therapy uses extended half-life rFIX protein and the product incurs high cost and requires life-time application. Stem cells transplantation is an alternative treatment for HB. Mesenchymal stem cells (MSCs) have been extensively studied in cell therapy with wide differentiation potential and easy to access. We propose to investigate F9 gene therapy based on MSCs using an advanced lentiviral vector system (NHP/TYF) and tissue specific promoters to drive the expression of a codon-optimized F9 gene (optF9), and evaluate the expression and function of the tissue specific gene therapy approach. We investigated three promoters including an universal promoter (EF1α) and two endothelial specific promoters (VEC and KDR). The three lentiviral vectors, TYF-EF1a-optF9, TYF-VEC-optF9, and TYF-KDR-optF9 were transduced into two telomerase modified primary human MSC cells, FT902 and 1207-9. The F9 RNA expression was examined by RT-PCR and quantitatively analyzed in relation to internal control GAPDH RNA, and we detected the following relative levels of F9 expression: EF1α-F9:1.0~1.2, VEC-F9: 0.4~0.5, and KDR-F9:0.7~0.9. We also quantified F9 protein expression by ELISA and detected secretion kinetics of F9 as following: EF1a-F9: 1300~1500 ng/10^6/24h , VEC-F9:200~500 ng/10^6/24h , and KDR-F9: 500~800 ng/10^6/24h. The functional activity of F9 was then determined by chromogenic assay (2-stage methods), and we observed similar trend for F9 activities as for the protein expression: EF1a at 5~10%, VEC at 0.5%~1.5%, and KDR at 2%~4% of the normal blood serum F9 activity.

Thus, the function of VEC and KDR tissue-specific promoters in MSCs has been confirmed. Further *in vivo* analyses of F9 activity, expression duration and immune response of these lentiviral-F9 gene modified MSCs are underway using F9 gene knockout mice.

388. Parameters Governing the Inhibitor Response to AAV Gene Therapy fVIII

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Clinical trials of liver-directed adeno-associated virus (AAV) vector gene therapy for hemophilia A have yielded multi-year, therapeutic fVIII activity levels. Importantly, no patients have developed anti-fVIII neutralizing antibodies (termed "inhibitors"). However, these trials restricted enrollment to previously-treated patients without a history of inhibitors. Preclinical data show that fVIII-naïve hemophilia A mice develop inhibitors following administration of certain liver-directed AAV gene therapy constructs and doses. Similarly, in the absence of immune suppression, non-human primates demonstrate a dose-dependent inhibitor response following AAV-fVIII administration. These observations point to potential challenges for expansion of AAV-fVIII gene therapy to previously untreated and inhibitor patients. The goal of this study was to identify mechanisms that govern the immune response to AAV-fVIII gene therapy. This information should enable strategies to promote tolerance, as well as help define parameters both for therapeutic development of AAV gene therapy and for evaluating and predicting the immune response when treating patients. To investigate the immunogenicity of AAV-fVIII gene therapy based on transgene cassette engineering, hemophilia A mice were screened with AAV2/8 vectors dosed at 1.6x10^13 vector genomes/kg (vg/kg). The vectors employ two independent, synthetic liver-specific promoters (HCB: Brown *et al. Mol Ther. Meth. Clin. Dev.*, 2018; E06.TTR: Greig, J.A. *et al., Hum Gen Ther,* 2017), expressing either a B domain deleted human fVIII, termed "HSQ", or a bioengineered high-expression fVIII, termed "ET3". By 9 weeks post-administration, the only vector to cause an immune response resulting in undetectable fVIII activity was E06.TTR-ET3 (n=3; IgG titers: 31, 7700, 9170 arbitrary units). By 17 weeks, one mouse treated with E06.TTR-HSQ developed anti-fVIII antibodies and fVIII activity was undetectable (IgG titer: 3840). None of the mice treated with HCB promoter vectors developed inhibitors. We predict that there is a dose threshold for each vector at which the probability of an inhibitor response approaches 1, perhaps due to a more rapid increase in fVIII. A dose response study capped at the highest clinically tested dose (6x10^13 vg/kg) with a 3-fold decrease ranging down as far as 2.46x10^11 vg/kg (4 mice per group) was conducted. Plasma fVIII activity was measured every 5 days for the first 30 days to characterize the importance of early kinetics in determining inhibitor development. FVIII activity from the E06.TTR-ET3 vector dosed at 2x10^13 vg/kg peaked at day 10 between 3.8-6.4 IU/mL and decreased rapidly by day 25, reaching undetectable levels by week 6 with an IgG titer as high as 14000. The same fVIII activity result was observed with the HCB-ET3 vector only when dosed 3-fold higher at 6x10^13 vg/kg; however, the IgG titers were lower, ranging from 5 to 580. In contrast, the HCB-ET3 vector dosed at 2x10^13 vg/kg reached peak fVIII activity at a later time point (day 20; 4.2-6.2 IU/mL) and did not fall to undetectable levels until week 8 (IgG titer: 24). Additionally, as these are synthetic engineered promoters, and off-target expression at varying vector doses has not been characterized, the biodistribution of these vectors to different cell types with different immune statuses may also play a role. To address the biodistribution question, we validated a bicistronic AAV2/8-Luciferase-P2A-GFP reporter vector for each promoter *in vitro* and will track vector expression patterns *in vivo* at immunogenic and non-immunogenic doses. Collectively, these data show a differential immune response dependent upon vector design and dose, and their impact on fVIII production rate.

389. Engraftability of Gene-Edited Sickle Cell Disease CD34+ Cells in Xenograft Mouse and Rhesus Transplantation Models

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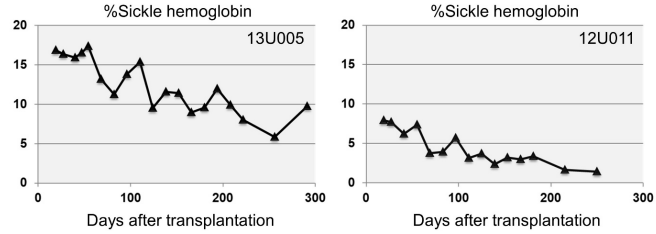
Sickle cell disease (SCD) is caused by a 20A>T mutation in the β-globin gene (βs-globin). Genome editing technologies have the potential to correct the SCD mutation in hematopoietic stem cells (HSCs), producing adult hemoglobin (Hb) while simultaneously eliminating sickle Hb. We have previously demonstrated efficient gene correction in SCD CD34+ cells with SCD mutation-specific guide RNA, Cas9 mRNA/protein, and single-stranded donor DNA, resulting in ~30% gene correction and ~50% indels at the DNA level, and ~60% normal β-globin production at the protein level in *in vitro* erythroid differentiation. Here, we evaluated engraftment of gene-edited CD34+ HSCs in xenograft mouse and non-human primate transplantation models. We electroporated SCD CD34+ cells using the GMP-compliant, FDA Master File-supported, and scalable MaxCyte GT® System to deliver editing tools. Gene-edited cells were transplanted into immunodeficient mice. In the first 6 months post-transplant, ~10% of human cell engraftment was observed; however, while ~20% gene correction was detected at early timepoints, these levels decreased over 6-month follow-up. Next, to model SCD gene correction in rhesus macaques, we designed normal β-globin to βs-globin gene conversion in the rhesus genome (n=2, 13U005 and 12U011). We also added an adjuvant to improve gene conversion efficiency. Mobilized rhesus CD34+ cells (3.4-3.8e7) were pre-stimulated for 2 days, electroporated to deliver rhesus β-globin-targeting guide RNA, Cas9 protein, and single-stranded donor DNA including the SCD mutation (20A>T), and cryopreserved after electroporation. Small aliquots of edited cells (before and after cryopreservation) were differentiated into erythroid cells *in vitro*, resulting in 17-26% gene conversion and 57-71% indels at the DNA level, and 50-100% β-globin production at the protein level, with no difference observed between aliquots taken before and after cryopreservation. Following 9.5 Gy total body irradiation, the frozen edited CD34+ cells (1.6-2.2e7) were injected into autologous macaques. Robust recovery of blood counts in 13U005 was observed, while peripheral blood recovery was delayed in 12U011, who was supported by serial whole blood transfusion. We detected 7-11% and 1-2% of

gene conversion as well as 44-54% and 45-51% of indels 1 month post-transplant in 13U005 and 12U011, respectively. Approximately 14% and 6% of sickle Hb production in red blood cells was detected by Hb electrophoresis 3 months post-transplant in 13U005 and 12U011, respectively, and these values gradually decreased post-transplant (Figure). Interestingly, ~10% of fetal Hb production was observed in 12U011 following transplantation, likely due to stress hematopoiesis during blood recovery. In summary, we developed xenograft mouse and rhesus transplantation models for HSC-targeted genome editing. The gene-edited CD34+ cells were engraftable for at least 3 months post-transplant in both models, while gene correction levels gradually decreased over the follow-up period. These findings will be helpful in the design of future HSC-targeted gene correction trials. (LL, CA, and MP are employees in MaxCyte.)



390. Scurfy CD4 T Cells Converted to Regulatory T Cells by *FOXP3* Gene Transfer Rescue Scurfy Mice after the Onset of the Disease

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IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked) syndrome is caused by mutations in the transcription factor *FOXP3*, which lead to the loss of function of regulatory T cells (Treg). The absence of functional Tregs induces the development of autoimmune manifestations in the first months or years of life. A gene therapy approach designed to induce a Treg program in CD4$^+$ T cells by *FOXP3* gene transfer could be a promising potential cure for IPEX. A CD4$^+$ T cells gene therapy for IPEX syndrome would require a stable and strong expression of FOXP3 with a correlated expression of a cell surface marker to allow selection of transduced cells. Due to a spontaneous mutation in *Foxp3*, Scurfy mice develop a disease very close to human pathology and therefore constitute a valuable model of IPEX syndrome. We developed a lentiviral vector comprising a bidirectional PGK-EF1α promoter driving expression of a codon-optimized human Foxp3 cDNA together with a ΔLNGFR cell surface reporter allowing selection of engineered T cells. This vector was selected for its efficiency in term of vector titer, transduction, FOXP3 expression, and correlation between FOXP3 and ΔLNGFR expression, in both WT and Scurfy murine CD4+ T cells. We established an *in vivo* assay to evaluate the functionality of Tregs based on their adoptive transfer in Scurfy mice coupled to preconditioning and IL-2 treatment promoting Tregs expansion. This model unraveled for the first time the possibility to cure Scurfy pathology after the onset of the disease and therefore constitute an optimal preclinical model. Using this model, we demonstrated that the adoptive transfer of Scurfy CD4$^+$ T cells converted to regulatory T cells by *FOXP3* gene transfer allows long-term rescue of Scurfy autoimmune disease with an efficiency close to WT Tregs. Moreover, transduced CD4+ T cells persisted *in vivo*. Strikingly, the transcriptomic profile of transduced CD4+ T cells, isolated from lymph nodes, after *in vivo* expansion in Scurfy mice, recapitulate nearly 70% of the Treg-specific transcriptomic signature. These results pave the way to gene therapy approaches of CD4 T cells to cure IPEX syndrome through the development of a global therapeutic strategies to tip the balance between Tregs and Tconvs in tolerance induction.

391. Liquid-Biopsy-Integration-Site-Sequencing for the Retrieval of Vector Integrations from Cell-Free DNA and Detection of Early Premalignant Expansions Hidden in Solid Tissues

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Clonal tracking techniques enable monitoring of the fate of thousands cells in tissues of gene therapy (GT)-patients and allow the assessment of the safety and efficacy of these procedures. However, the limited number of cells that can be tested and the impracticality to study cells residing in solid organs without performing invasive biopsies provides only a superficial snapshot of the clonal repertoire of genetically modified cells and reduces the predictive power of these tools as safety readout. Here, we describe Liquid-Biopsy-Integration-Site-Sequencing (LiBIS-Seq), a PCR technique optimized to quantitatively retrieve vector integration sites (IS) from cell-free DNA (cfDNA) released into the bloodstream by necrotic and apoptotic cells residing in diverse tissues. We applied LiBIS-seq to cfDNA purified from plasma samples harvested at different time points post-transplant from 7 patients of the LV-based hematopoietic stem cell (HSC) GT trial for Metachromatic-

Leukodystrophy and retrieved >10,900 IS. The genomic distributions and clonal abundances of cfDNA-derived ISs showed a polyclonal reconstitution and a positive safety profile as previously described. Since only 40% of the IS retrieved are shared with those retrieved from cell-DNA, we hypothesize that a fraction of IS could derive from vector-marked clones embedded in solid organs. To confirm this hypothesis, LiBIS-seq was applied to serum cfDNA obtained from 3 dogs treated by LV-mediated liver directed GT and harvested at different time points from injection. More than 11000 ISs were retrieved over 500 days of follow-up and without the need of solid-tissue biopsies. IS analyses showed a polyclonal pattern in all the treated and in agreement with the IS results observed from liver genomic DNA. Furthermore, it revealed that thousands of cfDNA IS (~10-20% total IS) were repeatedly captured across different time points, indicating the existence of a long lived and slow proliferating cell population composed by 5000-10000 clones on average. Moreover, LiBIS-seq enables the earlier detection of pre/malignant T-ALL clones triggered by LMO2 insertions in γRV-based clinical trials for X-linked severe combined immunodeficiency and Wiskott Aldrich syndrome. Similar results were obtained also in a mouse model of T-cell lymphoma, where LiBIS-seq was more efficient in revealing vector-marked neoplastic clones expanding in thymus than conventional methods based on cell-derived IS. Finally, by applying LiBIS-seq on cfDNA collected over time from Zap70 immune-deficient mice treated by intra thymic AAV GT we observed the same cluster of AAV ISs within the TCR-a chain gene obtained by analyzing genomic DNA of the targeted tissues and, due to the high level of somatic recombination occurring in this genomic locus, during T-cell development. Overall, our data showed that LiBIS-Seq enabled longitudinal clonal tracking studies in in-vivo directed GT and HSC-GT patients, improved our understanding of the clonal composition and turnover of genetically modified cells in solid tissues and allows the early and highly sensitive detection of non-circulating premalignant clones triggered by insertional mutagenesis residing in solid organs which are undetectable by conventional IS analysis.

392. Lentiviral Vector-Mediated Gene Complementation for the Diagnosis of LAD-I

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Leukocyte Adhesion Deficiency Type I (LAD-I) is a severe primary immunodeficiency caused by mutations in the *ITGB2* gene that encodes for CD18, the common subunit of β_2 integrins. Reduced or absent expression of CD18 prevents normal leukocyte trafficking and extravasation to infection sites, thus these patients suffer from recurrent and severe bacterial and fungal infections, which cannot be properly resolved by phagocytic cells. A 5 year old boy born to consanguineous parents of Pakistani origin was referred by the dentists with a history of severe chronic gingivitis and periodontitis. He did not have any other significant infectious history, nor delayed separation of cord. Peripheral blood count showed raised white cell count (17-22x10^6/L) and neutrophil count (10-13x10^6/L). Flow cytometry analyses showed less than 1% of CD18+ peripheral blood (PB) leukocytes. Although no $CD11a^+$ or $CD11b^+$ cells were found in PB, the presence of $CD11c^+$ was identified in these samples. Strikingly, no mutations have been found in *ITGB2* exons, while CD11a mRNA levels were detected by RT-PCR. Detection of CD18 membrane expression revealed differences among different antibody clones, indicating a possible non-functional protein as the cause of the defect. To confirm the implication of CD18 in the cell phenotype of this patient, T cells were transduced with the therapeutic Chim.hCD18 lentiviral vector (LV) used for the *ex vivo* gene therapy (GT) of LAD-I patients. Transduction of PB T cells with the Chim.hCD18-LV restored CD18 and CD11a expression in the membrane of these cells, regardless of the antibody clones used in the flow cytometry analyses. Recovery of β_2 integrin expression was accompanied by the restoration of its functionality, measured as specific CD18:CD11a binding capacity to sICAM *in vitro*. Sequencing of the full gene has been initiated in order to determine the mutation causing the LAD-I disease of this patient. These results suggest the convenience of performing complementation assays for a consistent LAD-I diagnosis, particularly in those cases in which mutation screening does not confirm the diagnosis of the disease.

393. Using a Microfluidics Device to Evaluate FVIII and vWF Production Under Conditions of Physiologic Flow

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Hemophilia A is a genetic disorder caused by a reduction or complete absence of the clotting protein Factor VIII (FVIII). In plasma, FVIII circulates bound to von Willebrand Factor (vWF) in a tight complex. The interaction between vWF and FVIII plays a critical role in the function, immunogenicity, stability, and clearance of FVIII protein, with changes in vWF concentration affecting overall FVIII levels. vWF responds to flow by changing both conformation and protein activity. However, little is known about FVIII responses to shear stress from fluid flow. We demonstrated that human placental cells (PLC) constitutively produce vWF and FVIII, and that FVIII production is significantly upregulated in these cells by transduction with a lentiviral vector encoding mcoET3, a bioengineered FVIII transgene (PLC-ET3). Here, using PLC-ET3, we investigated the effects of shear stress on FVIII production and secretion. Thus, we

created a microfluidic device by laser-cutting PMMA into a base, a channel, and an inlet layer that were adhered with double-sided tape, followed by adding a layer of gel to each channel before seeding with PLC-ET3. To accurately model the physiological shear stress in the sinusoids of the liver, where FVIII production occurs *in vivo*, a shear rate of 0.5 dyne/cm^2 was used. A lower shear stress of 0.05 dyne/cm^2 and a static culture were also included as controls. Supernatants were collected from cells grown under each of these shear conditions, and FVIII activity was measured by activated partial thromboplastin time (aPTT) test. vWF concentration in the supernatant was measured by ELISA. These analyses demonstrated that FVIII activity was reduced by about 50% in flow when compared to static conditions, and that vWF concentration decreased to about 60% at the moderate flow rate and 30% at the high flow rate. Evaluation of endogenous FVIII mRNA by qPCR analysis showed that FVIII mRNA was reduced to 9% and vWF mRNA was reduced to 13% both when compared to static conditions, while mRNA for the vector-encoded mcoET3 was not altered by flow. In order to determine the mechanism responsible for the observed decrease in FVIII and vWF mRNA, PCR arrays were performed to evaluate the effects flow exerted on expression of shear-responsive integrins and other genes involved in shear mechanosensing pathways. These analyses demonstrated that the SRC gene was upregulated in PLC under flow conditions. Because the SRC gene has been shown to induce expression of Kruppel-like factor 2 (KLF2), which can, in turn, upregulate miR10a and miR30c, we conducted further studies to investigate these post-transcriptional regulators. We found that flow conditions led to a 2-fold increase in KLF2 mRNA levels and a 3-fold increase in miR10a levels. Analysis of miR30c levels are ongoing. In conclusion, we show for the first time that shear stress due to fluid flow represses the production and secretion of both endogenous FVIII and vWF in PLC. This is likely to be caused, at least in part, by the mechanosensitive SRC pathway via upregulation of KLF2. The upregulation of this transcription factor then increases the levels of miR10a, which targets GATA6, thereby silencing the vWF promoter. Moreover, KLF2 has also been shown to increase levels of miR30c, which binds to the UTR of FVIII mRNA and thereby represses its production, providing a possible explanation for the decreased FVIII production we observed in PLC under conditions of flow.

394. Targeted Correction of Severe Combined Immunodeficiency (SCID) of Athabascan-Speaking Native Americans

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Severe combined immunodeficiency (SCID) is a group of primary immunodeficiencies (PID) characterized by an impaired immune system. They are made distinctive by the absence or a low abundance of mature T and B lymphocytes, while natural killer (NK) cells are not affected. One specific subtype of SCID is Athabascan-speaking Native American SCID (SCID-A). The protein responsible for this phenotype has been identified as Artemis, an enzyme which performs hair-opening activity during V(D)J recombination, and if mutated, causes hypersensitivity to double-stranded DNA breaks (DSB). Current treatment for SCID-A, and other subtypes, is hematopoietic stem cell transplantation (HSCT), but this comes with a significant risk of morbidity and mortality. Thus, developing new treatments for SCID-A that are safer and more effective is of high priority. In preliminary studies, we have achieved highly efficient targeting of Artemis using CRISPR/Cas9 and donor template for site directed DSB induction and homology-directed recombination (HDR). In addition to performing HDR, we have deployed the adenine base editor (ABE) and prime editing (PE3) approaches for introduction of site-specific nucleotide changes without the induction of DSBs. Our data demonstrates base conversion at the site of the premature stop (pmSTOP) codon that leads to Artemis deficiency in patient-derived fibroblasts and a SCID-A model K562 cells we developed. Using ABE we have been unable to demonstrate full pmSTOP reversal, though with the advent of PE3 we have successfully demonstrated pmSTOP reversal (Figure 1). Once we have achieved gene correction of Artemis in SCID-A CD34+ hematopoietic stem cells (HSCs), we plan to perform functional studies in vitro and in vivo. Our current results represent a new potential therapy for correcting the Artemis stop codon in SCID-A patient-derived HSCs using genome editing approaches that do not utilize toxic DSBs.

Figure 1

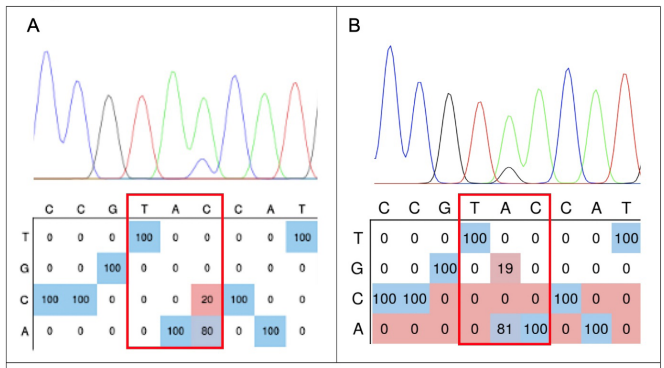

Figure 1. Efficiency attained by Sanger sequencing in clonal K562s in with PE3 (A) and ABE 7.10-VRQR (B) systems. Traces were analyzed by EditR (baseEditR.com).

Immunological Aspects of Gene Therapy and Vaccines

395. Removal of IgG Antibodies with the Bacterial Protease IdeS - A Potential Strategy to Circumvent Pre-Existing Immunity to AAV Vectors

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The successful application of AAV gene therapy technology has achieved clinical benefit in patients with inherited blindness, neuromuscular disease and hemophilia. A major obstacle to successful

gene transfer in humans is the presence of pre-existing neutralizing antibodies to the AAV capsid which can affect gene transfer, and the therapeutic potential of a clinical vector. Moreover, pre-existing neutralizing antibodies (NAbs), to AAV in patients may result in their exclusion from enrollment in AAV gene therapy trials. Furthermore, neutralizing antibodies generated in a patient who has received an AAV gene therapeutic, will likely prevent successful re-administration of a second vector dose. For these reasons, it is necessary to develop strategies to manage the humoral immune response to AAV vectors to ensure clinical success. Towards this goal, we evaluated the potential of a systemically delivered IgG-specific protease, IdeS, to degrade neutralizing antibodies to AAV *in vivo*, and provide a transient antibody-free window for AAV vector delivery. The immunoglobulin-degrading enzyme from *Streptococcus pyogenes* (IdeS), is a highly specific cysteine protease that cleaves Immunoglobulin G (IgG) and provides a novel treatment for autoimmune conditions and antibody mediated transplant rejection. A clinical grade IdeS, IDEFIRIX™, has been developed as a treatment to enable kidney transplantation in highly sensitized patients. In the study described here, in the context of a mouse, we show that pre-existing NAbs to an AAV2 capsid can be reduced to a level that allows successful systemic administration of an AAV2 vector expressing a secreted transgene. Passive transfer of human IVIG (2 mg/mL) to C57Bl/6 mice resulted in an anti-AAV2 neutralizing antibody titer of (1:32 - 1:64) that could be depleted with systemic administration of 3,000 U of IdeS (Promega). AAV2-sFLT02 liver transduction and serum sFLT02 transgene expression was higher in mice that were passively immunized with IVIG and treated with 3000 U IdeS compared to passively immunized mice that were not treated with IdeS, prior to vector delivery. Treatment with IdeS protease was safe and well tolerated and provided a complete, rapid, but temporary removal of pre-existing NAbs to AAV. The translational fidelity of this approach will be validated in the primate, but our data suggests IdeS treatment maybe a potential strategy to reduce pre-existing neutralizing antibodies to AAV capsids in the clinic, with the added benefit of avoiding long term immune suppression in patients.

396. The Effect of Innate Immune Response MAVS Sensor on AAV Long-Term Transduction

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Recombinant adeno-associated viral (rAAV) vectors have been extensively used as favored delivery vehicles for treatment of multiple diseases in consideration of their safety and long-term efficacy. However, after AAV vector administration, immune response remains a major concern in clinical trials, including innate immune response. Data from hemophilia B patients have demonstrated that transgenic coagulation factor IX expression decreased during 6-10 weeks after administration of a high dose of AAV vector (*Nathwani AC, et al. 2011;365 (25):2357-2365. N Engl J Med, Nathwani AC, et al. 2014; 371 (21):1994-2004. N Engl J Med).* Our recent study has shown that the activation of innate immune response triggered by double-stranded RNA (dsRNA) sensors may contribute to the decreased therapeutic expression at later time points following long-term AAV transduction (*Shao W, et al. 2018; 3(12): e120474. JCI insight).* In this study, we further explored the role of dsRNA activation pathway in AAV-induced innate immune response. MAVS is the common adaptor for cytoplasmic dsRNA sensors (MDA-5 and RIG-I). First, we studied the transgene expression and innate immune response activation in AAV transduced cells, and found that the significantly enhanced transgene transduction was observed in the MAVS knockout human hepatocyte cell line compared to that in control cell line. Additionally, dramatically decreased level of IFN-β mRNA was detected in MAVS knockout cell line at the later time points following long-term AAV transduction. Next, AAV vectors expressing shRNA specifically targeting MAVS (AAV/shRNA-MAVS) are constructed and tested for transduction efficiency and innate immune response activation in different cell lines *in vitro*. Preliminary results demonstrated that AAV/shRNA-MAVS induced a higher long-term transgene expression and lower innate immune activation when compared to AAV vectors expressing scramble shRNA. These results highlight the role of MAVS-dsRNA pathway in innate immune response activation and transgene expression at later time points following AAV transduction. Collectively, this study provides valuable insight in developing effective strategies to block the dsRNA innate immune response and enhance AAV transduction in future clinical trials.

397. Screening for miRNA Candidates and Investigating the Mechanisms of miRNA-Mediated Detargeting from Antigen-Presenting Cells *In Vivo*

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One of the primary causes of immunotoxicity following the administration of recombinant adeno-associated virus (rAAV) for gene therapy is the undesirable transduction of antigen presenting cells (APCs), which in turn triggers host immunity towards rAAV-expressed transgene products. Utilizing tissue-specific promoters fails to overcome this issue due to leaky expression in dendritic cells (DCs). Alternatively, the use of endogenous miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing immunogenicity. However, the mechanisms underlying miRNA-mediated modulation of anti-transgene immunity by APC detargeting are not fully understood. In this study, we identified in

the published literature endogenous miRNAs that are highly expressed in APCs, but not in muscle cells. Using the highly immunogenic ovalbumin (OVA) transgene as a proxy for foreign antigens, multiple binding sites of these miRNAs (miRBS) were engineered into the 3'-UTR of OVA, whose expression is driven by a strong ubiquitous promoter. Mouse DCs and macrophage cell lines were used to screen for miRBS-mediated APC detargeting, whereas myoblast cell lines were used to gauge the preservation of transgene expression in muscle. We confirmed that miR-142, a hematopoietic-specific miRNA previously tested in lentivirus vectors, as the leading candidate for efficient APC detargeting. Administration of rAAV1 vectors containing miR142-binding sites (miR142BS) allows for sustained transgene expression in skeletal myoblasts, with negligible anti-OVA IgG production. By using different mouse lines lacking one or more components of the immune system, we delineated that TLR9-mediated innate immunity and B cell humoral-mediated responses are for the most part dispensable for response against the OVA transgene. We also found that rAAV1 vectors containing miR142BS were able to repress costimulatory signals in DCs, blunt the cytotoxic T cell response, and attenuate clearance of transduced muscle cells in mice. Moreover, APC detargeting was also found to persistently downregulate transgene immunity upon redosing with a different serotype vector. Additionally, the blunting of humoral immunity against circulating OVA also correlates with detargeting of OVA expression from APCs. We hereby present evidence that the mechanism for APC-specific attenuation of transgene immunity by miRNA-mediated regulation involves circumventing cell-mediated immunity via the inhibition of cytotoxic CTLs. We have also engineered additional binding sites for other miRNA candidates in different combinations to test for their potential synergistic effects on transgene detargeting. Our study demonstrates that incorporating APC-specific miRBSs into rAAV vectors provides an effective approach for reducing transgene-specific immune response.
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398. Enhancing Anti-HIV CAR Therapy; Protection and Enrichment

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Chimeric antigen receptors (CAR) T cell immunotherapy was originally developed approximately 25 years ago to treat human immunodeficiency virus (HIV) infections, however lack of optimal CAR design was likely the reason the therapy did not show clinical benefits in subjects on anti-retroviral therapy (ART). With second generation CAR therapy's demonstrating dramatic responses in patients with B cell malignancies, the field has begun exploring using CAR therapy for targeting and eliminating HIV. Unlike B cell malignancies, HIV has three major challenges that need to be addressed in order to make an efficacious therapy. First, HIV can infect the CD4+ CAR T cells, and recent studies have shown that a lack of CD4+ population is related to decrease in vivo persistence and efficacy of CAR therapy. To address this issue, we introduced a short hairpin RNA (shRNA) against CCR5, a crucial protein for HIV entry into CAR construct. This shRNA demonstrated sufficient knockdown of CCR5 in primary T cells and prevented viral entry of R5-tropic virus in T cell reporter line. Although this strategy will prevent viral uptake, this shRNA does not address the issue of HIV latency and replication, as CD4+ T cells are a reservoir of HIV, which can be reactivated during *ex vivo* expansion. To address this issue, we introduced a shRNA against Tat/Rev, essential proteins for HIV replication, into the CAR construct. This shRNA protected against R5- and X4-tropic viral replication in a T cell reporter line as well as suppressed viral reactivation in HIV donor CAR T cells. Collectively, the combination of these two shRNAs in an anti-HIV CAR construct were capable of increasing expansion potential and retain memory phenotype when compared to unprotected CAR T cells. However, both protected and unprotected anti-HIV CAR T cells from HIV infected donors demonstrated decreased efficacy when compared to uninfected donor cells. We attributed this lack of efficacy to the final challenge HIV antigen overstimulation from untransduced T cells. Antigen expressed or released from the untransduced T cells stimulates the CAR T cells throughout the culture leading to an exhausted/anergic CAR T cell population. To address this issue of untransduced T cells, we added an shRNA against hypoxanthine-guanine phosphoribosyltransferase (HPRT) which results in the CAR T cells becoming resistant to 6-thioguanine (6TG). 6TG is an antimetabolite that is processed by HPRT and integrated into the DNA ultimately leading to cell death. We demonstrate here that repression of HPRT in CAR T cells protects the cells from 6TG-induced cell death allowing for efficient enrichment of anti-HIV protected anergic resistant CAR T cells. Notably, the repression of HPRT did not appear to impact CAR T cell functionality. Collectively, the strategy presented here may significantly improve antiviral therapy of CAR T cells in people living with HIV/AIDs.

399. Repeat Dosing of AV2.5T to Ferret Lungs Elicits an Antibody Response That Diminishes Transduction in an Age-Dependent Manner

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Repeat-dosing of recombinant adeno-associated virus (rAAV) may be necessary to treat cystic fibrosis (CF) lung disease using gene therapy. However, little is known about rAAV-mediated immune responses in the lung. Recently, a novel rAAV vector, AV2.5T, was developed for *CFTR* (cystic fibrosis transmembrane conductance regulator) delivery with a capsid variant that is highly tropic for human airway epithelium. AV.TL65-hCFTRΔR vector (human *CFTR* minigene) efficiently transduced both human and ferret airway epithelial cultures, and complemented CFTR Cl^- currents in CF airway cultures. *In invo* transduction assay also found that AV2.5T efficiently transduced ferret lung airway. Delivery of AV2.5T-hCFTRΔR to neonatal and juvenile ferret lungs produced h*CFTR* mRNA at 200-300% greater levels than endogenous f*CFTR* (ferret *CFTR*), indicating that ferret is a suitable species for the preclinical testing of AV2.5T for CFTR delivery to the lung. Then, we characterized the humoral immune response and reporter gene transduction after AV2.5T infection in ferret airways. Two ages groups of neonatal and juvenile ferrets were received single-dose with reporter vector AV2.5T-gLuc (Gaussia Luciferase) or repeat-doses with AV2.5T-fCFTRΔR (ferret *CFTR* minigene) prior 3 weeks or 4 weeks to reporter vector. Repeat-dosing significantly reduced

(11-fold) transgene expression of gLuc in bronchioalveolar lavage fluid (BALF) and increased BALF derived NAbs in juvenile ferrets but not in neonatal ferrets, despite near equivalent plasma NAbs responses and almost no significantly difference of gLuc levels at 14-days post-repeat-dosing in both age ferrets. Notably, both age groups demonstrated a reduction in BALF anti-capsid IgG, IgM, and IgA antibodies following repeat-dosing. However, plasma anti-capsid IgG, IgM and IgA varied between different dose groups in different age ferrets. Plasma anti-capsid IgG kept increase in both single- and repeat-dose groups in both age ferrets. Unique to juvenile ferrets was a significantly suppression of plasma anti-capsid IgM following repeat-dosing. While plasma anti-capsid IgA was significantly inhibited following repeat-dose in both age animals as well. Thus, age-dependent immune system maturation and isotype switching may impact the development of high-affinity lung NAbs following repeat-dosing of AV2.5T. This may provide a path to blunt AAV neutralizing responses in the lung. Although repeat-dosing elicited high levels of antibody in both plasma and BALF, gene transduction not totally impede by antibody. These results suggest that it may be feasible to re-dose at least once. Effective strategies to counteract the neutralizing response, such as via immunosuppression or binding of NAbs, are currently under investigation.

400. Therapeutic Antibody Secretion by Hematopoietic Stem Cells in the Non-Human Primate Model

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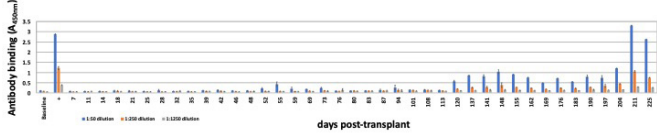
Antiretroviral therapy is the only FDA approved treatment for HIV but is associated with viral rebound when withdrawn. As an alternative, broadly neutralizing antibodies (bNAbs) administration has been promising in recent clinical trials. However, the limited half-life of these bNAbs has hampered their development. To address this issue, we have engineered human hematopoietic stem and progenitor cells (HSPCs) to secrete bNAbs. The property of these cells to persist indefinitely and to differentiate into different lineages with the ability to traffic to various tissue of interest address several issues faced with intravenous delivery of therapeutic bNAbs. This strategy efficiently limited HIV viremia in humanized mice. To test antibody secretion in an immune competent environment, an autologous transplantation was performed in uninfected non-human primate (NHP) using gene modified HSPCs to secrete VRC01 bNAb and express GFP. Antibody secretion and detection of modified cells in vivo were analyzed by ELISA and flow cytometry from 7 days to 8 months post-transplant. Secreted VRC01 antibody was detected in the blood 7 days post-transplant and reached a peak one month later at 30ng/ml. However, 60 days post-transplant no VRC01 antibody could be detected. This is in opposition with our observations in the immunodeficient humanized mouse model and suggest a potential immune response against the transgenes. Indeed, anti-GFP antibodies were detected as early as 6 weeks post-transplant. Anti-VRC01 (Figure 1) as well as anti-VSVg antibody responses were also detected at 6 months post-transplant despite the absence of VRC01. Modified cells could be detected up to seven months following the transplant as demonstrated by PCR detection of the integrated lentiviral construct and of the GFP gene. This was also supported by evidence of VRC01 mRNA detection at 6 months post-transplant in peripheral white blood cells. All together these data support the persistence of gene-modified cells in the peripheral blood but absence of antibody secretion and/or detection more than two months post-transplant, possibly due to the rise of an immune response against these exogenously expressed proteins. Further analyses of cell-based immune response as well as tissue analyses are in progress to understand the mechanisms explaining these immune responses. Additionally, secondary transplant of isolated bone marrow HSPCs has been performed in humanized mice to determine if VRC01 could be detected again in an immunodeficient mouse model. HSPC-based delivery of bNAbs efficiently decreased HIV-1 viremia in immunodeficient humanized mice. However, such strategy should be assessed in a more relevant immunocompetent model such as NHP. bNAb secretion by modified HSPCs could facilitate local delivery of functional proteins to the tissues of interest including HIV reservoir sites. The indefinite renewal potential of HSPCs and ability to differentiate into lineages migrating to tissues of interest address sustained antibody secretion and delivery to the sanctuaries. Importantly, HSPC-mediated bNAb secretion could be applied to other infectious or non-infectious diseases whose treatments require persistent antibody expression through multiple injections for sustainable efficiency. Figure 1: VRC01 bNAb detection by ELISA at the indicated days following the transplantation of gene modified HSPCs.



401. Novel Approaches in Engineering a Multipurpose AAV Vector Genome to Evade Both Innate and Adaptive Immune Responses

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Recombinant adeno associated virus (rAAV)-based gene therapy vectors have shown substantial promise in recent years for treating monogenic diseases. Owing to their excellent safety profile and high transduction efficiencies in a range of tissues, rAAVs have emerged as the platform of choice. Injection of rAAVs in muscles can also serve as "biofactories" for secreted proteins in prophylactic and therapeutic scenarios. Despite the low immunogenicity of rAAVs, as compared to other viral vectors discovered to date, some significant immunological obstacles still remain to be overcome before their transition towards becoming widely available therapies. Numerous reports have demonstrated a vital role for Toll-like receptor 9 (TLR9)

in sensing rAAV genomes and triggering innate and adaptive immune responses. TLR9, a pattern recognition receptor, is an innate immune DNA sensor that normally recognizes unmethylated CpG motifs in the DNA of pathogenic viruses and bacteria. Binding of CpG motifs dimerizes TLR9, leading to MyD88 activation and further induction of Type I interferons (IFNs) and pro-inflammatory cytokines, which promote anti-capsid T cell responses. We have recently developed a novel approach of incorporating TLR9-inhibitory (TLR9i) sequences derived from mammalian telomeres into AAV vector genomes to effectively block TLR9-mediated immune responses to AAV vectors. Separately, the use of miRNA binding sites for cell type-specific detargeting to attenuate transgene immunogenicity has been described to suppress transgene expression in different hematopoietic lineages. miR-142 is a hematopoietic-specific miRNA and is expressed in antigen presenting cells (APCs). We have previously shown that insertion of miR-142 binding sites into the 3'-UTR of the highly immunogenic ovalbumin (OVA) transgene effectively inhibited adaptive immune responses to the rAAV treatment. In this study, we have combined these two elements into a vector design that delivers the OVA gene to foster inactivation of both innate and adaptive immune responses against the transgene DNA and protein product, respectively. We hypothesize that the combination of two orthogonal immunomodulatory strategies may lead to synergistic benefits for AAV gene therapy. This novel multipurpose AAV vector system serves to silence the TLR9-sensing pathway, inactivate the adaptive immune response against the expressed transgene, and enhance the onset and expression level of small transgenes via the ssAAV genome design. In this report, our experimental findings will be presented, and vector biology of such novel designs will be discussed. We found that intramuscular injection of vectors, containing both the elements, leads to further boost in transgene expression as compared to either of these elements by itself. This effect was revealed to be the result of an initial inhibition of local, tissue specific suppression of innate immune markers (TLR9i sequence) followed by inhibition of T cell activation (miR-142 binding sites). Our approach not only widens the therapeutic window of gene therapy, but also paves the path to engineer new rAAV vectors with improved safety and potency for gene therapy studies. [a] Co-corresponding authors

402. Sensitivity of Different AAV Serotypes to Pre-Existing Human Nabs, In Vivo Impact

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While it is clear that pre-existing neutralizing antibodies exclude many potential patients from gene therapy treatment with AAV-based vectors, current exclusion levels are very rigorous (usually titres of >1:5). Testing a large cohort of serum samples from healthy volunteers and using the same experimental conditions, the approximate percentage of eligible subjects with NAbs below 1:5 was determined for both AAV8 and AAVAnc80 serotypes. 300 serum samples of individuals of different geographical and ethnic origin, age and equal gender distribution were tested *in vitro*. Approximately 37% and 40% of the individuals were seronegative for AAVAnc80 and AAV8, respectively. Subsequently, the sensitivity of AAVAnc80 and AAV8 to NAbs was tested *in vivo*. C57BL/6 were passively immunized with human sera of different serotype-specific titres a day before AAV vector administration (i.v). AAVAnc80 was found to be more sensitive to NAbs than AAV8, i.e. much lower existing NAb titres could partially or fully inhibit transduction. The high sensitivity of AAVAnc80 to NAbs was further confirmed in NHPs. Immunoadsorption was required for an efficient transduction of NHP liver, even in animals with low NAbs. Confirming the potential of the immunoadsorption technique to remove anti-AAV NAbs allowing an efficient liver transduction. Transcription levels and expression of the reporter gene were in line with the amount of vector genomes present in liver. Based on the findings, we can argue that the real impact of a NAb positive signal obtained using an *in vitro* assay should be further tested in a more relevant biological assay and the seroprevalence values need to be reconsidered accordingly.

403. Developing Immunomodulatory Viral Vectors for Gene Therapy Based on the Hepatitis C Virus NS5A Protein

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Viral vector mediated gene therapies have a promising future to treat many human diseases; however, host immune responses to viral vectors and their components pose a safety risk and can reduce therapeutic efficacy. Thus, novel strategies to reduce host immune responses are critical for advancing these therapies. Since T cell activation (TCA) is required for development of a T cell response, we hypothesized that incorporation of factor(s) that inhibit TCA in the design of a viral vector may reduce T cell response and enhance vector and transgene persistence during gene therapy. To test this hypothesis, we generated lentiviral vector (LV) to express a full-length protein (LV-NS5A) or a short 20 amino acid (aa) peptide (LV-sNS5A) derived from the hepatitis C virus (HCV) NS5A protein, which has been previously shown to inhibit Lck, a key enzyme required for TCA. GFP expression and inhibition of T cell function were compared between control vector expressing GFP alone or vector expressing GFP and NS5A. Following transduction of human T cells (Jurkat), GFP expression was comparable between LV and LV-NS5A; however, only LV-NS5A inhibited TCA. LV-NS5A inhibited activation of proximal TCR signaling events as measured by phosphorylation of Lck, ZAP-70 and LAT. Expression of a short 20aa NS5A peptide was sufficient to inhibit TCA as LV-sNS5A also inhibited TCA compared to a control vector. LV-sNS5A did not cause global T cell signaling dysfunction as TCR-independent TCA was unaffected in LV-sNS5A transduced T cells. Furthermore, synthetic 20aa NS5A peptides inhibited TCA in primary human T cells. These data suggest that incorporation of an immunomodulatory NS5A peptide in viral vectors may modulate T cell function. Current studies are underway to assess the ability of NS5A peptide to reduce T cell responses in the context of gene therapy vectors such as AAV.

404. Neutral Lipopolyplexes for In Vivo Delivery of Conventional and Replicative RNA Vaccine

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Nucleic acid vaccination relies on injecting DNA or RNA coding antigen(s) to induce a protective immune response. RNA vaccination is being increasingly used in preclinical and clinical studies. However, few delivery systems have been reported for in vivo delivery of RNA of different sizes. Using a tripartite formulation with RNA, cationic polymer, and anionic liposomes, we were able to encapsulate RNA into neutral lipopolyplexes (LPPs). LPPs were stable in vitro and successfully delivered conventional RNA and replicative RNA to dendritic cells in cellulo. Their injection led to reporter gene expression in mice. Finally, administration of LPPReplicon RNA (RepRNA) led to an adaptive immune response against the antigen coded by the RepRNA. Accordingly, LPPs may represent a universal formulation for RNA delivery.

405. Preferential Trafficking to Liver Parenchyma Cells and Tolerogenic Signal Induction by ImmTOR™ Nanoparticles Leads to Higher AAV Vector Transduction and Transgene Expression after Initial and Repeated Administrations

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We have previously shown that tolerogenic ImmTOR nanoparticles encapsulating rapamycin block adaptive immune responses against the AAV capsid, thereby enabling repeat administration of AAV vectors. Here we further demonstrate that ImmTOR also enhances transgene expression after the first dose of AAV vector in naïve mice and provide evidence for augmented AAV trafficking to liver and ImmTOR-driven tolerization in liver milieu. The transduction benefit of ImmTOR at the first AAV administration is independent of its effects on adaptive immunity; it is seen in beta 2-microglobulin- and Rag2-deficient mice and cannot be achieved in vivo by free rapamycin. Admixing ImmTOR and AAV is important for enhanced transgene expression after the first dose but not for inhibition of the antibody response to AAV. ImmTOR and AAV admixture facilitates AAV trafficking to the liver and results in increased vector copy numbers and transgene mRNA expression in hepatocytes in a manner that appears to be independent of the AAV receptor. Furthermore, ImmTOR induces a tolerogenic profile in all major liver cell types, but especially in liver sinusoidal endothelial cells (LSEC), resulting, in turn, in elevation of hepatic Tregs. Finally, ImmTOR appears to enhance autophagy in hepatocytes. Collectively, ImmTOR affects multiple aspects of AAV biology and immunology at the first dose, including trafficking, antigen presentation and autophagy. This multi-pronged mechanism of ImmTOR action makes it an attractive candidate to enhance systemic gene therapeutic applications. The first dose benefit of adding ImmTOR to AAV gene therapy is immediate, dose-dependent and not mouse strain-specific. It can also overcome low levels of pre-existing antibodies to AAV. The rapid and enhanced transgene expression may enable faster onset of therapeutic effects achieved at lower AAV doses and coupled with the inhibition of antibodies and T cell responses against AAV enable multiple vector redosing.

406. In Vivo Expression of Nucleic Acid-Encoded Human Monoclonal Antibody Against Dengue Virus by Electroporation

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Dengue virus (DENV) infection becomes the important public health problem especially in tropical and subtropical countries. The ideal dengue vaccine are equally give protection to all serotypes. Nevertheless, the vaccine alone is not adequate to prevent dengue infection. The therapeutic antibody is one alternative option for immune passive therapy, especially neutralizing human antibody showed cross protection to all four serotypes. One of obstacle of anti-dengue antibody is the infection enhancement phenomenon, called antibody dependent enhancement (ADE). In this study, the 1G7C2-variable heavy (VH) and light chain (VL) genes of parental neutralizing human mAb, which fused to the Kozak leading sequence, were constructed as expression plasmid DNA in pFUSE vector. The gene encoding the CH2 antibody domain on heavy chain was genetically modified by Leucine to Alanine substitutions at positions 234 and 235, which lacked binding to Fcγ receptors for prevents ADE. Here we demonstrated that a single electroporation of DNA encoded immunoglobulin gene via intramuscular can express modified human monoclonal antibody against 4 serotypes of dengue virus without enhancing activity in BALB/c mice. The new antibody expressing plasmids are possible to be a candidate therapeutic antibody against dengue virus infection.

407. Adenovirus Capsid-Based Anti-Fentanyl Vaccine

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Fentanyl is a powerful synthetic mu-opioid receptor agonist originally developed in the 1960s for pain management of cancer. Despite its clinical efficacy, fentanyl possesses a substantial abuse liability and is significantly more potent than heroin, another mu-opioid receptor agonist. Fentanyl is relatively simple and inexpensive to produce,

facilitating widespread illegal manufacture and distribution. Illicit fentanyl is typically injected, snorted, smoked, or ingested in order to produce an intense, short-term euphoric high. Fentanyl's potency, coupled with it often being added to or covertly substituted for heroin, results in a significant risk of overdose and subsequent death for users; more than 50% of opioid-related fatalities in the United States during 2016 were caused by fentanyl or its derivatives. While mu-opioid receptor antagonists, such as naloxone, can attenuate overdose symptoms and reduce fatality rates, it requires multiple doses to overcome fentanyl. As with many drugs of abuse, fentanyl penetrates the blood-brain-barrier in order to reach its cognate receptors in the brain and induce the perceived reward for the user. Thus, a therapeutic able to effectively sequester fentanyl in the blood and prevent its access to the brain would not only serve as a viable prophylactic intervention for overdose, but as a potential treatment option for fentanyl addiction. Since $E1^{-}E3^{-}$ adenovirus serotype 5 (Ad5) gene transfer vectors are potent immunogens, we have developed a novel vaccine platform for addictive drugs by direct covalent conjugation of the normally immune-silent small molecule drug analog to the capsid proteins (hexon, fiber, penton base) of a noninfectious, disrupted Ad5 vector (dAd5). A fentanyl analog, carfentanil (CF), was linked to dAd5 capsid proteins via an EDC, S-NHS crosslinker to produce the anti-fentanyl vaccine (dAd5-CF). BALB/c female mice were administered dAd5-CF (4 μg; intramuscular) formulated in Adjuplex adjuvant at week 0, 4, and 11. Naive mice were administered PBS at the same time points. Blood collected bi-weekly from wk 0 to 12 was assayed for anti-fentanyl antibody titer. Serum anti-fentanyl titers increased from time 0 to 6 wk to reach a plateau of $2x10^4$ maintained from 6 to 12 wk, the last time point tested. At wk 13, dAd5-CF and naive mice were intravenously administered a dose of 1 μg of fentanyl spiked with 2 μCi of ^{3}H-fentanyl and sacrificed 1 min post-injection to assess fentanyl distribution in brain *vs* blood. The dAd5-CF immunized mice sequestered ^{3}H-fentanyl in the blood relative to the brain compared to naive mice ($p<0.04$). In summary, capitalizing on the immunogenicity of the Ad5 capsid, we have developed an anti-fentanyl vaccine that has the potential to treat fentanyl addiction and prevent inadvertent future overdose.

408. Neutralizing Antibodies Against Selected AAV Serotypes in a Colombian Population

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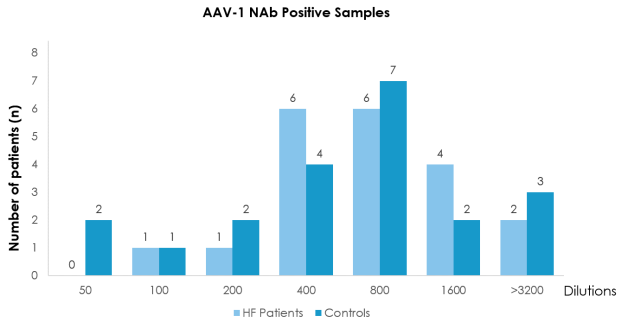
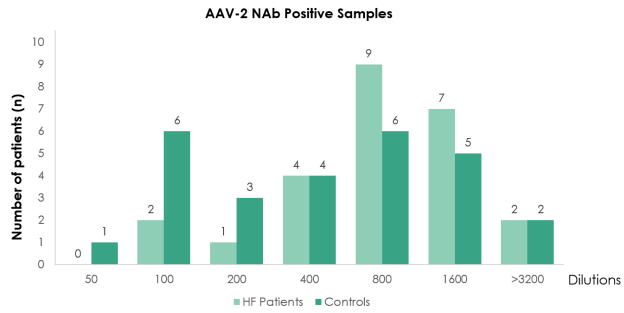
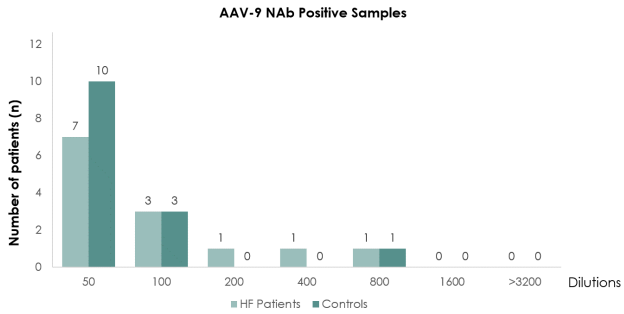
Neutralizing antibodies (NAb) activity against the viral capsid of adeno-associated viral (AAV) vectors decreases transduction efficiency, thus limiting transgene expression. Several reports observed a variation in NAb prevalence due to age, AAV serotype, and, most importantly, geographic location. There are no recent reports describing the anti-AAV NAb prevalence in Latin America. Here, we describe the prevalence of NAb against different serotypes of the AAV vector (AAV1, AAV2, and AAV9) in Colombian patients with Heart Failure (HF) (referred as cases) and healthy individuals (referred to as control). The levels of NAb were evaluated in serum samples from 120 subjects (60 from each group) using an *in vitro* inhibitory assay. HEK 293 cells were plated on a 96-well plate at 20K density and then infected with an AAV vector expressing the green fluorescent protein (GFP) under the control of the CMV enhancer/beta-actin (CB) promoter (rAAV-CB-GFP) that was previously incubated with serial dilutions of each participant's serum. The plate was evaluated 24 hours after the infection. The neutralizing titer of the sample was reported as the first dilution at which at least 50% inhibition of the GFP transgene signal was achieved. A neutralizing titer of > 1:50 was considered positive. The highest prevalence of NAb in the case and control groups was, as expected, to the serotype AAV2 (43 and 45%, respectively), followed by AAV1 (33.3% in each group) and AAV9 (20 and 23.2%, respectively) (Figure). The presence of NAb for two or more of the serotypes analyzed was observed in 25% of the studied samples. Most of the positive samples for AAV1 (55-75%) and AAV9 (93%) were also positive for AAV2, suggesting coinfection or cross-reactivity. Although there were no significant differences in individual or combined prevalence of NAb for any serotype between case and control groups, the participants in the control group had a different distribution of the combined prevalence, with a lower proportion of positive samples for the three serotypes (18.3 % in cases vs. 6.7% in controls), favoring other combinations not seen in the HF cases. Finally, the presence of NAb was correlated with previous exposure to toxics, hospitalization, and surgery in a logistic regression model. These results constitute the first report of the prevalence of NAb against AAV in the HF population in Latin America. Outstandingly, the prevalence of NAb in our population is higher than the one reported for the same serotypes in the US but lower than in other locations such as China or Africa, a context that may have considerable implications when implementing therapeutic strategies based on AAV vectors in our population.







409. AAV-Binding Antibodies in Cats Living in the Northeastern United States Lack Neutralizing Activity

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Adeno-associated virus (AAV) vectors are promising tools for in vivo gene delivery in experimental animals and humans. Cats are an important preclinical animal model for both studying pathogenesis of human diseases and testing new therapeutic approaches, including AAV vector-mediated gene therapy. A prerequisite for conducting preclinical studies of AAV gene therapy in large animal models is an understanding of pre-existing humoral immunity against AAVs because anti-AAV neutralizing antibodies (NAbs) have the potential to significantly limit the treatment efficacy. However, our knowledge of pre-existing humoral immunity against various AAV serotypes in cats is severely limited. Notably, a recent study demonstrated that approximately half of domestic cats living in Switzerland harbor pre-existing NAbs against one or more AAV serotypes. This observation underscores the importance of more detailed investigation into pre-existing humoral immunity against various AAV serotypes in cats. Here, we show that prevalence of anti-AAV NAbs in cats living in the Northeastern United States is very low, despite the fact that a large fraction of the cat population carries antibodies that bind multiple AAV serotypes. These findings are in stark contrast with the high prevalence of anti-AAV NAbs in cats living in Switzerland. We previously reported at the ASGCT 2015 meeting that an analysis of 85 serum samples collected from 35 client-owned cats, 20 feral cats, and 30 specific pathogen-free (SPF) cats living in the Northeastern United States revealed 9% to 90% prevalence of antibodies that bind various AAV serotypes as detected by an AAV-binding antibody ELISA. Since AAV-binding antibodies do not necessarily neutralize infectivity, we carried out in vitro NAb assays against AAV2, AAV6, and AAV9 on 20 and 44 cat serum samples that were negative and positive for AAV-binding antibodies, respectively. In brief, each cat serum was diluted at 1:2.5, 1:5, and 1:40 and incubated with 1 x 10^9 vector genomes of AAVx-CMV-luciferase vector before infection of reporter cells (note: x=AAV serotype 2, 6, or 9). Two days post-infection with the reporter AAV vector pre-incubated with cat sera, a chemiluminescence-based luciferase assay was performed to quantify transduction efficiency and assess the levels of NAbs present in each serum sample. Consequently, we found that none of the 44 AAV-binding antibody-positive cat sera tested here showed neutralizing activity. Instead, AAV6 and AAV9-binding antibodies exhibited a transduction-enhancing effect. This enhancing effect was not observed for AAV2-binding antibodies. In addition, the assay revealed that AAV6-binding antibody-negative cat sera enhanced reporter cell transduction, indicating that cat serum by itself contains an undefined AAV6 transduction-enhancing factor. Moreover, we found that anti-feline panleukopenia virus (FPV) antibodies that cross-react with AAV6 can be induced by preventive FPV vaccination. We identified a potential amino acid residue on the AAV6 capsid that is responsible for interacting with anti-FPV antibodies. In summary, these new observations significantly further our understanding of pre-existing humoral immunity against AAVs in cats, and highlight a significant difference in the nature of AAV-binding antibodies in cats living in geographically different regions.

410. Pharmacokinetic Comparison of *In Vivo* Expressed Monoclonal IgG/MEDI8852 Antibody vs. Recombinant IgG MEDI8852 Delivered by Various Routes of Administration in Mice

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Through our collaboration with the Defense Advanced Research Projects Agency (DARPA), we are developing novel technologies for delivering nucleic acids constructs into patients for *in vivo* expression of therapeutic proteins. These platforms (mRNA encapsulation within lipid nanoparticles (mRNA), DNA via electroporation (DNA), and adeno-associated virus vector (AAV)) were evaluated using an anti-

influenza A human mAb (MEDI8852) to lay the groundwork for the development of a rapid response to any future pandemics. Although preliminary MEDI8852 pharmacokinetics (PK) results showed that all three platforms can achieve MEDI8852 serum antibody levels >10 μg/mL, platform-dependent differences in the clearance phase of the mAb was due to anti-drug antibodies (ADA) responses. To further understand these PK profiles and ADA responses associated with *in vivo* expressed antibody, we developed an ELISA-based MEDI8852 ADA assay and performed a comparative PK study using recombinant MEDI8852 protein delivered via the same administration routes (IM and IV) as our three platforms. When we assessed the ADA responses, we observed that ADA levels were inversely proportional with antibody expression levels during the clearance phase of the studies. Despite expressing high peak MEDI8852 serum antibody levels, ranging from 32-243 μg/mL, the timing of ADA induction was slightly different between the *in vivo* expressed antibody platforms. Additionally, the timing of ADA induction for the platform was different relative to the recombinant groups, trending towards faster induction in the *in vivo* expressed antibodies. Interestingly, even though the timing of ADA induction was faster than that seen with recombinant protein, the overall average level of ADA was lower in AAV, compared to the other platforms, which may partially explain the long-term circulating levels observed through at least week 28. These studies show that high levels of therapeutic human mAb expression can be achieved with our nucleic-acid-based delivery platforms despite enhanced clearance due to the presence of ADA. Funding source: This research was developed with funding from the DARPA under HR011-18-3-001. The views, opinions and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government. This document was cleared by DARPA on January 27, 2020. All copies should carry the Distribution Statement "A" (Approved for Public Release, Distribution Unlimited). If you have any questions, please contact the Public Release Center.

411. Validation of an Anti-AAV9 Human IGG Screening ELISA

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Due to their versatility, optimal safety profiles and high transduction efficiency, Adeno-Associated Virus (AAV)-based vectors have become instrumental for recent advancements in the gene therapy field. However, pre-exposure to wild-type AAV can trigger humoral immune responses which can pose significant challenges to the intended gene delivery. Ultimately, pre-existing immunity to AAV can have implications for in-/exclusion of patients from clinical trials and/or therapies. One of the standard methods employed to evaluate anti-AAV antibody responses is a binding ELISA. Here, we describe the validation of an anti-AAV9 human IgG ELISA and the results thereof. Briefly, AAV9 empty capsids were coated overnight onto ELISA plates. Wells without AAV9 were also included on each plate to measure the background signal for each sample. Following the coating step, the plates were blocked to prevent non-specific binding of antibodies. After incubation with human serum and plasma samples, plates were washed and an enzyme-labelled IgG-detection antibody was added. Subsequent to substrate addition and color development, the sample optical density was measured. A sample dilution was deemed positive based on criteria set around the measured signals in the AAV9-coated and uncoated wells.Eight individual assay runs were performed for validation of the following parameters: relative accuracy, intermediate precision, repeatability, linearity, specificity, sample stability, as well upper and lower limit of quantitation (ULOQ and LLOQ, respectively). Up to thirteen validation samples were tested, including known positive and negative samples, as well as samples that were generated by dilution of a high positive sample in negative serum matrix. Samples were tested three times per run by different operator teams on different days. The validation data obtained demonstrated that all tested parameters met the acceptance criteria. Concluding, with the validation of AAV9-binding ELISA, we established a robust working frame that can be harnessed as screening platform for assessing pre-existing immune responses to AAVs.

412. CD47 Minipeptide Modification Paradoxically Enhances Anti-AAV Capsid Humoral Immunity Following Systemic Delivery

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A major hurdle of AAV gene therapy is the immune response. The uptake of the AAV vector by professional phagocytes initiates the immune recognition process. Strategies that can minimize phagocytic AAV uptake hold promise to attenuate AAV immunity. The CD47 minipeptide was previously shown to prevent phagocytosis of lentivirus and nanoparticles by macrophage. We hypothesize that incorporation of the CD47 minipeptide into viral capsid can prevent AAV uptake by antigen presenting cells, hence reduce immunity. We engineered His-tagged mouse CD47 minipeptide to AAV.rh32.33 VP2 with or without a flexible peptide linker. Non-denaturing slot blot, denatured silver stain and capsid protein specific western blot confirmed CD47 minipeptide incorporation and its presence on the capsid surface. To study the biological properties, we generated recombinant vectors to express EGFP under a ubiquitous promoter. CD47 minipeptide insertion did not significantly alter viral yield (p=0.665). Intriguingly, the linker containing vector showed significantly reduced infectivity in 293 cells (p=0.006) and was not studied further. Next, we injected 1e12 vg particles of modified and unmodified vectors to the tibialis anterior muscle of normal mice. At harvest, the CD47 modified vector resulted in higher EGFP expression. To investigate systemic effect, we injected 1e12 vg particles of vectors via the tail vein. At 4 days post-injection, we observed robust B cell and CD4+ T cell accumulation in the peripheral blood in mice injected with the CD47 modified, but not the parental AAV.rh32.33 vector. Nevertheless, unmodified AAV.rh32.33 induced a greater accumulation of total and CD44+ CD8+ T cells which peaked at 14 days post-injection. To determine if the robust B cell response to the modified vector resulted in enhanced humoral immunity, we measured capsid specific IgM, IgG1 and IgG2a in the serum by ELISA. Mice injected with the CD47 modified, but not the parental, vector developed a significantly pronounced IgM (peak 14 days) and IgG2a (peak 21 days) response. IgG2a remained elevated

until the study end-point at 70 days post-injection. No significant changes were seen with the IgG1 levels. Our results suggest that CD47 modification may paradoxically increase capsid humoral immunity. Rational modification of the AAV capsid through genetic engineering may have unpredictable and variable effects (Supported by NIH, Hope for Javier and Jackson Freel DMD Research Fund).

413. Recombinant AAV Serotype 8 Doesn't Trigger Viral Gene Pathways in Human Monocyte-Derived Dendritic Cells

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Recombinant Adeno-Associated Virus-derived vectors (rAAV) are the most used *in vivo* viral gene transfer platform with numerous therapy clinical trials worldwide for genetic diseases. Already three rAAV products have been approved in the market. Nevertheless, their clinical use has been tempered by rising concerns over their immunogenicity. Most of previous studies described host adaptive humoral and cellular immunity against the viral capsid and/or the transgene product. Very few studies have been interested in rAAV-crosstalk with the innate immune system. Particularly, little is known about the interaction of rAAV with dendritic cells (DC). Here, we have investigated the ability of the rAAV serotype 8, currently under evaluation in phase 3 clinical trials for liver and muscle genetic diseases, to transduce and activate *in vitro* primary human monocyte-derived DC (moDC). Using a quantitative RNAseq-based method, we have found that rAAV8 does not trigger anti-viral or inflammatory gene expression in moDC from 12 healthy donors, as compared to adenovirus-derived vectors. This lack of moDC reactivity was observed despite the presence of AAV8 capsid in moDC cells as well the detection of transgene transcripts. In addition, autologous transduced moDC were not able to activate memory T cells that were reactive towards AAV8 capsid-derived peptides. Using RNAseq strategies in DC-based cell models might contribute to the evaluation and characterization of rAAV serotype immunogenicity.

Cell Therapies

414. Selecting Potent Guide RNAs with Minimal Off-Target Activity for the CRISPR/Cas9 Engineering of KSQ-001, An Engineered Tumor Infiltrating Lymphocyte (eTIL™)

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Adoptive cell therapy (ACT) with *ex vivo* expanded Tumor Infiltrating Lymphocytes (TIL) offers a potentially transformative treatment for refractory solid tumors. However, the immunosuppressive tumor microenvironment (TME) limits the effectiveness of TIL therapy. To identify gene targets capable of enhancing anti-tumor T cell function, we performed a genome-wide CRISPR screen in T cells *in vivo*. We discovered and subsequently validated CT-1 as a top target to improve T cell function for ACT. KSQ-001 is an engineered TIL (eTIL™) therapy with the CT-1 gene inactivated by CRISPR/Cas9. KSQ-001 is under development as an autologous ACT for treatment-refractory solid tumors. We describe herein the identification of potent and selective sgRNAs used to target the CT-1 gene during the manufacture of human TIL into KSQ-001. To identify sgRNAs suited to engineer KSQ-001, we systematically evaluated all potential SpCas9 CT-1 sgRNAs for potency and selectivity. We rank-ordered sgRNA potency by screening sgRNA tiling libraries targeting CT-1 using a functional read-out in primary human T cells. Top hits were independently validated by assessing editing efficiency at the genomic cut-site and by performing CT-1-dependent functional assays in primary human T cells. To identify selective sgRNAs able to potently inactivate CT-1 with minimal off-target edits, an *in silico* approach was paired with unbiased experimental mapping of off-target cut sites in primary T cells using GUIDE-Seq. Identified CT-1 sgRNA off-target sites were then verified using targeted amplicon sequencing and target capture technology, with CT-1 sgRNAs further triaged. Using these assays, we identified a sgRNA targeting CT-1 with an editing efficiency at or above 90% in human TIL that translated to robust pathway modulation and possessed minimal off-target edits. Together, these data demonstrate the discovery of a potent and selective CT-1 sgRNA that will be used for the manufacture of KSQ-001eTIL™ in the clinic.

415. The Impact of Donor Sex on Umbilical Cord Tissue Mesenchymal Stromal Cells

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Background: Umbilical cord tissue (CT) is rich in mesenchymal stromal cells (MSCs) and can be collected in a non-invasive manner following delivery. Given the potential utilization of MSCs in regenerative medicine applications, many newborn stem cell banks offer cryopreservation of CT, alongside cord blood, for future cell-based applications. MSCs are found in various adult tissues, including bone marrow and adipose tissue. However several reports suggest increased age and certain chronic disease conditions negatively impact MSC function; there is also evidence donor sex may impact the degree of therapeutic benefit of adult MSCs in certain experimental disease conditions. Given the potential impact of donor variability, we sought to examine the influence of donor sex on characteristics of newborn MSCs isolated by explant outgrowth from cord tissue cryopreserved as a composite tissue in a newborn stem cell bank. **Methods**: Research donated umbilical cords from 10 full term neonates (5 female, 5 male) were collected from consenting mothers. Upon receipt, a portion of the cord was excised to evaluate the metabolic status of the tissue (Skiles et al, 2018). Each cord was then cut into small pieces and prepared for cryopreservation and later isolation of MSCs by explant outgrowth or plated as fresh explants (Skiles et al, 2018). After storage in the vapor phase of liquid nitrogen for at least a week, thawed composite tissue was explanted in MSC-selective medium for 2 weeks (end of P0). MSCs were expanded to the end of the third passage (P3) and cell characteristics compared. **Results**: Quality of CT was assessed by metabolic activity assay of composite tissue using an AlamarBlue based assay, with neither fresh nor thawed tissue resulting in a difference in dye reacted based on donor sex (Table 1). Cell yield of male and female derived CT was similar when plated fresh and when isolated from previously cryopreserved tissue (Table 1). A small difference in the viabilities of cells isolated from thawed female and male donor cords was noted (90.1%±6.0 and 81.5%±5.8 respectively, p=0.05), however the difference was no longer apparent by the end of P3 (94.3%±2.5 and 94.8%±1.4, p=0.7). Cell populations isolated from female and male donor cords expressed surface markers CD73, CD90, and CD34/45 at 98.5%(±1.9%), 95.0%(±4.6%), and 1.2%(±1.9%) vs. 99.5%(±0.4%), 93.4%(±5.9%), and 0.4%(±0.3%), respectively, with no significant differences (p=0.3, p=0.7, p=0.4). Doubling time during P3 growth was 1.1(±0.26) and 1.3(±0.28) days for cells from female and male donor cords respectively, with no significant difference (p=0.4). **Conclusion**: Umbilical cords from full term females and males are similar in their cell yield when utilizing explant outgrowth to isolate cells from composite tissue and the cells obtained are phenotypically indistinguishable with similar proliferation and growth characteristics. This study is limited in size; additional analysis using a larger data set, including cytokine profiling and functional attributes relevant for regenerative medicine, is in progress. In the setting of personalized medicine and private newborn stem cell banking, every newborn tissue is considered an inherently unique resource. Exploring the impact of donor sex, as well as maternal and paternal characteristics to the newborn whose tissue is stored, is of interest as we seek to ensure maximum clinical utility of cord tissue cryopreserved for future cell-based therapies in a large scale stem cell bank setting.

Table 1.

	Fresh Tissue		Frozen Tissue	
	Average alamarBlue % Reduction	Average Cell Yield/Gram (Std Dev)	Average alamarBlue % Reduction	Average Cell Yield/Gram (Std Dev)
Female (n=5)	62.5%	5.9x10^6 (8.8x10^5)	54.9%	4.9x10^6 (5.5x10^6)
Male (n=5)	65.6%	6.3x10^6 (2.4x10^6)	63.4%	5.8x10^6 (4.7x10^6)
p-value	0.54	0.75	0.40	0.79

416. Glutamate-Cysteine Ligase Modifier Subunit Overexpression Confers Resistance Against Busulfan

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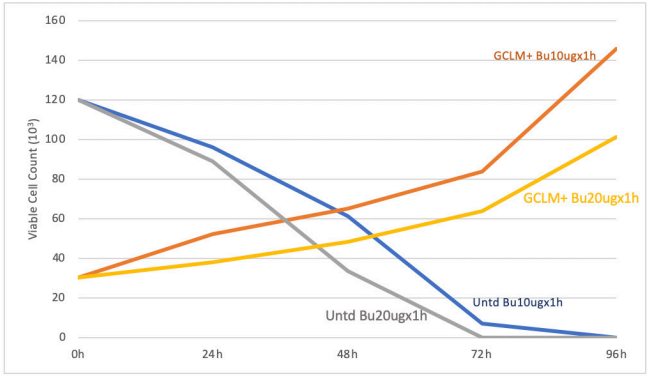
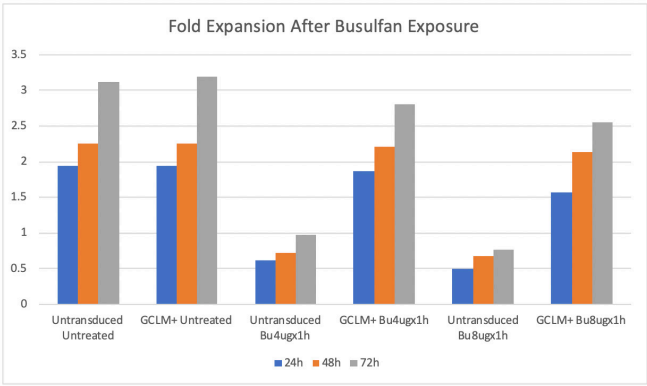
Background: The biggest obstacle in gene-modified stem cell therapies is lack of sufficient engraftment. One proposed solution is giving these cells chemo-resistance against cytotoxic agents to select them in vivo. However, finding a single resistance gene that confers sufficient resistance against agents particularly toxic to hematopoietic stem/progenitor cells (HSPCs) has been challenging. Busulfan is used for conditioning regimens in HSPC transplants due to its profound toxicity to HSPCs. Glutamate-cysteine ligase (GCL) is an enzyme functions at the first step of Glutathione (GSH) synthesis pathway, which contributes to busulfan clearance. We evaluated GCL modifier subunit gene expression as a busulfan resistance factor.**Methods:** We transduced Jurkat, CEM, and Tf-1a cell lines with a lentiviral vector that expresses GCLM and green fluorescent protein (GFP) genes. Chemoprotection experiments were done by incubating untransduced or mixed populations of (20% transduced, 80% untransduced) cells with busulfan in concentrations ranging from 0 to 200ug/ml for 1 or 2 hours. Cells were then incubated in busulfan-free culture for 96h. Area under the concentration-time curve (AUC) was estimated by using trapezoidal rule from different times and concentrations of incubation. Pre and post-exposure GSH levels were measured with fluorometric assays. Cell survival and proliferation at different time-points were measured by Nucleocounter NC-200 and flow cytometry using PI and 7-AAD staining protocols. The enrichment of protected GFP+ cells were evaluated by flow cytometry. We also measured baseline GSH levels and GCLM expression in primary human HSPCs harvested from 3 different donors with fluorometric and RT-PCR assays. **Results:** GSH level changes between untransduced and transduced Jurkat, CEM and Tf-1a cells were 2.1, 1.7, and 1.9-fold respectively. Untransduced CEM cells were found to be most sensitive to busulfan exposure. Cell death and proliferation in all cell cultures were in an AUC-dependent manner. The 1.7-fold GSH activity in CEM cells conferred at least 3.5-fold protection against busulfan. At 72h of the culture following 10ug/ml busulfan exposure for 1h, the purity of transduced CEM cells increased from 20% to 88%, and the viable transduced cell count increased from 30x103/ml to 84x103/ml. At doses higher than 10ug/ml or longer exposure than 1h, the transduced cells were 100% of the cultures after 72h (Fig 1). Fold expansion in viable transduced cells at 72h was slightly lower than untreated controls but much higher than treated

groups, suggesting a low degree busulfan-related toxicity in transduced cell population (Fig 2). GSH levels in transduced cells remained stable throughout the experiment, whereas GSH in untransduced cells was depleted after busulfan exposure. RT-PCR analysis showed that GCLM is not highly expressed in these cells.





Conclusion: Increased expression GCL modifier subunit alone confers substantial chemoprotection against busulfan. This indicates that GCLM transgene expression can be used for post-transplant in vivo selection in gene modified autologous HSPC transplants.

417. Pigment Epithelium-Derived Factor Peptide Stimulates the Proliferation of Hair Follicle Stem Cells Through its Effect on Dermal Papilla Cells

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Two types of hair stem cells, hair follicle stem cells (HFSCs) and dermal papilla cells (DPCs) are responsible for hair follicle cycling. Our previous work in stem cells has uncovered that pigment epithelium-derived factor short peptide (PDSP) facilitates full-thickness cutaneous wound healing by promoting HFSC proliferation. It still remains to be investigated whether PDSP has a significant role for DPCs. In response to androgens, especially a more active form, dihydrotestosterone (DHT), dermal papilla secretes diffusible factors that cause decrease of the proliferation and induction of apoptosis in follicular keratinocytes, leading to androgen-potentiated balding. In this study, topical application of PDSP reversed the DHT-induced growth suppression of follicular keratinocytes and hair premature transition from anagen to catagen in two mouse models of alopecia. In addition, PDSP suppressed DHT-induced growth suppression of follicular keratinocytes in cultured hair follicles, supporting the hair growth-protective effect of the PDSP. Our data provide early evidence that PDSP protects DPCs or its progenitors to against DHT-induced androgenetic alopecia.

418. Second-Generation Targatt™ System Generates High Efficiency and Safe Large Fragment Knock-In in Human iPS Cells

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Gene editing in patient-specific induced Pluripotent Stem Cells (iPSCs) offers one of the most promising approaches for personalized therapy in regenerative medicine. CRISPR/Cas9 works most efficiently for gene knockout through the non-homologous end joining (NHEJ) pathway, and for point mutation/ correction through homology directed repair (HDR). However, efficiency for large fragment DNA knock-in through nuclease mediated HDR is very low. Previously, we published an alternative technology platform TARGATT™ to efficiently generate large fragment knock-in using a "Docking-site-ready" iPSC master line (first generation TARGATT™ system). The master cell line was generated by inserting an integrase recognition "attP" site into a specific genomic safe harbor locus using CRISPR/Cas9. Here, to further improve this technique and streamline transgene integration for easy adaptation to large-scale protocol needed for clinical application, we present the second-generation cell lines with higher efficiency, simpler procedure and safer clinical application. Frist, we optimized the "attP" sequence in the docking-site and integrase variants. Second, we strategically re-designed the docking site construct to leave no antibiotics genes after transgene integration. Using this second-generation system, we can knock-in any transgene (e.g. 20 kb) at the preselected docking site with high efficiency, and without drug selection or cell sorting. Our data support that second-generation TARGATT™ provides a valuable new platform for efficient genome editing in human iPSCs.

419. Regional Gene Therapy for Bone Healing Using 3D Printed Scaffolds

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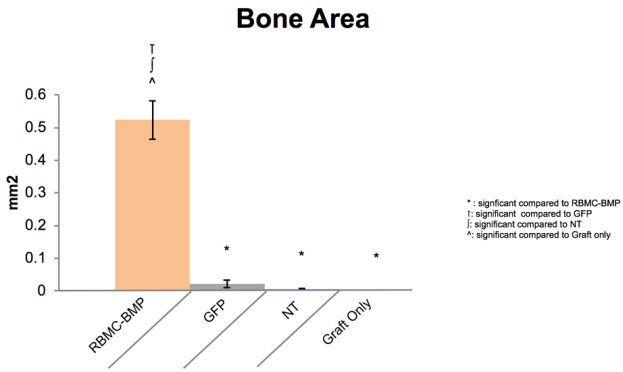
INTRODUCTION: Regional gene therapy utilizing an ex-vivo strategy to express BMP-2 has been demonstrated to be effective in pre-clinical models. The cells need to be delivered in a carrier, and one possible option is a 3D printed scaffold. The purpose of this study was to evaluate the viability of a custom 3D-printed osteoconductive scaffold as a cellular delivery vehicle in a rat regional gene therapy model. METHODS: A well-established, rat femoral critical-sized bone defect model was utilized. A femoral sleeve scaffold was designed to match

the dimensions of the defect and 3D-printed from a hydroxyapatite-composite, osteoconductive material. The scaffolds were then loaded with rat bone marrow-derived mesenchymal stem cells transduced with a lentiviral vector carrying cDNA for BMP-2 (RBMC-BMP). This experimental group was compared against 3 control groups - empty scaffolds (Graft only), scaffolds loaded with nontransduced rBMCs (NT), and finally scaffolds loaded with rBMCs transduced for green fluorescent protein (GFP) expression. Bone formation was assessed using serial radiographs over 12 weeks, microcomputed tomography (uCT), and histomorphometry.RESULTS: Treatment with BMP-2 expressing rBMCs with the 3D-printed scaffold demonstrated a significant increase in new bone formation. Blinded scoring of radiographs showed excellent healing with RBMC-BMP (12 of 14 fully healed) versus the control groups (none healed). The experimental group had a mean bone area of 0.52mm^2 versus 0-0.02mm^2 in the controls as measured by histomorphometry (Figure 1). On uCT, the experimental group demonstrated significantly more bone volume at the defect site versus the controls (34.75 mm^3 versus 8.9 - 13.9 mm^3, respectively, $p < 0.01$).DISCUSSION/CONCLUSIONS: A 3D-printed osteoconductive hydroxyapatite-composite scaffold was found to be an effective carrier for bone marrow cells transduced to overexpress BMP-2. The scaffold alone was not sufficient to promote adequate healing, suggesting that it is not substantially osteoinductive. The combination of gene therapy with 3D-printed scaffolds is promising, but additional work is required to optimize cell dosage and delivery.



420. Abstract Withdrawn

421. CS1 Targeted CAR-T Cells (MB-104) for the Treatment of Multiple Myeloma Shows Antitumor Activity Sparing Normal T-Cells Despite the Common Expression of CS1

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Multiple myeloma (MM), a plasma cell malignancy, remains a significant therapeutic challenge due to frequent relapses and resistance to targeted therapies. There are several antigens being targeted for treatment of MM, of which BCMA targeting chimeric antigen receptor (CAR) T cell therapy has shown remarkable response rates in clinical trials. Despite the high response rate, relapses are frequent, and durability of response remains to be seen. Mechanism of relapse include, outgrowth of antigen negative tumors, lack of CAR-T persistence and inhibitory tumor microenvironment. Hence, there is a clear need for developing CAR-T cells targeting novel antigens that can increase treatment efficacy and durability. Mustang Bio has acquired an autologous CS1 CAR-T cell therapy for treatment of relapsed or treatment-resistant MM. CS1 (also known as CRACC, SLAMF7 and CD319) antigen is a promising target, as it is highly expressed on the surface of malignant plasma cells and at relatively lower levels on other immune cell types (such as NK cells, T cells, and monocytes). The presence of CS1 on immune cells could pose challenges, such as, CAR-T manufacturing challenges (including fratricide), as well as on target off tumor cell lysis. CS1 gene deletion in CAR-T cells addresses fratricide, but it will not prevent the CS1 CAR-T from targeting endogenous immune cells. CS1 CAR-T administration resulted in significant survival benefit and tumor regression against CS1+ tumor cells in orthotopic NSG mouse model for MM. Here, we characterize and assess the impact of MB-104 (CS1 CAR-T) on T cells due to shared CS1 antigen expression. Methods: Expression of CS1 on normal blood cells and multiple myeloma cell lines was determined using anti-CS1 antibody from BioLegend. Healthy donor T cells were transduced with lentivirus expressing anti-CS1scfv-41BBZ-T2A-tEGFR and expanded using commercially available T cell stimulation reagent plus cytokines to generate MB-104. Non-transduced, GFP transduced or CD19 CAR transduced cells were used as controls. T cell viability, growth kinetics and phenotype were assessed over time using the Mustang Bio CAR-T cell manufacturing process. T cell fratricide was assessed via a flow cytometry-based assay. Results: Here we demonstrate MB-104 does not confer biologically significant fratricide and can be successfully manufactured as evident by viability, growth kinetics and fold expansion despite the shared antigen expression between tumor cells and T cells. CS1 positive T cells are present in culture during the expansion of MB-104 suggesting absence of fratricide. Finally, MB-104 can induce potent anti-tumor cell lyse and proliferate in response to tumor cells. Taken together our results demonstrates MB-104 is a novel CS1 targeting CAR that shows potent anti-tumor cell lysis but spares normal T cells, despite the shared CS1 antigen expression on tumor cells and T cells.

422. Optimized Culture Medium for Enhanced *Ex Vivo* Expansion of Human Hematopoietic Stem Cells

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A major limitation in *ex vivo* expansion of harvested human hematopoietic stem-progenitor cells (HSPCs) is the rapid differentiation of HSPCs at the expense of the most primitive pluripotent hematopoietic stem cells (HSCs). A culture system that expands pluripotent HSCs would benefit clinical HSC applications including transplantation and gene therapies. To address this challenge we developed a xeno-free, serum-free medium - CTS™ StemPro™ HSC Expansion Medium. In a Design of Experiments approach, media constituents were systematically altered and media iterations were evaluated with the goal of maximizing *ex vivo* expansion of HSCP immunophenotypes.

Culture of normal primary human CD34+ cells immunopurified from cord blood, mobilized peripheral blood and bone marrow in HSC Expansion Medium supplemented with FLT3L, SCF, TPO, IL3, and IL6 (FKT36), resulted in higher numbers of immunophenotype-defined HSPCs, as compared to either uncultured day 0 cells or cells cultured in industry-standard culture media containing FKT36. For example, culture of primary human CD34+ cells from mobilized peripheral blood (mPB) for 7 days in FKT36-containing HSC Expansion Medium resulted in ~100-fold increased numbers of CD34+CD45+Lin- cells and ~2000-fold increased numbers of CD34+Lin- CD90+CD45RA- cells (an early HSPC immunophenotype), as compared to uncultured day 0 cells. $CD34^+$ cells expanded in this medium maintained differentiation capacity in *in vitro* colony forming assays, forming erythroid and non-erythroid cell colonies. In addition, using a directed differentiation method we showed that CD34+ cells were able to differentiate into erythroid lineage which generated red blood cells at greater than 80% efficiency as indicated by co-expression of CD71 and CD235a markers. We demonstrated that CTS™ StemPro™ HSC Expansion Medium enables transduction of CD34+ cells with lentiviral vector generated using CTS™ LV-MAX System. Additionally, this medium supports gene editing CD34+ cells with CRISPR-Cas9 Gene editing tools that are suitable for cell and gene therapies. Following gene editing using TrueCut Cas9 Protein v2 and electroporation CD34+ cells cultured in HSC Expansion Medium maintained the CD34+CD90+ CD45RA- immunophenotype and were capable of forming erythroid and non-erythroid cell colonies in *in vitro* colony forming assays. Crucially, $CD34^+$ cells expanded with CTS™ StemPro™ HSC Expansion Medium exhibited long-term engraftment potential and multilineage chimerism at 6 months post-transplant in a murine model. We demonstrated that *ex vivo*-cultured $CD34^+$ HSPCs generate cell dose-dependent engraftment in NRG mouse bone marrows and spleens at 26 weeks post-transplant. By expanding HSPCs that are well-suited for translational purposes such as transplantation and gene therapies, CTS™ StemPro™ HSC Expansion Medium can be a key tool for clinical HSC applications.

423. Targetable Micropharmacies: Cells that Produce Small-Molecule Drugs *In Situ* at the Tumor Site

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Small-molecule drugs, particularly cytotoxic cancer therapeutic agents, are fundamentally limited by their toxicities when administered systemically. Thus, local production of a drug *in situ*, only at the site of disease, provides a strategy to increase this therapeutic index. Here we leverage the emerging cell-based therapeutic technology of CAR-T cells (chimeric antigen receptor T-cells) to develop living micropharmacies, termed SEAKER (Synthetic Enzyme-Armed KillER) Cells, which can produce small-molecule drugs *in situ* at the site of disease. SEAKER cells are genetically encoded with enzymes that can unmask non-toxic prodrugs. The SEAKER cells are administered first, interact with tumor antigen, clonally expand at the tumor site, and produce high local concentrations of the enzyme. The inert prodrug is then administered systemically, but is only activated at the tumor site by the SEAKER cells. The active drug can then diffuse into tumors and kill cells regardless of antigen expression. This novel approach provides targeted, synergistic anticancer activity of both the small-molecule drug and T-cell. We demonstrate *in vitro*, *in cellulo*, and *in vivo* activity of the SEAKER platform using two classes of enzymes and several classes of prodrugs. This establishes a modular therapeutic approach for a variety of cancers as well as other diseases.

424. Melanocortin Receptor-4 Regulates Expression of Anti-Angiogenic Molecules in Plasmacytoid Dendritic Cells and Reduced Suture Induced Corneal Neovascularization

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The avascular cornea is endowed with resident plasmacytoid dendritic cells (pDCs). We have recently shown that corneal pDCs are involved in maintaining cornea avascularity through expression of angiostatic proteins, including endostatin (ES), platelet factor (PF)-4, tissue inhibitor of metalloprotease (TIMP)-3 and thrombospondin (TSP)-1. The purpose of this study was to examine if corneal pDCs neuropeptide receptors may regulate the angiostatic activity of pDCs and to determine the potential therapeutic use of pDCs to suppress pathological angiogenesis. We first examined the ability of trigeminal ganglion (TG) neurons, which innervate the cornea, to induce pDC angiostatic activity. Primary splenic pDCs from DPE-GFP×RAG1-/- transgenic mice (pDC-GFP) were isolated by fluorescence activated cell sorting (FACS) and cultured with primary C57BL/6 TGs, isolated by Percoll differential gradient, in Ham's F-12 with 10% heat inactivated FBS. Corneal pDC gene and protein expression of ES, PF-4, TSP-1, TIMP-3 were quantified by single cell qRT-PCR as well as by flow cytometry. At 24 hours of pDC and TG neuron co-culture, expression of ES, TSP-1, PF-4, and TIMP-3 by pDCs at the gene and protein levels was significantly increased compared to baseline pDCs and TG neurons alone ($p<0.05$). We next examined the role of neuropeptide receptors such as the melanocortin receptors on pDC angiostatic activity. Expression of melanocortin (MC-4) receptor and angiostatic molecules of splenic and corneal pDCs was quantified by flow cytometry ± MC-4 agonist (THIQ) [10ug/mL]. The expression of MC receptors 1-5 was assessed on pDCs and revealed that pDCs express the gene and protein of the MC-4 receptor at higher levels compared to conventional dendritic cells and macrophages ($p<0.05$). pDCs incubated for 24 hours with a highly selective agonist for MC-4, THIQ, demonstrated significantly greater mRNA and protein levels of ES, PF-4, TSP-1, and TIMP-3 compared

to baseline non MC-4 activated pDCs ($p<0.05$). Next, we examined the in vivo role of MC4 agonist THIQ on pDC mediated suppression of corneal neovascularization (CNV). Utilizing an established suture induced model of corneal neovascularization, 3 sutures were implanted in C57BL/6 animals for 14 days. THIQ [10ug/mL] was injected subcutaneously every 2 days for 14 days. Murine corneas were excised, stained for CD31, and quantified by confocal microcopy and ImageJ. Animals treated with THIQ [10ug/mL] showed significantly reduced CNV when compared to saline treated animals ($p<0.05$). Our findings demonstrate co-culture of pDCs with TG neurons increases pDC expression of proteins that suppress neovascularization and pDCs expression of angiostatic molecules is, at least in part, mediated by MC-4. We then show pDC MC4 activation by THIQ reduces suture induced CNV. Together, our data suggest a potential novel pDC based therapy to reduce CNV. Further studies are required to examine the role of pDCs as a cell based therapy to suppress pathological angiogenesis.

425. Abstract Withdrawn

426. Acoustofluidic Sonoporation Gene Delivery for Cancer Immunotherapy

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T-cell-based immunotherapies that leverage the collection and genetic modification of patients' T-cells to express chimeric antigen receptors (CAR) specific for tumor antigens represent a significant advance in the treatment of relapsed and refractory leukemia and B-cell lymphomas. The effective manufacturing of CAR-T cell products for patient care requires target cell populations to be engineered rapidly, efficiently, safely, and cost effectively. Toward this goal, we report an acoustofluidic strategy to deliver a CD19-specific CAR transgene to T-cells *in vitro* by shearing cells against a glass microcapillary wall. Mechanistic investigations of cell membrane permeability post-acoustofluidic treatment reveal that the cell and nuclear membranes are disrupted in model cell types, promoting the entry of DNA and other biomolecular cargoes for gene-editing into cells. To further enhance the performance of engineered T-cells and achieve uniform CAR expression, we have configured our platform to deliver CRISPR/Cas9-based cargoes that direct the CAR to the T-cell receptor α constant (*TRAC*) locus. Overall, this acoustic-based transfection strategy represents a versatile platform technology that potentially enables a wide range of therapeutic payloads to be delivered to target cells for applications spanning both the cancer immunotherapy and stem cell-based gene therapy spaces.

427. Bridging NK Cell Expansion Methods Towards a Feeder-Cell Free Scalable GMP Production of Hyperfunctional NK Cells

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Natural Killer (NK) cells are lymphocytes that detect and kill virally-infected or malignant cells in a non-HLA restricted manner. Such attributes potentially allow the development of a potent, allogeneic, anti-cancer cellular immunotherapy. Clinical potential of NK cell-based therapies has been recognized for a range of malignancies and various approaches for clinical application are being explored. For broad accessibility however, a safe, scalable, affordable production platform is key. A feeder-cell (FC) based NK cell expansion platform using K562 cells modified to express mbIL-21 and 41BBL (FC21) has been previously developed to efficiently expand PBMC-derived NK cells to allow therapeutic application of high doses of NK cells. NK cells expanded with FC21 (FC21-NK) have hyperfunctional phenotypic & functional attributes and were successfully administered in a Phase 1 trial (NCT01904136) to 24 patients as adjunctive treatment to HSCT for AML, without significant safety concerns. More recently a FC-free method was developed using membrane particles presenting mbIL-21 & 41BBL (PM21), derived from plasma membranes of FC21. In contrast to FC, these PM21 particles can be irradiated at 150-fold higher level to achieve terminal sterilization. The resulting PM21 particles could be considered a lower risk Tier 3 ancillary material compared to FC. While PM21 particles similarly allows for high scale expansion of NK cells with similar hyperfunctional phenotype & function, the PM21 particle production method eliminates the risk of residual tumor feeder cells in the final drug product. Finally, the PM21 particle expansion process has been adapted to GMP compliant closed, semi-automated processing on scalable production platforms, which allows affordable mass-production. This report demonstrates the suitability of PM21-based large scale manufacturing of therapeutic NK cells (Kiadis NK cells, K-NK) and the similarity in expansion rate, phenotypic & functional characteristics, relative to both FC21-NK and PM21-NK generated at academic lab-scale. FC21-NK were produced with the same feeder cell line and general method used for clinical manufacturing in study NCT01904136. PM21-NK were grown from same donors as for FC21-NK and directly compared for NK cell expansion yield, cytotoxicity, cytokine release (IFNγ and TNFα) and expression of key receptors, and were generally found to be similar. In-vivo therapeutic potential of FC21 and PM21 cells was also similar, both increasing median survival in a rapid tumor model by 10 days relative to controls in a murine ovarian-cancer model. K-NK cells produced through the large scale manufacturing process were tested with similar characterization assays and were found to be very similar to the FC21-NK and PM21-NK cells produced at academic scale, and showed further improved expansion rates thanks to optimized process conditions. These data establish the feasibility of large scale production of highly functional K-NK cells expanded with PM21. The GMP-compliant process for PM21-expanded K-NK cell production is currently being applied in a broad development program to further establish safety and efficacy in the clinic.

428. Machine Learning-Driven Label-Free Flow Cytometry for Cell Manufacturing

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Flow cytometry is a methods useful for analyzing phenotypic characteristics of cells by measuring purity of target cells in

heterogeneous cell populations for clinical diagnosis, as well as for controlling quality of therapeutic cell products and monitoring them in blood samples. One hurdle to analyze the cell products using flow cytometry is, however, that it requires cell labeling by specific antibodies/reagents under GMP compliance, and the process is time-consuming and costly. Here we introduce label-free ghost cytometry (LFGC), a new label-free flow cytometry that analyzes cell morphology based on cell-specific waveform signals without image production using machine learning. LFGC may become a simple, fast and cost-effective method for cell analysis. In this study, we assessed the potential applicability of LFGC to characterize the characteristics of cell products. In the first case, we developed a supervised machine learning model from a training data set comprising of label-free GC waveform signals of T cells that were labeled with markers of Annexin V plus PI. The T cells were then classified into live or dead states by applying the trained model to the label-free GC waveforms without observing the fluorescent ground truth labels. Similarly, we assessed the ability of LFGC for identification of T cells in human PBMC using CD3 labeling. Lastly, we applied the LFGC system for discriminating in-vitro activated T cells from resting T cells using CD25 labeling. Our results show that LFGC accurately classified the live and dead T cells with area under the receiver operating characteristic curve (AUC) of 0.954. LFGC also classified the CD3 positive T cells from PBMC with AUC of 0.967 and also the activated T cells from the resting ones with AUC of 0.987. These results support that LFGC can be used to accurately predict the viability, purity and possibly functions of T cells without molecular labeling and will be thus useful for characterizing cell products.

429. Optimizing Gamma Delta (γδ) T Cell Manufacturing from Peripheral Blood Mononuclear Cells (PBMCs)

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Clinical applications for the use of gamma delta (γδ) T cells have advanced from initial interest in expanding γδ T cells *in vivo* to interest in the development of a manufacturing process for the *ex vivo* expansion of γδ T cells. Cryopreservation is a necessary step in the development of a commercial manufacturing process, yet little is known about the effects of cryopreservation on γδ T cells. Our lab has previously shown that γδ T cells can be expanded from a pool of peripheral blood mononuclear cells (PBMCs) in serum-free conditions using zoledronate and IL-2 in OpTmizer medium. Our current method for storing expanded γδ T cells consists of freezing the cells in a controlled-rate freezer in a solution comprised of 5% Albutein (90%) and dimethyl sulfoxide (DMSO) (10%). Our preliminary data show that when γδ T cells are cryopreserved and thawed in growth medium, as little as 20% of the cell product is viable by 24 hours post-thaw. Therefore, we used our γδ T cell expansion method to determine the effects of cryopreservation on γδ T cell health and functionality and optimize the initial thawing process. When we compared 5 good-manufacturing practice (GMP) quality media specific for cryopreservation, we found none provided an added benefit in terms of 1) the number of cells recovered or 2) the percentage of cells recovered post-thaw or 3) the percentage of apoptotic γδ T cells up to 4 hours post-thaw. Surprisingly, we found that thawing the cell product directly into human serum albumin (5%) (HSA), but not human serum, human platelet lysate (HPL), or OpTmizer media, significantly decreased the number of apoptotic γδ T cells by an average of 24% at 4 hours post-thaw. Specifically, after thawing in HSA, we found less than 40% of cells were apoptotic at 4 hours whereas greater than 60% of cells were apoptotic after thawing in the other media. The addition of IL-15 and/or IL-21, added no benefit in terms of increasing post-thaw viability. Efforts were then focused on manipulating the product prior to cryopreservation. Because γδ T cells appear to be especially sensitive to cryopreservation, we attempted to provide some protection by condensing the chromatin prior to freezing. We found that condensing the chromatin in solutions with high osmotic concentrations prior to freezing decreased the percentage of apoptotic γδ T cells immediately after thawing. For example, 1x PBS in media, which has been reported to induce chromatin condensation, increased viability by an average of 10%, as compared to γδ T cells frozen under standard conditions. Taken together, these data suggest that manipulating γδ T cells prior to freezing with various GMP compliant freezing media has no effect on cell viability, but the choice of post-thaw media can provide some benefit. Our results further suggest it is possible to manipulate the cell product prior to freezing to decrease the negative effects of cryopreservation, and we were able to achieve greater than 70% viability after thawing our cryopreserved γδ T cell product candidate. Further work will investigate the impact of chromatin condensation on the post-thaw health and functionality of γδ T cells.

430. Building Capabilities for an Allogeneic Cell-Based Cancer Immunotherapy Approach with T-iPSC as the Renewable Intermediate

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The immuno-oncology field has grown immensely since the FDA approval of CAR-T therapy to treat B-cell leukemias. However, currently approved CAR-T manufacturing methods utilize autologous cells, which present challenges including heterogeneity in the starting cells which come from unhealthy donors, and a high cost of production. These downsides have prompted investigation into improved methods to develop therapeutic T-cells, including using induced pluripotent stem cells (iPSC) as the starting material. iPSC can be differentiated into T-cells, providing a near infinite source of these cells, and iPSCs derived from antigen-specific T-cells will preserve the genetic signature of the unique T-cell receptor upon differentiation. However, these cells can be difficult to reprogram to iPSC. Previous data has shown that Sendai virus based reprogramming methods can efficiently generate iPSCs from CD3+ pan T-cells. Here, we show that these methods can also efficiently generate iPSC from isolated CD4+ or CD8+ cells. These iPSC were analyzed for immune repertoire using an NGS-based assay, and characterized for genetic stability, pluripotency, differentiation capacity. The workflow presented here demonstrates a comprehensive suite of media, reagents, and characterization assays that can enable the generation of iPSC for use in cell therapies.

431. Increased Engraftment of Gene Modified HSPCs Overexpressing ALDH1 Using *In Vivo* Chemoselection

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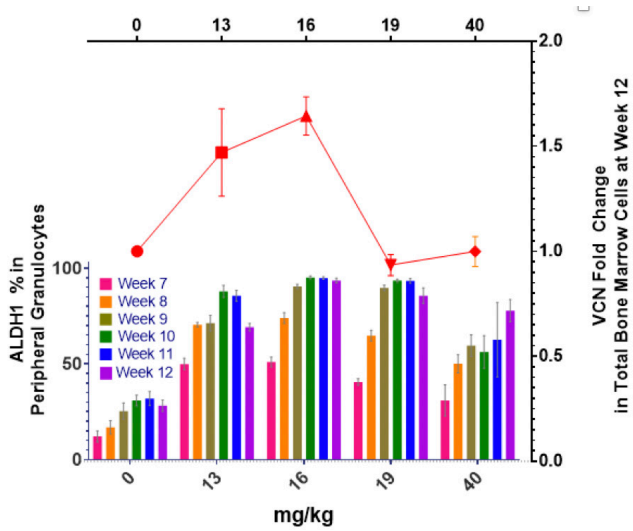
Background: Allogeneic hematopoietic stem cells transplants (HSCT) can cure chemotherapy-resistant leukemia and lymphoma including those with HIV infection using CCR5-Δ32 homozygous donor cells. However, significant risks prevent broad use, and current use of autologous HSCTs lack sufficient bone marrow engraftment. High intracellular levels of ALDH1 confer protection against cyclophosphamide (CY) and thus allow the selection of gene-modified cells overexpressing ALDH1 with CY. As a potential engraftment-enhancing strategy, we designed a lentiviral vector (HV01) that causes ALDH1 overexpression along with CCR5 knockdown and C-peptide expression (fusion inhibitor) for HIV protection. We characterized each expression cassette *in vitro* individually and evaluated multilineage engraftment of HV01-modified hematopoietic stem cells (HSPCs) in C57BL/6 mice using CY as a post-HSCT chemoselective agent. **Methods:** Expression of ALDH1 and C-peptide and knockdown of CCR5 were measured by FACS, rtPCR and qPCR. Mice underwent autologous HSCT with HSPCs transduced with lentivirus expressing ALDH1 or with a control vector after non-myeloablative conditioning. Both groups then received daily CY (0, 13, 16, 18, 40 mg/kg) between weeks 1 and 10 post-HSCT and engraftment was monitored by peripheral blood FACS analysis weekly beginning at week 7. End-of-study bone marrow analysis was done using FACS and measurement of vector copy number (VCN) by qPCR. Bone marrow engraftment was calculated by comparing the VCN of samples from the test groups to the 0mg CY treatment group. **Results:** Two-and-a half-fold increase in CY resistance and up to 90% knockdown of CCR5 expression was observed by *in vitro* characterization studies. C44 expression data will be presented. At week 11, peripheral blood granulocytes overexpressing ALDH1 increased to an average of 94% (range 83%-98%) in mice treated with 16 mg/kg/d of CY. End-of-study bone marrow VCN analysis demonstrated 163% increase compared to control, which correlated with peripheral blood FACS analysis. (See Figure)



Conclusion: We demonstrate proof-of-concept of substantially increasing engraftment of gene-modified HPSCs overexpressing ALDH1 using post-HSCT CY chemoselection in a syngeneic mouse model. This approach, combined with genetic modifications to protect cells from HIV infection or insert therapeutic genes, could allow for successful autologous SCT as a potential cure for HIV or monogenic disorders.

432. Abstract Withdrawn

433. Abstract Withdrawn

Vector and Cell Engineering, Production or Manufacturing

434. Developing a Potency Assay for AAV-Based Genome Editing Vectors

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The GeneRide platform utilizes the natural process of homologous recombination to achieve targeted genome editing without the use of exogenous nucleases or promoters. Developing a potency assay for this unique technology poses additional challenges over those for canonical gene therapy products, including the requirement of a highly sensitive detection method to measure low levels of genome integration. The first GeneRide candidate, LB-001, is currently under clinical development for the treatment of methylmalonic acidemia. LB-001 targets site-specific integration into the albumin locus to allow the gene of interest (*MMUT*) to be expressed concomitantly with albumin. In order to control LB-001 product activity and assess lot-to-lot consistency, a cell-based assay was developed to measure fused mRNA expression as a surrogate of biological activity. The assay was developed in a cell line

that naturally expresses albumin and can thus drive expression of the *MMUT* gene upon site-specific integration. Fused mRNA is quantified using primers overlapping the host genome and the transgene DNA to ensure that only the integrated product is detected. The results show that AAV-driven homologous recombination is reproducible in vitro, which allows for the qualification of assay control material. The method was tested for linearity, repeatability, and specificity. Examination of the assay data demonstrates that this method is suitable for assessing the relative potency of integrating GeneRide vectors.

435. A Benchtop Analyzer for the Rapid Analysis of Amino Acids, Water Soluble Vitamins, Biogenic Amines and Dipeptides in Cell Therapy Media

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There is a need for advanced process analytic technologies that can monitor important media nutrients and cellular metabolites at the point-of-need alongside bioreactors and other culture vessels in process development and manufacturing site. Cell culture analysis is routinely conducted in core laboratories with liquid chromatography instruments by absorbance detection (e.g. HPLC-UV). Since many common components in cell media do not have fluorophores, a chemical derivatization step is required to add a tag to the molecules for detection. This additional sample preparation step slows the analysis cycle from when the sample is taken to when it is measured resulting in significant time delays for scientists. Shown here is an integrated analyzer that enables rapid turn-around-time with minimal sample volume to expedite process development decisions. The analyzer runs samples with just filtration and dilution which is much simpler than the lengthy and costly derivatization schemes and analysis times required of comparable approaches. The potential benefits of putting this analyzer at the point-of-need include reduced reliance on the "centralized laboratory" model, decreased capital and facility costs, more rapid (near real-time) analytics feedback, and improved product quality and/or titer. The low sample volume requirement for this analyzer (tens of microliters) allow for cell media optimization to occur earlier in the process development stage (at the microtiter plate and microbioreactor scale) where total bioreactor volumes are small, and the scientist desires to run assays every day during the process. Examples will be shown including screening different viral vector and T cell media formulations to ascertain changes in composition depending on the source and nutrient profile.

436. Construction and Evaluation of a Bacteriophage Lambda-Based DNA-VLP Vaccine against HIV

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Background: Bacteriophages "phages", bacterial viruses, can and have been engineered for various applications, including many applications in the field of gene therapy. The outer protein capsid of phage lambda can be alerted through a strategy called "Phage Display", this strategy involves the engineered expression of a peptide(s) of interest fused to the coat protein of a tolerant phage. Additionally, phage lambda has a packaging capacity of 48.5 kb, which can be modified to carry a dsDNA cargo of interest. Phage-based vaccine strategies benefit from a) the protection of their DNA cargo by the capsid and b) their ability to stimulate both cell-mediated and humoral immune responses. Phages also offer a safe alternative to their mammalian viral-based counterparts as they cannot infect mammalian cells. **Methods and Results**: Display peptides, including TAT, gp120, and the SV40 peptides were displayed on a specialized λ phage to target the phage to cells of interest, including dendritic cells, macrophage, and fibroblasts. Furthermore, genetic recombination strategies known as functional immunity assays were used to engineer the phage genome where a specialized HIV Gag-Env genetic construct was sub-cloned into a eukaryotic expression vector to ensure mammalian host expression then recombined into the phage genome. The Gag-Env construct forms harmless, yet highly immunogenic self-assembling virus-like particles (VLPs) resembling HIV. DNA encapsulated within the phage vector was assessed through DNase sensitivity assays and the extent of DNA degradation, and was analyzed by gel electrophoresis. Cell targeting and transfection efficiency were assessed using eGFP as an initial GOI using flow cytometry on treated cell lines. The immunological effects of engineered phage and parental plasmid treatments on target cell-lines will be explored by analyzing gene expression via transcriptomics and confirmed with RT-PCR. The expression of HIV-VLPs will be visualized through electron microscopy and confirmed by Western Blotting and confocal imaging.

437. Large Scale Suspension Production of AAV Capsid Variant Libraries from Stable Recombinant HEK293 Cell Banks

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Directed evolution is an established process for generating large diverse libraries of AAV capsid mutants and selecting for novel AAV variants with improved capsids, in terms of interactions with the tissues of interest and immuno-neutralization profiles. These capsid libraries are typically produced in adherent HEK293 cell systems, which, as a result of their two-dimensional format, are difficult to scale, and often do not yield sufficient material for systemic screening in vivo. Current adherent cell methods utilize limiting-dilution transfection of the capsid gene to reduce genome cross-packaging, which also reduces viral output. Iodixanol gradient purification steps further reduce this output, and these libraries are only single-use due to their transient transfection methods. To address the limitations of adherent single-use library systems, we created HEK293 library cell lines in which each cell carries a single capsid variant coding sequence stably integrated into its genome. We established our library platform in HEK293 suspension cells, enabling scalable production of AAV capsid libraries in bioreactors. Transfection conditions were adapted for optimal virus yield in suspension culture,

maintaining a constant concentration of PEI and DNA per mL of culture. Once created, these stable producer suspension cells can be used immediately to generate viral libraries with generic AAV replicase and adenovirus helper plasmids, or cryo-preserved for future use. AAV capsid variant diversity in these libraries was estimated to be in the E+05 range, determined by next generation sequencing. To rapidly expand from established 3L bioreactor parameters to 50L, we adjusted agitation set points to maintain a constant volumetric mass transfer coefficient (k_La) and scaled aeration rates by maintaining a constant vessel volume per minute. We report n=3 50L bioreactor production batches yielding an average of 1.4E+14 total vector genomes (vg) prior to purification. Productivity at this scale was consistent with those of small scale (3L) pilot batches, ranging from 2E+03 to 3E+03 vg/cell, and typically 10x higher than yields of adherent libraries, which yielded ~2E+02 vg/cell. To enable purification of one 50L vessel in a single working day, we modified our existing iodixanol gradient method to accommodate higher concentrations of cell lysate. Following purification, we obtained yields of 3E+12 total vg/50L vessel. This method enabled us to obtain research-grade AAV capsid libraries of suitable quantity, viral titer and purity for robust screening of novel AAV in multiple animals, i.e. systemically in ~30 mice or ocular screening by bilateral intravitreal dosing of ~15 nonhuman primates (NHP). We have demonstrated successful scale-up of our AAV capsid library suspension HEK293 platform to 50L bioreactors with 10X higher productivity than established adherent methods. Our approach overcomes the limitation of adherent HEK293 systems and enables systemic dosing in mice or intravitreal dosing in NHPs with a single 50L bioreactor production. Because systemic NHP screening of novel variants in high throughput libraries is more relevant for human use than screening in mice, we are currently developing a 200L process to achieve intravitreal dosing 1 NHP per 200L batch.

438. An Optimized and Simplified Process for Automated Manufacturing of Gene Engineered T Cells for Adoptive T Cell Therapy

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Adoptive immunotherapy using gene-modified T cells redirected against cancer has proven clinical efficacy and tremendous potential in several medical fields. However, such personalized medicine faces several challenges in the complexity associated with the current clinical manufacturing methods. Conventionally, the preparation of autologous gene-modified T cells is labour intensive and comprises many open handling steps including the critical final formulation of the drug product for the patient. Therefore an all-in-one automated solution starting from T cell enrichment to final formulation of the drug product with intermediate in-process control is of high demand for clinical cell manufacturing. We have developed a new T Cell Engineering (TCE) system including the automated sampling and filling process for gene-modified T cells to enhance the standard T Cell Transduction (TCT) process on the CliniMACS Prodigy. A Formulation Unit as accessory and a new single-use closed tubing set (TS521) with several attached product bags allow the filling of cell products for fresh use and/or for cryopreservation. A refined software including automated in-process sampling and final formulation of the drug product dependent on drug substance and patient-specific characteristics enable a simplification of the process with less user interactions. Here, we demonstrate that the new TCE application has a comparable performance as the standard TCT process. Furthermore, the automated final formulation step generates cell products with similar characteristics that matches patient-specific requirements. Taken together, the automated TCE application on the CliniMACS Prodigy is capable of manufacturing a gene-modified cell product from initial cell enrichment to a final formulated drug product for patient treatment.

439. Development of an Advanced Purification Platform Process for Adeno-Associated Viruses Using a Novel Impurity Removal Technology and a New Affinity Matrix

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Adeno-associated virus (AAV) vectors are promising tools for gene therapy, and some products have already been approved as commercial drugs. However, a standardized and efficient AAV manufacturing process does not exist like the platforms available for manufacturing monoclonal antibodies. The downstream process for AAV vectors normally consists of four parts: clarification of cell lysate, purification of the AAV vector, removal of empty particles, and concentration and buffer exchange. During a typical clarification process using depth filtration, large insoluble cell debris are removed, but soluble impurities, such as host cell proteins (HCP) and DNA including plasmid vectors, are not sufficiently reduced. The AAV concentration in crude clarified cell lysate is extremely low compared with the impurities and therefore multiple purification steps are required. Tangential-flow filtration (TFF), and ion-exchange and/or hydrophobic interaction chromatography are generally used to remove impurities. The process development for each product is time-consuming and the purification yield is sometimes not very satisfactory. Affinity chromatography is a powerful tool for AAV capture and impurity reduction, but an appropriate resin for each serotype should be selected and the purification yield is sometimes low because of the high impurity content in the loading material. When affinity chromatography is used for capture purification step, anion exchange chromatography is normally selected for polishing and empty particle removal step. Thus, we focused on improving the clarification and affinity purification steps to develop an advanced and scalable platform process for AAV vectors. A novel impurity removal technique was developed to improve the clarification step. The improvement was evaluated in terms of process simplification and productivity of the whole AAV downstream process as compared with the general clarification process using depth filtration and TFF. This new technology was based on unique characteristics of "Additive D" to adsorb process related impurities, such as HCPs and DNA. The new process using the technology was simple, efficient and scalable. The addition of "Additive D" to the endonuclease treated cell lysate of

AAV expressing HEK293 cells followed by filtering the mixture after agitation for 1 hour resulted in efficient removal of impurities to the same degree as the general process. The new process removed DNA and HCPs, and the amount of expensive endonuclease needed for nucleic acid degradation was also reduced to less than 1/10 that required to obtain the same residual DNA level from the general process. We also evaluated the affinity purification step by commercially available resin using the clarified solution from the new process and achieved a higher purification yield than the general process. Herein, we summarize our results of a new affinity matrix for AAV capture with better performance than current resins. This combination of a novel impurity removal technology and affinity matrix should be a powerful tool to construct a new AAV purification platform process.

440. POROS AAV9: Optimization of Resin Capacity and Recovery

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Gene therapy is a rapidly growing sector; Adeno-associated virus (AAV) is the predominant vector of which AAV9 remains one of the more commonly used serotypes used to deliver the desired gene of interest. The cost and efficiency of manufacturing AAV9 drug substance has significant opportunity for improvement. Historically, downstream processes utilized ultracentrifugation. Although ultracentrifugation remains an effective means of purification, incorporating this tool into a large-scale process has significant scale-up limitations. AAV Affinity resins have emerged as effective initial capture, have the advantages of greatly improving purity with one robust column step, and are more tolerant to a variety of load conditions compared to non-affinity options. However, the AAV binding specificity provided by the affinity resin comes at a much higher cost compared to non-affinity options. Thus, developing an AAV9 affinity purification method that maximizes product recovery and minimizes cost of goods is of great interest. AAV9-specific POROS CaptureSelect AAV9 resin was investigated in this study. Typical chromatography processes perform the entire run in downflow mode. While this can enhance separation via the theoretical plate model of chromatography, on-column separation is not needed for AAV affinity chromatography. Furthermore, after loading the sample on the column, open ligand sites increase towards the bottom of the column due to dynamic versus static binding. This study shows that the AAV9 recovery can be improved by eluting in up-flow mode, which avoids exposure of AAV9 to the open binding sites towards the bottom of the column. In addition, this study shows factors leading to higher binding capacities and lower cost of goods for large scale purification processes

441. Creation of a High-Yield AAV Vector Production Platform in Suspension HEK293T Cells Using a Design of Experiment Approach

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Despite advances in the rAAV gene therapy field, vector manufacturing remains a challenge. Transient transfection is the most commonly used method for rAAV production and significant research has been conducted to optimize transfection conditions and improve viral-vector yields. In this study, we used a Design of Experiment (DOE) methodology to optimize rAAV8 production in suspension HEK293T cells. We simultaneously varied the ratios of transgene, packaging, and helper plasmids, total DNA concentration, and cell density across 52 conditions to identify the optimal parameters of viral vector production. The DOE-optimization significantly increased the yield of rAAV8-EGFP production and revealed a unique set of parameters with a lower concentration of transgene plasmid, a higher concentration of packaging plasmid, and higher cell density comparative to the previous parameters determined by using the OFAT (one-factor-at-a-time)-based method. Using this DOE-optimized protocol we achieved an unpurified yield approaching 3×10^{14} viral genomes (VG) per liter of cell culture for rAAV8-EGFP. Moreover, we found the DOE-optimized method could be applied to major rAAV serotypes and capsid variants. Additionally, we incorporated PEG-based virus concentration, pH-mediated precipitation, and affinity chromatography to our downstream processing enabling average purified yields of $>1\times10^{14}$ VG/L for rAAV-EGFPs across 13 serotypes and capsid variants as verified using published rAAV Reference Standard Stock (RSS). Using the DOE-optimized protocol, we have generated 220 rAAVs of different serotypes and genes of interest with an average production yield of 8.92×10^{13} VG/L demonstrating the reproducibility of this protocol.

442. Magnetic Bead-Free Targeted Cell Selection from PBMCs Using Phase-Change Hydrogel Particles

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Bio-Techne, WOBURN, MA

Chimeric Antigen Receptor (CAR) T cell therapies have demonstrated encouraging outcomes for cancer treatments. As their approval rates with the regulatory bodies increase, the need to generate specific T cells via a scalable, robust process will grow significantly. The current positive cell selection processes use magnetic beads which introduce an extra down-stream processing step to remove the beads. Hence, we have developed a magnetic bead-free, positive cell selection platform that uses dissolvable hydrogel particles. This ease of operation for positive cell selection has the potential to generate expedited cellular outputs, with highly specific cell populations allowing for companies to achieve an optimal cellular ratio for their respective therapies. Cloudz dissolvable microspheres (30 μm) were manufactured from an ionotropic co-polymer hydrogel. Cloudz microspheres were subsequently modified to incorporate streptavidin (SA) onto their surface. Next, cells tagged with biotinylated antibodies for CD4 or CD8 were combined with the SA-conjugated Cloudz microspheres and the respective cells were selected through a 20 μm mesh filter. The targeted $CD4^+$ and $CD8^+$ T cells were harvested using a release buffer that dissolves the Cloudz particles. Selections were performed using $1x10^6$ (small scale) or $100x10^6$ (large scale) peripheral mononuclear cells (PBMCs) in order to investigate the scalability of the process. Next, we examined whether the selection process resulted in T cell activation by characterizing for CD25/CD69 co-expression. Finally,

the purified T cells were cultured for 9 days at different cellular ratios with the Cloudz T Cell Expansion Kit, featuring Cloudz CD3/CD28, to study the T cell activation potential post-selection. Flow cytometry was used to analyze the target cell uptake, recovery, purity, and viability. SA-conjugated Cloudz microspheres were able to efficiently capture target cells from a heterogeneous cell population. More specifically, at the small-scale level ($1x10^6$ cells) (*Figure 1*), we were able to reliably recover greater than 70% of the target cells in our elution/released fraction. Irrespective of the scale of the selection, we were able to consistently bind more than 80% of the biotinylated target cells to the SA-conjugated Cloudz microspheres. Moreover, bound cells that were eluted were above 93% positive for the specific target cell population, while maintaining a viability of 90% regardless of the scale of the selection. Lastly, a sequential selection for $CD4^+$ and $CD8^+$ T cells from PBMCS resulted in a purified final population which demonstrated low levels of CD25/CD69 co-expression (< 8%) at Day 0. This low level of co-expression shows that the selected cells are not being stimulated prior to being cultured at different ratios of CD4:CD8 with our Cloudz T Cell Expansion Kit. Overall, the SA-conjugated Cloudz microspheres demonstrate great potential as an efficient and scalable platform to positively select target cells with high purity from a heterogeneous starting cell population. The SA-conjugated Cloudz microspheres are a versatile cell selection unit that provide the possibility to eliminate the use of magnetic beads from not only cell selection processes utilized for CAR-T cellular therapies, but also across other platforms and different cell types. Additionally, this magnetic bead-free selection method will allow researchers to study the most optimal combinations of cells to improve efficacies of CAR-T therapies.

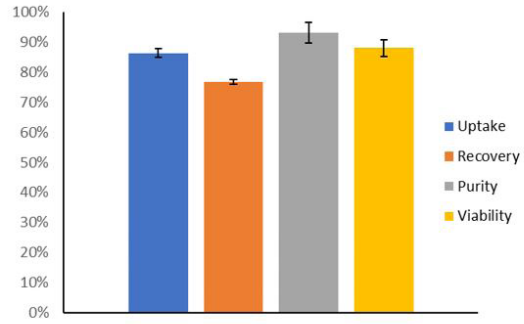

Figure 1: Small Scale Cell Selection from PBMCs using SA-conjugated Cloudz microspheres (n=3). Uptake = Percent of target cells bound to Cloudz microspheres. Recovery = percent of target cells recovered following release from Cloudz microspheres. Purity = % purity of selected target cell population. Viability = % of viable target cells following selection.

443. Perfusion Enables Increased Lentivirus Production Using the iCELLis® Bioreactor System

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Historically, there have been no manufacturers using Good Manufacturing Practices (GMP) to produce lentivirus in Canada, which has hampered gene therapy and CAR-T clinical programs in this country. The Ottawa Hospital recently developed a lentivirus manufacturing process using adherent cells in a flatware culture system, and used this process to produce sufficient amounts of clinical-grade lentivirus in a GMP manner for a Canadian CLIC-1901 CD19-CAR T Phase I/II clinical trial. While our previously established lentivirus manufacturing process used a flatware culture system, we plan to develop a larger-scale production process for clinical-grade lentivirus using the iCELLis bioreactor system. The iCELLis bioreactor system provides a large surface area for adherent cell growth, has a small physical footprint, and recently emerged as the leading scalable, single-use bioreactor technology for clinical manufacturing of viral vectors and vaccines. Our initial estimates suggest we could produce lentiviral vectors for approximately 2000 clinical doses for CAR-T cell therapy applications in a full-scale iCELLis 500+ bioreactor system; however, observed experimental titers in the first several iCELLis Nano batches were low. We hypothesized that this low titer was due to product instability in the bioreactor for long exposure times, and therefore implement a perfusion-based feeding system to remove product from the bioreactor for storage at more stable conditions. In this poster, we demonstrate that this perfusion strategy successfully resulted in bioreactor titers greater than the legacy flatware process for n=4 batches.

444. Determination of Viral Titer and Host Cell Proteins to Support Viral Vector Production in Gene Therapy Using an Automated, Miniaturized Immunoassay

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The rapid development of new gene therapy treatments has led to a great demand for assays with rapid turnaround times to support optimization of the production of viral vectors based on rAAV (recombinant adeno-associated virus) and the lentiviruses, HIV-1 (human immunodeficiency virus 1) and EIA (equine infectious anemia). Gyrolab® Systems have become established analytical immunoassay tools for performing bioanalysis in bioprocess development and manufacturing. The unique microfluidic, flow-through assay format dramatically reduces reagent and sample volumes and eliminates incubation times common in ELISAs. Features that increase the productivity in bioprocess workflows include automated analysis, broad analytical range, the generation of high quality results using small sample and reagent volumes, and 21 CFR part 11 compliant software. Here we demonstrate the development of three assays for the Gyrolab System: for determination of the AAV2 capsid titer using commercially available reagents, lentivirus vector titer analysis by quantitating the p24 viral capsid protein using Gyrolab p24 Kit, and an assay for the determination of host cell proteins (HCP) from the human embryonic kidney cell line (HEK 293) that is frequently used to manufacture viral vectors for gene therapy applications. Assay method development for AAV2 capsid titer was rapid and only required 6 runs over 1½ days. This Gyrolab assay had a broad analytical range: 1×10^7 to 1×10^{11} particles/mL, or 4 logs compared to 1.5 logs for ELISA. Gyrolab AAV2 assay was high throughput - 96 data points/h, or up to 960 data points/working

day. The performance of the AAV2 quantification assay on Gyrolab System was comparable with the commercial Progen AAV Titration ELISA (PROGEN Biotechnik GmbH). The immunoassay developed for Gyrolab system using the p24 kit delivered a broad, three-log working range that can minimize the number of dilutions needed to analyze bioprocess samples with lentiviral concentrations ranging from µg/mL down to ng/mL. The Gyrolab assay for HEK 293 HCPs generated precision data over a broad working range of 10 - 10000 ng/mL. These fully automated Gyrolab immunoassays generated high-quality data with a roughly one-hour assay time on-instrument, using 10 times less sample volume compared to ELISA. This performance, together with an efficient workflow makes the open Gyrolab platform a valuable tool in the development and production of gene therapeutics, for example in the measurement of viral titer and HCP impurities.

445. Molecular Design and Characterization of Packaging Plasmid Sequences for Improved Production of Novel Clade F AAVHSCs

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A scalable, transfection-mediated production process for novel Clade F AAVHSC vectors has been developed, demonstrated up to 500L production scale (3x500L), and is being utilized for both gene transfer and gene editing programs. The AAV plasmid production system involves sequences specific to the adenovirus helper genes, AAV replicase and capsid genes, and AAV ITRs flanking the promoter and therapeutic transgene. To further optimize these components, molecular characterization and engineering of the packaging plasmids has been evaluated to improve design, targeting higher vector productivity, improved genome packaging, and better product quality. Improvements to the AAV Rep sequence demonstrated up to a 50% improvement in productivity, an increase in the number of full capsids packaged, and a reduction in host cell DNA content. Purified AAVHSC genomes between 2.3-2.5 kb contained minimal double packaged vectors while non-Clade F capsids contained over 2-fold greater double packaged vectors as measured by analytical ultracentrifugation. We have demonstrated that AAVHSCs can efficiently package high quality vector with genomes from 2.3- 4.8 kb in length. Additionally, evaluation of multiple plasmid backbones was undertaken for three design alternatives to standard antibiotic selection. While each backbone showed some similar characteristics with the current transfection platform, one alternative design showed improved productivity while reducing the total amount of DNA required for transfection and demonstrating the utility of improved plasmid design for a reduction in cost. AAVHSC productivity and product quality was improved by optimizing the genetic sequence of the recombinant genome and packaging components to allow for high quality production of a wide range of transgene sizes.

446. Viral Clearance: An AAV Case Study Utilizing MVM, XMuLV and a Non-Infectious MVM Surrogate during Downstream Development

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Viruses can arise during the manufacture of biopharmaceuticals through contamination of exogenous viruses or endogenous expression of viral sequences. Regulatory agencies therefore require "viral clearance" validation studies for each biopharmaceutical prior to approval. These studies demonstrate the manufacturing process' ability at removing or inactivating virus and are conducted by challenging scaled-down manufacturing steps with a "spike" of live virus. For gene therapy, the choice of a "relevant" spiking virus has been the subject of much discussion. In this work, we sought to demonstrate if a popular AAV-affinity chromatography resin, AAVX (ThermoFisher), could distinguish between AAV and Minute Virus of Mice (MVM), a common parvovirus used for mAb viral clearance validation studies, thereby demonstrating effective clearance of a "worst case" contaminant. In addition, we sought to analyze the clearance of Xenotropic Murine Leukemia Virus (XMuLV), a common model retrovirus used for mAb spiking studies. Due to the cost of viral clearance spiking studies, we also employed the use of a novel and economical feasible non-infectious MVM surrogate. This MVM - Mock Virus Particle (MVM-MVP) has been demonstrated for use in mAb process applications and we report here its first use in an AAV process. Data from this study shows that not only did the AAVX resin effectively remove both viral challenges, but did so during worst case parameter testing. In addition, MVM-MVM vs MVP comparability data demonstrated the potential for using this non-infectious surrogate for downstream process development and characterization activities.

447. Novel Approach for Quantitative Real-Time Particule Analysis of Lentiviral Vectors

Victoire Barailles
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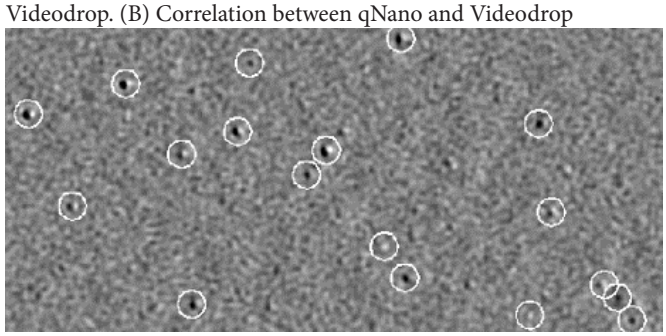
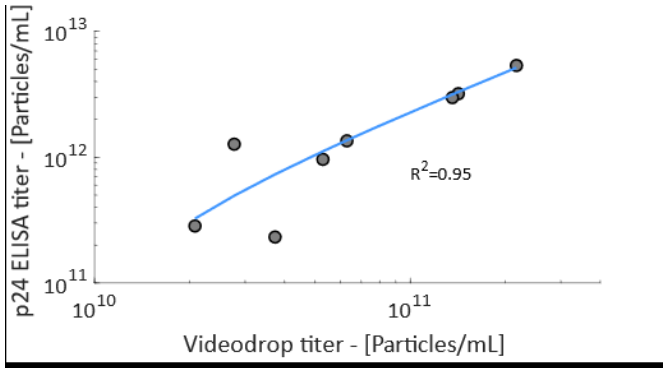
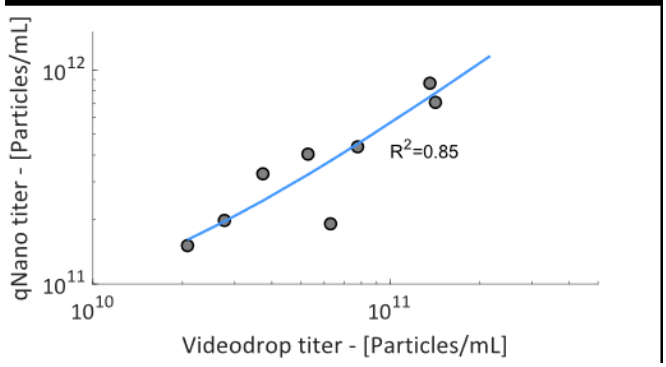
Lentiviral vectors are efficient vehicles for stable gene transfer in dividing and non-dividing cells. They tend to be increasingly used as a powerful tool to introduce genes into cells ex vivo, for instance in CAR-T cell therapies. During manufacturing and production of lentiviral vectors, relevant quality control is necessary to allow batch release. Among standard quality control methods that can be used, quantification of lentiviral vector particles - or physical titer - is one of the most important. Up to now, this characterization can be achieved either indirectly with p24 protein quantification or with physical methods like Tunable Resistive Pulse Sensing (TRPS) for example, both methods implying prior preparation of samples (lysis, dilution or filtration). These two methods thus show important limitations as they cannot accurately reflect the true nature of the product, in addition to being relatively time-consuming. Myriade, a French company created in 2017, is developing Videodrop, a new optical technique performing real-time, user-friendly, and label-free measurement of lentiviral vector physical titer. This method, based on full-field interferometry, was tested on various lentiviral vector samples: in a context of Drug Product (DP) release as well as in-process

controls. We compared three lentiviral physical titration methods on aratinga.bio productions: p24 ELISA, qNano and Videodrop - Myriade instrument. The correlation between Videodrop analysis and the other two methods appeared to be robust, with high R^2 values. These results suggest that Myriade technology is relevant for DP release as well as in-process controls, offering the ability to be a tool for continuous improvement. It is an easy-to-use and fast alternative to the standard more complex and time-consuming physical titration methods. "1graphic_" Figure 1 - Lentiviral vector sample image obtained with Myriade technology.

"2graphic_" - Figure 2. Comparison of methods measuring physical titer on Drug Products. (A) Correlation between p24 ELISA and Videodrop. (B) Correlation between qNano and Videodrop







448. Abstract Withdrawn

449. Chemically-Defined, High-Density Insect Cell-Based Expression System for Scalable AAV Vector Production

James Kurasawa[1], Andrew Park[1], Carrie Sowers[1], Rebecca Halpin[1], Andrey Tovchigrechko[1], Claire Dobson[2], Albert Schmelzer[1], Changshou Gao[1], Susan Wilson[1], Yasuhiro Ikeda[1]

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Recombinant adeno-associated virus (AAV) vector is one of the most utilized viral vectors in gene therapy due to its robust, long-term in vivo transgene expression and low toxicity. One major hurdle for clinical AAV applications is large-scale manufacturing. In this regard, the baculovirus-based AAV production system is highly attractive due to its scalability and predictable biosafety. Here, we describe a simple method to improve the baculovirus-based AAV production using the ExpiSf Baculovirus Expression System with a chemically-defined medium for suspension culture of high-density ExpiSf9 cells. Baculovirus-infected ExpiSf9 cells produced up to 5×10^{11} genome copies of highly purified AAV vectors per 1 mL of suspension culture, which is up to 19-fold higher yield than the titers we obtained from the conventional Sf9 cell-based system. When mice were administered the same dose of AAV vectors, we saw comparable transduction efficiency and biodistributions between the vectors made in ExpiSf9 and Sf9 cells. Thus, the ExpiSf Baculovirus Expression System would support facile and scalable AAV manufacturing amenable for preclinical and clinical applications.

450. A Novel Flow Cytometry-Based Method to Characterize Transfection Efficiency in Genetically Engineered Mesenchymal Stem Cells

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Regenerative Medicine, The Ottawa Hospital Research Institute, Ottawa, ON, Canada

The immunomodulatory and regenerative activities of mesenchymal stem cells (MSCs), the most commonly used therapeutic cell product, can be enhanced via nonviral transfection to overexpress therapeutic proteins. Traditionally, transfection efficiency is assessed by using a plasmid encoding a reporter gene, which may not accurately reflect expression levels of the therapeutic protein. We have developed a method that allows for simultaneous quantification of both plasmid copy number and target protein expression, without the need for a reporter gene. We demonstrated the utility of this technique by comparing the transfection efficiencies of two candidate nonviral DNA vectors, containing minimal (or no) bacterial plasmid sequences, but expressing identical target proteins (vector A vs. vector B). The vectors were chemically labeled with fluorescent probes and then used to transfect MSCs. Twenty-four hours post-transfection, MSCs were probed with target protein-specific antibody, and levels of target protein expression and labeled vector content were assessed by standard (Fortessa) and imaging (Amnis) flow cytometry. With vector A, 33.0% of cells contained labeled vector, yet only 1.7% of these vector-positive cells expressed target protein. The transfection efficiency with vector B

was superior, as 52.8% of MSCs were transfected, and 11.9% expressed target protein. Thus, using this approach we have shown that less than 25% of vector-positive MSCs produce detectable target protein, possibly due to improper subcellular localization of the vector. Our method allows simultaneous quantification of the total transfected-MSC population and the protein-expressing subpopulation, which has important implications for the potential therapeutic efficacy of a genetically enhanced cell product.

451. Rapid CMC Development and Pre-Commercial Considerations for rAAV Gene Therapy Products for Rare Diseases

James Warren

Pharmaceutical Development, Ultragenyx Gene Therapy, Woburn, MA

Despite significant progress over the past several years in the process and analytical development of rAAV vectors for numerous clinical gene therapy applications, fundamental challenges of low productivity, poor downstream yield, immature product quality characterization, and limited external manufacturing capacity continue to pose challenges for the biopharmaceutical industry in the CMC development of these products. Ultragenyx Gene Therapy has established a fast-to-clinic CMC development strategy leveraging two distinct production platforms, implementation of high-throughput centers of excellence, and a state-of-the-art pilot plant to streamline and standardize the development and technology transfer of pre-clinical and clinical candidates to external manufacturing partners. Process Development has resulted in greater than 5-fold volumetric productivity increase and significant improvement in yield across chromatography and filtration steps. Scalability to 250L has been demonstrated within 2-3 months, which significantly decreases time and risk toward GMP manufacturing at up to 2000L scale. Significant development in analytical characterization methods have enabled product quality assessment to maintain comparability and enhance product understanding as we transition toward pre-commercial development. Finally, a risk-based strategy has been implemented for characterization and late-stage development and qualification of the processes and analytics.

452. Improved Proliferation and Transduction Efficiency of CAR-T Cells Using a Novel Xeno-Free Medium

Asma Ayari[1], Francisco Martinez-Becerra[1], Yitao Tu[2], Yinan Lu[2], Roderick O'Connor[2]

[1]R&D, Nucleus Biologics, San Diego, CA,[2]Dept. of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Chimeric antigen receptors (CARs) are a promising approach to redirect T cell specificity against cancer. In this form of adoptive immunotherapy, patient-derived T cells are activated, transduced with a CAR transgene, and expanded *ex vivo* for several days prior to reinfusion. Effective CAR-T cell therapies are dependent on cell culture methods conducive to each phase of this ordered process. Remarkably, commercially available media formulations have not been optimized for lentiviral transduction or T cell expansion for adoptive immunotherapy and are supplemented with serum from animal or human origin. There are inherent limitations in their inclusion as declining availability and lot-lot inconsistencies will impede future progress in this arena. Thus, there is a veritable need to replace components of animal origin used in the culture or preservation of cells replaced with relevant protein sources and concentrations. We recognize that the use of components of human blood cell fractions that are absent in serum might be a xeno-free alternative with valuable therapeutic potential. In the present study, we first investigate the capability of Physiologix™ XF (Phx) Human Growth Factor Concentrate (hGFC) (*a cGMP, xeno-free media supplement able to replace standard serum supplements such as FBS and human serum*) in improving T cell growth and transduction. Our *in vitro* data show that Phx supports T cell proliferation and significantly enhances lentiviral-mediated gene expression 2-3-fold across a wide range of multiplicity of infections (MOIs). We also compared the ability of anti-GD-2 CAR-T cells expanded *ex vivo* in medium conditioned with either Phx or HS to clear tumor burden in a human xenograft model of neuroblastoma. We show that CAR-T cells expanded in Phx have superior engraftment and potency *in vivo* (Mouse model). Secondly, using a combination of metabolomics and process optimization we identified a new formulation named NB ROC. NB ROC is designed to be used along with Phx. We tested NB ROC on T cells isolated from the blood of three donors using standard protocols. We show that NB ROC improved T cell proliferation compared to other current proprietary media formulations. This increase in proliferation did not compromise early memory phenotypes; providing an attractive solution for allogeneic products. GFP Lentivirus transduction was highly enhanced when cells were grown in NB ROC. Optimization of virus usage in this manner will reduce CAR-T cell manufacturing costs considerably. In summary, we developed a customized medium formulation (NB ROC) for CAR-T cell therapies. Conditioning NB ROC with our serum replacement Physiologix enhanced T cell expansion and CAR-T cell memory cell differentiation. We are currently focused on developing collaborations to adapt NB ROC to the needs of the CAR-T cell manufacturing space.

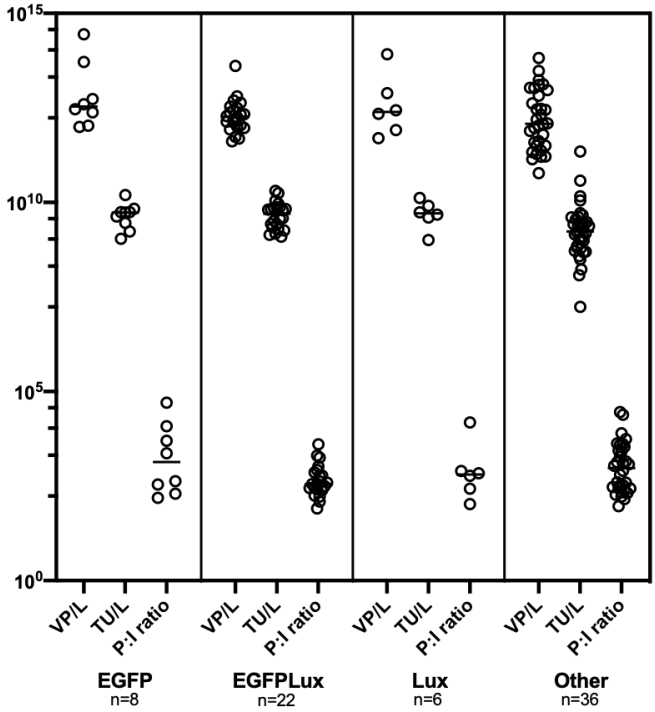
453. Large-Scale, High-Throughput Production of rSIV.F/HN Vectors

Mariana F. A. Viegas, Toby Gamlen, Catriona C. Conway, Rebecca J. Dean, Kamran M. Miah, Stephen C. Hyde, Deborah R. Gill

Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

Our laboratory is developing non-viral and viral vectors for the treatment of a range of airway disorders. We have previously demonstrated that a recombinant simian immunodeficiency virus (rSIV) vector, pseudotyped with the fusion (F) and hemagglutinin-neuraminidase (HN) envelope proteins from Sendai virus (together termed rSIV.F/HN), has high tropism for multiple cell types found within both the conducting airway epithelium and the gas exchanging alveolar regions of the mouse, sheep and human lung. To facilitate clinical development, we are routinely producing a large number (multiple lots/week; target >1e10 TU) of highly purified (anion-exchange/TFF), high-concentration (target >1e9 TU/mL) preparations of rSIV.F/HN. Historically, we have used an upstream production workflow centred on the use of suspension-adapted HEK293T cells cultured in rocking (WAVE, GE) bioreactors. The approach is robust

and generates consistently high yields (median 2.7e9 TU/mL, n=68 preparations), with similar yields observed for a range of vector configurations:



In a bid to simplify our upstream production workflow and increase throughput, we compared our standard process with the Thermo Fisher LV-MAX™ Lentiviral Production System using a standard rSIV.F/HN-EGFP vector. We used the LV-MAX™ system essentially as described by the supplier, with a workflow centred on the use of suspension-adapted Gibco™ Viral Production Cells cultured in shake-flasks. When using the LV-MAX™ system at the 1.5L scale, EGFP vector yield was similar to that observed at 5L using our in-house WAVE approach (p=0.51). Although the LV-MAX™ system is considerably more expensive than our in-house approach, the use of the LV-MAX™ system reduced the overall lot process time from 7 to 4 days and the use of simpler USP equipment and reduced DSP volume handling demands facilitated substantially greater throughput within the same facility. However, further consideration may be needed when using the LV-MAX™ system for production of vectors carrying disease-specific transgenes; as, in our hands, the volumetric yield advantages shown for simple EGFP vectors have not been consistently observed for other transgenes.

454. Development of a Stable Helper Virus-Free Producer Cell Line for Scalable Adeno-Associated Virus Vectors Production Based on Human Suspension Cells

Ines do Carmo Goncalves, Kerstin Hein, Ben Hudjetz, Nikola Strempel, Nina Riebesehl, Helmut Kewes, Thu Bauer, Israa Jamaladin, Corinna Bialek, Sabine Schmidt-Hertel, Juliana Coronel, Aishwarya Patil, Ahmad Al-Dali, Nicole Faust, Silke Wissing
Viral Vectors, CEVEC Pharmaceuticals, Cologne, Germany

Over the past decade, gene therapy applications have revolutionized the landscape of clinical medicine for inherited and acquired human diseases. Encouraging safety profile, target efficiency and long-lasting transgene expression established recombinant adeno-associated virus (rAAV) vectors as an ideal vehicle for transgene delivery. Nonetheless, this growing interest in rAAV-based gene therapy underscored a critical need for scalable manufacturing processes capable of increasing both yields and quality of rAAV particles.To bridge the production gap in gene therapy, we combined our expertise in cell line development technologies with an in-depth understanding of the rAAV basic biology to generate our innovative stable helper-virus free rAAV production platform. Development of this platform involved genetic engineering of our proprietary animal component-free growing suspension cells to stably express all the components essential for rAAV production as well as the transgene flanked by the ITRs. As a first step, we generated pre-packaging pools harboring Tet-inducible expression of the Rep genes as well as the adenoviral helper functions E2A, E4orf6 and VA RNA. Following single cell-cloning, we were able to identify multiple top rAAV pre-packaging clonal cell lines that produced high-titer rAAV vectors upon transient transfection of the capsid genes and the transgene flanked by ITRs. During cell stability evaluation, these cells sustained stable expression of Rep and Helper functions and comparable rAAV productivity both in the absence or presence of selective pressure over time. Importantly, these tests validated the nonexistence of leaky expression in the Tet-controlled induction of the toxic Rep and Helper functions. Lastly, the capsid genes and the transgene flanked by the ITRs were stably integrated into the best-performing rAAV pre-packaging clonal cell lines, generating an inducible rAAV stable helper virus-free producer cell line. Upon induction, the producer cells yielded high-titer and high-quality rAAV product, demonstrating the feasibility of our stable helper-virus free rAAV production platform. Upstream process development and scale-up to 200 L is currently in progress.In conclusion, our stable rAAV production platform tackles the challenges posed by the high demand for rAAV production at industry scale by offering a system that grants reproducible, scalable, cost-efficient and GMP-compliant manufacturing of rAAV product of high quantity and quality.

455. Epigenetic qPCR Assay for Immune Cell Identity and Purity Testing of Cellular Therapies

Jerry Guzman, Uma Lakshipathy, Mark Landon
Thermo Fisher Scientific, Carlsbad, CA

Thorough testing is required for product release during the production of cell therapies in which contaminating byproducts may be present. Characterization of these biological products include determination of biological activity, identity, purity and impurities. Identity and purity assays most commonly utilize cell surface markers or secreted molecules which are measured by flow cytometry. Some of the critical challenges with flow cytometry is the lack of standardization due to user and technical variability, and the need for live samples. PureQuant are cell specific epigenetic qPCR-based assays and offer an appealing alternative to overcome the challenges with flow cytometry. CAR-T cells generated using a second generation (CD3ζ and 4-1BB) anti-CD19 CAR lentivirus were evaluated using PureQuant methylation assays specific for CD3+, CD4+, CD8+ T cells, and contaminating B cells and

monocytes. Enriched B cells and monocytes were spiked in CAR-T cells of the same donor at known cell numbers and interrogated using flow cytometry and PureQuant assays to establish sensitivity. Each assay includes internal controls to check for assay performance, assay-specific differences, and standards to deduce copy number. The study results between flow cytometry and PureQuant corroborate; however, PureQuant offers a direct result reported as percent cell type without the need for gating strategies and subjectivity.

456. Enhancement of Helper-Dependent Adenoviral Vector Production

Ranmal Avinash Bandara, Rongqi Duan, Jun Li, Jim Hu
Translational Medicine, The Hospital for Sick Children, Toronto, ON, Canada

Cystic Fibrosis (CF) is a genetic disease seen mainly in Caucasian populations. CF is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Although CF is a multiple organ disease, lung failure is the major cause of the current mortality and morbidity. CF patients show defects in fluid transport in the lungs resulting in mucus stagnation, which leads to chronic infection followed by chronic inflammation. Due to medical advances, the median life span of individuals with Cystic Fibrosis (CF) increased to over 40 years in the US. However, better therapies are needed to extend the life of patients. Delivery of the CFTR gene to cultured cells and in vivo has shown promise in restoring CFTR function. Since lung surface epithelial cells are renewed, albeit slowly, site-specific integration of the therapeutic gene into airway stem cells is required to achieve long term therapeutic efficacy. Using our helper dependent adenoviral vector (HD-Ad), we deliver the CRISPR/cas9 system with donor DNA to achieve site-specific gene integration. CRISPR/cas9 induces a double stranded break (DSB), which allows the integration of the CFTR gene containing homology arms complimentary to either side of the DSB. The presence of CRISPR/cas9 decreases the production of HD-Ad in our packaging cell line. Although we can produce enough for small animal studies, the yield is too low for large animal studies and clinical trials. Therefore the production of HD-Ad must be enhanced. We have now identified reagents that can enhance HD-Ad vector production.

457. A Dissolvable Growth Capsule System for Expansion and Genetic Engineering of Cellular Therapeutics

Lauren Timmins, Matthew Teryek, Ayesha Aijaz, Biju Parekkadan
Biomedical Engineering, Rutgers University, Piscataway, NJ

Within the rapidly growing field of engineered cell-based therapies, a major obstacle for continued success is the lack of a scalable, cost-effective manufacturing platform. Here, we present a cell encapsulation technology using a polyethylene glycol (PEG)-based matrix material to expand and genetically-modified encapsulated cells in situ. The PEG capsule was designed to provide mechanical protection from shear injury in high-speed bioreactor culture. A dissolvable PEG capsule matrix was further developed to enable the recovery of the cells after culture by simple addition of an enzyme. The interior of the capsule was decorated with cell-specific conditions to expand human cell therapeutics. Mesenchymal stromal cells (MSCs) were initially studied and found to expand in an adhesive capsule environment where they attained their characteristic spindle-shaped morphology compared to non-adhesive capsules. Furthermore, adherent cells could be co-encapsulated with lentiviral particles and be genetically engineered with promising efficiency. All told, this 3D capsule system addresses the major gaps in expanding human cell therapeutics in conventional stirred bioreactors by: (1) protecting cells from mechanical agitation and shear stresses during stirred tank suspension culture, (2) quick and easy isolation of cells by simple degradation of the capsule, (3) ability to transduce cells through coculture with genetic constructs.

458. Development and Delivery of a Hands-On Short Course in AAV Manufacturing to Support Growing Workforce Needs in Gene Therapy

Caroline Michael Smith-Moore, Laurie Overton, Shriarjun Shastry, Cristiana Boi, Gary Gilleskie
BTEC, North Carolina State University, Raleigh, NC

The emergence of commercial adeno-associated virus (AAV) gene therapy products has led to a rapid increase in demand for a skilled workforce. As commercial AAV manufacturing is still new, the pool of personnel trained in manufacturing, quality control, process development, process engineering, and numerous other roles is insufficient to meet demand. Additionally, many of the processes currently utilized to generate AAV at the bench are not scalable for commercial production or cGMP compatible, further widening the training gap. In order to fill this gap, a four-day, short course has been developed to provide hands-on training in scalable technologies and analytical methods utilized for AAV manufacturing. Students are trained using an AAV production process that employs suspension cell lines (Sf9 and HEK293), single-use bioreactors, detergent lysis, scalable filtration, and affinity chromatography, as well as analytical assays for quantification and characterization. This course serves as a first of its kind example of a curricula specific for AAV manufacturing. The course has been delivered to post doctoral fellows as well as industry professionals. Details of the course design and development, feedback from students to date, plans to disseminate the course content to other institutions, and plans for developing additional courses in the area of gene therapy will be discussed.

459. Development of a Scalable Downstream Process for Lentiviral Vector Production

Eran Or
Process Development, MassBiologics, Boston, MA

Lentiviral vectors are a powerful tool for cell and gene therapy. Lentiviral vectors have the ability to integrate genetic material into both dividing and non-dividing cells, allowing them to transduce a variety of cell types. They are increasingly being used for ex-vivo gene transfer. Increasing demand for viral vectors, combined with a dependency on lab scale manufacturing processes for these vectors has led to production capacity becoming a bottleneck in delivering new cell and gene therapies to patients. In order to tackle this bottleneck new methods to produce lentiviral vectors must be developed. Therefore, purification steps that work well in a small scale (e.g. ultracentrifugation) need to be replaced by more efficient procedures that are amenable to scale-up. Here we present a novel downstream

processing approach, which purifies lentiviral vectors using two successive chromatography steps working in flow-through mode. These steps can be combined with single-pass TFF to concentrate the product and achieve the desired formulation. The set-up can be operated continuously, it minimizes exposure of the lentiviral vectors to destabilizing high salt concentrations and can be scaled-up.

460. Transient Transfection Parameters for Lentiviral Vector Production in 293Ts and VPC Cells

Alicia Diane Powers, Timothy D. Lockey, Michael M. Meagher

Therapeutics Production and Quality, St. Jude Children's Research Hospital, Memphis, TN

According to Gene Therapy Trials Worldwide, the use of lentiviral vectors in gene therapy clinical trials increased from 55 reported trials by 2012 to 196 reported trials by 2017. These lentiviral vectors are frequently generated by transient transfection, and, as vector demand grows, more efficient methods of transfection are needed. The replacement of calcium phosphate with PEI has simplified the transfection process, but newer transfection reagents optimized for specific vector types provide additional opportunities. Serum-free and animal origin-free medias allow for a more defined final product and easier downstream processing while generating additional opportunities for optimization. Lentiviral vectors have typically been produced by transient transfection of HEK293(T) cell lines, but HEK293 cell lines, such as the VPC cell line, are also available for transient transfection. Here we investigate transient transfection parameters in 293TS and VPC cells to produce a lentiviral vector for use in a clinical trial treating solid tumors. We examine the effect of cell seeding density, DNA concentration, transfection reagent, transfection reagent to DNA ratio, growth media, culture time post transfection, and the use of a supplement and enhancer in each of these two cell types. By transduction of HOS cells and ddPCR, we show that modification of these parameters increases our lentiviral yield from $5.7x10^7$ tu/mL to $2.2x10^8$ tu/mL.

461. Optimization of AAV Vector Production Using Statistical Design of Experiment

Xingcheng Chen[1], Nida S. Zubiary[1], Timothy Ryan[2], Dogan Ornek[1], Tao Xiang[1], Chi-Ming Yu[1], W. Roy Lin[1], Chih-Cheng Chen[1]

[1]Boston Institute of Biotechnology, Southborough, MA,[2]Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA

Applications of adeno-associated virus (AAV) in gene therapy has increased significantly in the last two decades, due to its remarkable safety profiles and efficiency of gene delivery into various tissues. Demand on the supply of recombinant AAV vectors has also increased in order to fulfill pre-clinical and clinical requirements. Furthermore, helper-virus-free mediated transient transfection has been widely adopted. Low titer and process scalability remain two key issues in AAV manufacturing. The productivity of AAV vectors is often affected by the AAV serotype, cell culture type, the size of Gene of Interest (GOI), cell density, transfection method, plasmid DNA concentration and other factors. Based on the risk assessment of these factors that may impact AAV production with helper-virus-free mediated transient transfection, we have evaluated 6 process parameters using JMP's Definitive Screening Design. In this study, critical process parameters (CPP) were identified and their operating ranges were established for optimum and robust AAV production. Findings will be discussed in this presentation.

462. Optimisation of Scalable Chromatographic Solutions to AAV Purification Using a Design-of-Experiments Approach

Sebastian J. Aston-Deaville, Kanchan Thapa, Janice Charlesworth, Adrian Sylvester, George Prout, Kevin Bowes, Steven Williams, Vera Lukashchuk, Tony Hitchcock, Darrell Sleep, Daniel Smith

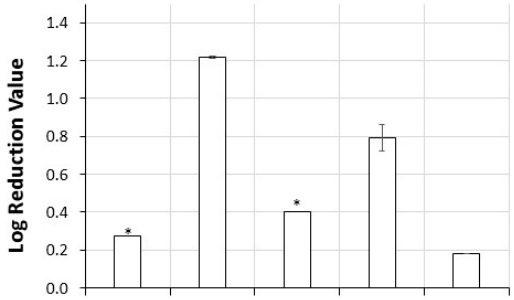
Innovation, Cobra Biologics, Newcastle-Under-Lyme, United Kingdom

Adeno-associated virus (AAV), as a viral vector for gene therapy, offers many benefits over the alternatives; in particular, its intrinsic low propensity for inducing a host immune response, as well as the existence of numerous serotypes that preferentially target particular tissue types. Downstream purification of AAV, as with all viral vectors, presents specific issues that are not seen with other biologics. The high binding capacity of available chromatography platforms, combined with the comparatively low production yields of viral vector products (milligrams of viral vector per litre of culture versus grams of monoclonal antibody per litre of culture), can make the design of a truly scalable chromatography purification process especially challenging.Mass flow chromatography platforms, such as monoliths and nanofibers offer a high-throughput production platform for large biologics such as AAV and are capable of processing at residence times lower than 0.5 minutes. However, the different serotypes of AAV vary in their specific physical characteristics, which means that the ideal chromatography parameters for one serotype are unlikely to be the same for the next.The "Design of Experiments" (DOE) approach is a way of efficiently exploring the available experimental design space of a process. Multiple factors are identified and simultaneously varied according to a defined layout, after which the results are analysed and a multivariate regression-based model is generated. This can then be used to interpolate (and to a limited degree extrapolate) the optimal conditions required to achieve a defined output.Herein, we describe the use of DOE to investigate and identify a set of cation-exchange chromatography parameters that offer maximal recovery of a recombinant AAV-9 model vector and elimination of residual host cell proteins. By varying buffer pH and [NaCl] in the Load, Wash and Elution phases, as well as the flow rate and loading level, it was possible to achieve a significant increase in step yield of the total vector particle content in the eluate fraction, as well as a concurrent decrease in HCP concentration. Furthermore, we propose that this same approach could be used to quickly adapt the chromatography parameters described here for model AAV-9 to alternative or novel AAV serotypes.

463. Nuclease Selection Affects Digestion of DNA in HEK293 Cells

Ashlee Maclean
Process Development, Mass Biologics, Mattapan, MA

Lysis and clarification is a common first step in the purification of adeno-associated virus (AAV). AAV based vectors are an increasingly relevant commercial biologic as it is a small non-enveloped virus capsid that packages linear single stranded DNA up to 4.7 kbp. To purify AAV, it must first be released from the cells via lysis. Lysis releases impurities such as host cell DNA and residual plasmid DNA, which can cause immunogenic responses in the patient, compromise product integrity including aggregation, and place a greater challenge on downstream purification processes. Nucleases can be added to lysate to reduce residual DNA, solution viscosity and increase product yield. Here, we evaluated residual DNA levels and recovery after digestion with two nucleases, Benzonase and Salt Active Nuclease High Quality (SAN-HQ). Human embryonic kidney 293 (HEK293) cells expressing AAV were digested with either Benzonase or SAN-HQ at various pH and salt concentrations. SAN-HQ digestion at pH 9 and 500 mM NaCl resulted in the greatest removal of residual DNA (1.2-fold log reduction). Therefore, use of SAN-HQ during the lysis step will ease the challenge placed on downstream processing resulting in greater residual DNA removal and improved product quality.



	1	2	3	4	5
Enzyme	SAN HQ	SAN HQ	SAN HQ	SAN HQ	Benzonase
NaCl (mM)	500	500	1000	1000	350
pH	7	9	7	9	8

LRV was calculated using the residual DNA concentration reported by Picogreen
Standard deviation derived from duplicate samples
*Experiments were performed in singlet due to material limitations

464. Establishment of a Scalable Production Process Using Stable Helper-Virus Free AAV Producer Cell Lines Based on Human Suspension Cells

Kerstin Hein, Inês do Carmo Gonçalves, Nikola Strempel, Jens Woelfel, Christian Niehus, Ben Hudjetz, Nina Riebesehl, Helmut Kewes, Thu Bauer, Israa Jamaladin, Aishwarya Patil, Ahmad Al-Dali, Corinna Bialek, Juliana Coronel, Silke Wissing, Nicole Faust
CEVEC Pharmaceuticals GmbH, Koeln, Germany

Within the last years, the gene therapy field made a huge leap forward seeing a number of products gaining regulatory approval and entering the market. Recombinant adeno-associated virus (rAAV)-based treatments had their share in it - making use of a vector with favorable safety profile, delivering long-term gene expression and offering a panel of serotypes with different tissue tropisms. Still, the application of rAAV-based gene therapy as viable and affordable treatment option, especially for more common indications requiring systemic administration, will critically depend upon substantial improvements in vector manufacturing in regard to scalability, consistent product quality and costs. Therefore, we developed a novel AAV production platform based on stable AAV producer cells, which overcomes these issues by enabling the manufacturing of viral vectors in suspension culture solely upon induction. For the generation of these stable rAAV producer cell lines, suspension CAP cells were genetically modified to stably express Rep proteins, as well as the adenoviral helper functions E2A, E4orf6 and VA RNA. Resultant pre-packaging AAV single cell clones were further genetically modified by stable integration of the capsid function and transgene flanked by the ITRs resulting in our inducible rAAV producer cell line system. These stable, animal-component free growing producer cell lines offer now completely new possibilities of process development in the field of viral vector production by abolishing the need of any transfection or infection step. Using the ambr15 system as scale-down model, initial experiments were testing the influence of different physical parameters and supplements in order to develop a robust and scalable process for high titer and quality rAAV production. This process was then successfully transferred into disposable stir tank bioreactors, while the scale-up to 200 L is currently ongoing. In conclusion, our stable helper virus-free AAV production platform provides a solution to the demand of high titer and quality vector production at industrial scale in a reproducible, scalable and cost-efficient manner.

465. Full or Empty: A CMC Perspective on Production and Purification of Empty Vector Particles

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Our clinical pipeline covers a broad range of genetic diseases targeting central nervous system, muscle, respiratory, and heart tissues with Adeno-Associated Virus (AAV)-mediated gene therapy. A major hurdle with this approach is the high prevalence of AAV-neutralizing antibodies in the general population often resulting in patients being excluded from the trial. Although there have been numerous clinical trials where AAV vectors have been successfully applied, there is a finite understanding of the feasibility to re-administer AAV and whether it will be necessary. One such focus is to understand the effect of "empty" AAV vector particles on the immune response in both pre-clinical and clinical settings. Empty vectors, or capsids that lack a full-length therapeutic vector genome, are an artifact in the production of recombinant AAV. Viewed as an impurity, residual empty particles are a concern as they may elicit unwanted host immune responses due to increased exposure to AAV antigen beyond the therapeutic dose; however, this remains a controversial topic. Here, we describe the steps taken to optimize serotype independent downstream process, with a focus on removal of in-process impurities as well as enrichment of

empty capsids via ultracentrifugation and chromatographic separation. Our data show high vector purity and significant enrichment of empty capsids, 75% (Bioreactor) to 90% (DS), and reduction of partial/full capsids from 22% (Bioreactor) to 6% (DS), measured by Analytical Ultracentrifugation. Our goal is to impact the future of how gene therapeutics can improve patient outcomes and enable them to benefit from potential gene therapy improvements in future.

466. Validation, Implementation, and Comparability of Rapid Sterility for GMP Testing

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Rapid microbiological methods (RMMs) are commercially available on multiple different types of platforms, many of which have a broad range of testing capabilities. Regarding sterility testing, RMMs are an increasingly common alternative to the traditional 14 day manual test as they can reduce testing time by up to 50%. Additionally, in many cases the RMMs have demonstrated increased detection sensitivity when compared against the manual 14 day sterility test method. The regulatory bodies are supportive of RMM technology as the quality of testing and sensitivity is improved over traditional culture-based methods as well as reducing product release timelines. The BacT/ALERT® is designed to detect carbon dioxide (CO_2) generated during the growth of microorganisms. If the sample contains microorganisms, the colorimetric sensor in the media bottle base changes colors. The instrument detects the color change as it scans the bottles every 10 minutes and monitors each bottle individually. This study compared the ability of the traditional 14 day culture method and the rapid sterility method to detect the presence of contaminating microorganisms in a representative cell product. In addition to the positive controls, each microorganism was tested at 3 different concentrations; with the lowest concentration below the limit of detection. While both methods detected the panel of challenge organisms, there was increased sensitivity and shorter time to positive when using the rapid detection method.

467. High Efficiency Downstream Processing and Enrichment of Full AAV Particles Produced from Suspension HEK293 Cells

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As part of a scalable AAV production process using suspension HEK293 cells, the downstream vector purification requires efficient removal of large quantities of cellular debris. Cell lysis is often required to release the AAV, which also releases impurities such as host cell DNA and residual plasmid DNA. These impurities could potentially be considered immunogenic when the product is administered to patients. Nucleases can be added to the lysate to reduce residual DNA, solution viscosity and increase product yield. Here, we evaluated residual DNA levels and recovery using different nucleases. In addition to cellular impurities, empty and partially filled capsids represent an additional antigenic burden that may impact treatment efficacy. Therefore, minimizing the levels of empty AAV capsids from the final product is extremely important. Ion-exchange chromatography takes advantage of the subtle differences in the net surface charge between the empty- and full-AAV capsids that can be modified by the pH and/or salt concentration of the mobile phases. Here we demonstrate that full-capsid enrichment >90% was achieved using a salt gradient.

468. Robust Scale-Up and Production of Multiple Gene Therapy Vectors for Pre-Clinical Applications at 250 L Scale with the Sf9:Baculovirus Expression System

Russell Udani, Patrick Carroll, Michael Chlumecky, Dominic Ciampa, Nripen Singh

Technical Operations, Voyager Therapeutics, Cambridge, MA

Voyager Therapeutics has established a fast-to-clinic CMC development strategy leveraging a state-of-the-art pilot plant to streamline and standardize the development and technology transfer of preclinical and clinical gene therapy candidates. Process development has resulted in a significant volumetric productivity increase and improvement in yield across chromatography and filtration steps. Scalability of multiple new gene therapy products to a 250L scale for pre-clinical applications has been demonstrated with the Sf9:baculovirus expression system.

469. Aratinga.Bio Versatile Platform for the Bioproduction and Characterization of Lentiviral Vectors: from R&D Quality up to Pilot-Grade Batches

Cécile Bauche, Victoire Barailles, Nicolas Delacroix, Marion Fichini, Justine Flaire, Alice Pailleret, Aurélie Perier, Yann Rault, Renaud Vaillant

Aratinga.Bio, Villejuif, France

Recent market approval of CAR-T cell therapies and the continuously growing number of gene-based *ex vivo*and *in vivo* therapies evaluated in clinical trials has created a huge constraint on the supply of high quality grade lentiviral vectors (LV) which results in higher manufacturing costs and longer timelines that are not sustainable for early-stage development programs.aratinga.bio has built up a versatile manufacturing platform for the manufacturing of high-quality grade LV material designed to cover customers' needs from R&D *in vitro* and *in vivo* evaluation up to regulatory preclinical studies. Our 350 m2 facility based in Paris employs 10 USP and DSP experts and offers manufacturing capacities in mammalian cell suspensions from 1 to 80 L of bulk that comply with the European regulations on the handling and use of genetically modified organisms. Here we report on our routine process that allows the bioproduction of 60 mL batches within 5 days with infectious and physical titers above 5x10E7 TU/mL (measured by qPCR and FACS) and 3x10E12 TU/mL respectively. This scalable process has been developed in bioreactors and synthetic medium (no bovine serum albumin needed). After two purification and concentration steps, the vector batches are sterile-filtered and stored at -80°C in adapted vials. Depending on the concentration factor used

during the DSP, LV titers can reach the final concentration of 10E9 TU/mL. Depending on the use of the product, different formulations can be proposed. The robustness of this process has been validated by dozens of bioproduction campaigns of pseudotyped LVs with different envelopes and encoding different reporter genes or Chimeric Antigen Receptors. Adapted quality controls have been developed, analytically validated and implemented to ensure the characterization of the products. These controls are used to define identity, potency (p24, qPCR…), purity (HCP, residual DNA…), safety (standard sterility tests, endotoxin, mycoplasma…), and stability (pH, osmolality, aggregates…) of the products. In addition, we have implemented biophysical methods compatible with in-process control (Biacore, qNano, Videodrop) that allow a tight management of operations and bioproduction costs. With the implementation of a Quality by Design approach and the input of an internal Quality Assurance service, we are able to constantly improve our bioproduction process, enable a process control strategy and enhance product safety, quality and efficacy. aratinga.bio is thus able to provide an integrated solution to support biotech companies and academic laboratories throughout the development of their disruptive gene therapy programs.

470. Abstract Withdrawn

471. Oncolytic Virus Production using MRC5 Cells in Pall's iCELLis® Nano Bioreactor is Equivalent in High and Low Compaction Beds

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[1]Merck, West Point, PA,[2]Pall Corportation, Westborough, MA

In the past five years, Pall's iCELLis bioreactors have established themselves in the industry for the development and production of viral products. The bioreactor's characteristics, mainly scalability, fixed-bed cell retention, closed-processing, advanced process control, single-use ease-of-implementation and footprint reduction, make the iCELLis bioreactor well suited for viral production. In this poster, we present our work on the use of the iCELLis Nano bioreactor to successfully culture MRC5 cells and to produce a high-titer proprietary oncolytic virus. Since the iCELLis Nano bioreactor is available in 6 different fixed bed geometries, questions arise if equivalent productivity can be maintained in the largest bed size, a height of 10 cm and a carrier compaction factor of 1.5x. This bed geometry is thought to be the most challenging in terms of obtaining a uniform cell distribution, even and efficient infection, and subsequent high productivity. In these experiments, we demonstrate with an n=3 that the virus productivity in the largest, high compaction, 4 m^2 fixed bed is equivalent or higher than the 2.7 m^2 low compaction fixed bed and legacy flatware process.

472. Cost Modeling Comparison of Static, Suspension and Fixed-Bed Bioreactors to Manufacture Commercial Gene Therapy Products

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Adeno-Associated Viral vectors (AAV) are powerful tools used for *in vivo* gene delivery to cure congenital diseases. AAV can be efficiently produced by transient transfection of HEK293 cultured in static 2D multitray system (MT) and this method is widely used at small scale for preclinical and clinical studies. However, use of this system at commercial scale (>200 L) comes with significant risk and cost. To overcome this scaling challenge, bioreactor based systems can be used but a choice must be made between suspension or adherent based processes. Cost modeling is a useful tool that can be used to determine the impact of particular process choices at large scale. In this study, we compare AAV manufacturing costs for three different upstream processes: static MT, suspension bioreactor and fixed-bed bioreactor; using transient transfection in HEK293 cells in serum-free media as expression system at the 200 and 1000 L scale. Utilizing the BioSolve♦ software for modeling entire processes in cGMP conditions, cost structure is determined and sensitivity analysis is used to identify parameters that can leverage the cost of goods (CoGs).

473. Use of a Scalable Lentiviral Vector Production System in Early-Stage Research to Enable Straightfoward Manufacture Toward Translation

Jason Sharp, Shikha Mishra, Meaghan McKavitt, Ryan Gonzalez, Mark Landon, Uma Lakshmipathy

Thermo Fisher Scientific, Carlsbad, CA

Lentiviral vectors are widely used for the delivery of genetic material to cells for the generation of cellular therapies. Feasiblity and early process development in such workflows such as CAR-T cells begin with the end product in mind. The profile of the virus and the ex-vivo modified cells are sensitive to the effects of cellular processes connected to the bioproduction process which in turn is critical to the function of the potential therapeutic. There is therefore a market need for scalable and regulatory-compliant reagents that can be used in early research all the way up to cGMP manufacturing.The CTS LV-MAX Lentivirus Production System is manufactured in conformity with cGMP for medical devices (21 CFR Part 820), meets the USP <1043> ancillary material supplier responsibilities for cell-, gene-, and tissue-engineered products, and European Pharmacopoeia (Ph. Eur.) 5.2.12 recommendations; with additional Drug Master File or a Regulatory Support File. These features make this platform an appealing choice for the transition and technology transfer from research-grade discovery through GMP-grade clinical and commercial manufacturing.Here, we assessed the scalability of CTS LV-MAX lentivirus production system from 1 mL high-throughput screen scale-out to larger scale lead scale-up workflows. For the scale-out, multiple libraries of over 100 constructs each were manufactured at 1 mL scale. These libraries were used to assess the function of various sequence modifications in a high-throughput, high-content screen. Individual constructs from this screen were selected for scale up to 100 mL with comparable titers at both scales. For scale-up, EF1a promoter driven CAR with CD19 (FMC63), 4-1BB, and EGFRt was used to generate virus from 30 mL up to 2 L in shaker flask and bioreactor formats. This scaled-up virus was successfully used ot generate CAR-T with confirmed functionaility. The results of this study suggest that utilization of CTS LV-MAX defined systems and viral production cells at the scale-out, research

stage can be carried forward through scale-up manufacturing, thus avoiding lentivirus profile changes and technology transfer issues that can occur with adaptation of non-GMP systems and enabling a more rapid transition from research to clinic.

474. Abstract Withdrawn

475. Implementation of the Aber Futura Biomass Probe in Pall's iCELLis Nano Bioreactor Provides a Robust and Reproducible Method to Assess Cell Density

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In the past five years, Pall's iCELLis bioreactors have emerged as the leading bioreactor technology for clinical manufacturing of viral vectors and vaccines. Pall and InVitria have previously demonstrated the successful implementation of OptiPEAK HEK293T blood-free media formulation in the iCELLis bioreactor, including rapid and uniform cell attachment to the iCELLis fixed-bed bioreactor and robust cell expansion kinetics found to be equivalent to those observed in serum-containing media. However, a major challenge in assessing cell growth is accurately and reproducibly measuring cell density. One legacy method is nuclei counting, but this method can have up to 20% variability, requires open aseptic bioreactor handling, and only provides a discrete set of data points. Another method is metabolic analysis, such as glucose consumption and lactate generation. While these measurements are less variable and can be performed sterilely, they are also discrete, and extremely sensitive to culture conditions. The Aber Futura Biomass Probe provides a third method to measure cell density, one that provides an accurate, continuous reading, while maintain sterility. In this poster, we demonstrate the implementation of the biomass probe to accurately and reproducibly count HEK293T cells, overcoming past implementation challenges, such as the interfering effect of transient air bubbles. Finally, we correlate the biomass measurements with legacy nuclei counting and glucose consumption methods.

476. Clinical Viral Vector Manufacturing Capacity Analysis to Support Product Development

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The number of Advanced Therapy Medical Products (ATMPs) in development has grown exponentially over the past five years. ATMPs typically use engineered viral vectors as a delivery vehicle to replace defective, disease-causing genes to compensate directly for a genetic defect or to encode a therapeutic protein construct (e.g., chimeric antigen receptor, CAR) for disease treatment. Growth in the number of product candidates has been spurred by dramatic clinical effectiveness in rare disease populations and the approval of three gene therapies and three genetically-modified cellular therapies over the last seven years. All licensed products either consist of adeno-associated virus gene therapy vectors (Glybera®, Luxturna®, and Zolgensma®) which are directly administered to the patient to replace a defective gene or rely upon lentiviral vectors to engineer cells that are then expanded and become the product delivered to the patient (Strimvelis®, Yescarta®, and Kymriah®). The manufacturing of viral vectors is a complicated endeavor that requires specialized facilities and procedures. As a result, there is a limited pool of Contract Development Manufacturing Organizations (CDMOs) capable of accommodating the growing demand. To understand better whether CDMO capacity is keeping pace with clinical viral vector demand, we built a model to forecast the quantity of viral vectors required to support ongoing and planned clinical trials. The projected vector requirements were compared with the CDMO manufacturing capacity available over time. Our analysis focused on adeno-associated virus (AAV) and lentivirus/retroviral vectors and took into consideration the ranges of vector product yield, potential batch sizes, vector requirements for disease/product type, target organs, number of trials, and patient enrollment. The results indicate that viral vector demand will exceed CDMO manufacturing capacity in 2020.

477. Efficient and Consistent Purification of a Variety Kind of Serotypes of AAV Vectors Both from Culture Supernatant and Inside of Host Cells Simultaneously

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Introduction: Recombinant adeno-associated virus vectors (rAAV) have been widely used as vehicles to deliver gene of interest for therapeutic purpose or pre-clinical study of gene therapy. In the early phase of development, efficient and consistent purification procedure of rAAV is desired. Since the rAAV manufactured by the helper virus free system accumulates both in culture supernatant and inside of the host cells, and the localization of rAAV varies depending on its serotypes or harvest time, we addressed to develop an efficient method to extract and purify rAAV both from culture supernatant and host cells simultaneously. **Methods:** rAAV harboring fluorescent gene ZsGreen1 were manufactured by the transient transfection using AAVpro® helper free system (Takara Bio) by the transient transfection of three plasmids into 293T cells. After transfection, rAAV were harvested from both cell lysate and cell culture medium by a detergent mediated extraction at a time. The crude lysate was further purified by using a part of components included in AAVpro® concentrator (Takara Bio) and AAVpro® purification kit (Takara Bio). The obtained viral genomic copy number was determined by the quantitative PCR using AAVpro® Titration Kit (for Real Time PCR) Ver.2 (Takara Bio). **Results:** At first, localization of rAAV was investigated in serotype 1, 2, 5 and 6 three or seven days after transfection. The localization of rAAV inside of the 293T cells varied depending on the serotypes; and there was a trend of significant decline of rAAV inside of the cells from day 3 to day 7, while total amount of rAAV did not changed significantly. Next, we tried to extract total rAAV by adding the detergent directly

to the culture fluid of 293T cells. rAAV was efficiently recovered with preserving the biological titer in all serotypes. The extracted rAAV was further purified by the combination of commercially available AAVpro® concentrator and AAVpro® purification kit. The obtained rAAV represented high purity with equivalent biological titer compared to the rAAV obtained by using the existing purification kit. **Discussion:** Development of efficient rAAV purification procedure with a consistent platform is necessary. As the localization of rAAV inside of the cells varies depending on the serotypes and harvest time after transfection, we addressed to extract rAAV both from culture supernatant and host cells simultaneously. In combination with the commercially available AAVpro® concentrator and AAVpro® purification kit for downstream purification, we have established a simple and efficient rAAV preparation method applicable to a variety kind of serotypes. This protocol would provide a powerful and an effective method with consistent recovery for preparing multiple serotypes of rAAV in the same platform.

478. Abstract Withdrawn

479. Understanding Scalability of Mustang Q Devices in Lentiviral Vector Purification Using Doe Approach

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Lentiviral vectors (LVVs) are increasingly used in treatment of acquired and inherited genetic diseases. Production and purification of the LVVs remains challenging due to low titers from upstream processes and the low stability of the virus. A typical LVV purification strategy might include an AEX step positioned after clarification and before tangential flow filtration and final filtration. Thus, anion-exchange (AEX) chromatography has a key role in purification of LVVs. As the AEX step is positioned directly after clarification, relatively large volumes have to be processed. This makes membrane chromatography an ideal choice as it can be operated at and above 10 membrane volumes per minute. Despite widespread application of AEX membrane chromatography, there has been little published effort to understand the scalability of membrane devices when transitioning from filter plate experiments to small and then larger capsules. Here we implemented a Design of Experiment (DOE) approach to optimize the bind and elute conditions of LVVs in Mustang Q filter plate experiments and then transferred these conditions to 0.18 mL and 1 mL Mustang XT Acrodisc Units. Gradient and step elution experiments were carried out to understand the role of salt and additives in desorption of LVV from the membrane surface. In addition, we compared the performance of Mustang Q devices to other AEX sorbents. Under optimized conditions, the observed capacity of Mustang Q capsules was 2.4×10^{11} to 1.5×10^{12} transfecting particles/mL of membrane. In summary, a scalable LVV purification protocol was developed for facile transition from Mustang Q filter plate to larger XT capsules.

480. Walk-Away Automation System without Centrifugation for Better Flow Cytometry Sample Preparation and Reliable Data Tracking

Ira Kim[1], Amanda McCabe[2], Felicia Ciuculescu[2], Roberta Zappasodi[3], Isabell Schulze[3], Christoph Eberle[4], Melvin Lye[1], Nadiezda Fernandez Oropeza[1], Ann Wang[1], Chyan Ying Ke[1], Kong Leong Cheng[1], Reese Wong[1], Ih Chin Kon[1], Royce Pek[1], Taha Merghoub[3], Myriam Armant[2], Namyong Kim[1]

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Recent advances in gene and cell therapy have led to a new and exciting era to treat patients, offering innovative treatment options to many debilitating and incurable diseases. As such, the need for quantitative and reproducible cell analysis is rapidly growing, particularly for clinical studies. Multi-parameter flow cytometric analysis is a widely used technique for immunophenotyping of specific cell subsets in both academic and clinical settings due to its high-throughput and multiplexing capabilities with relatively low costs. Despite such advances, however, consistent sample preparation prior to analysis through automation remains as a major challenge. The Laminar Wash™ (LW) AUTO system is a fully automated cell preparation platform without the use of centrifugation. LW AUTO enables sample preparation, incubation and cell washing within an area approximately half of a conventional automation cell washer, rendering it suitable for adaptation in both research and clinical studies. This is accomplished by the integrated LW HT1000 system, which consists of a novel wall-less plate and laminar-flow cell washer that performs automated washing of suspension cells and retains up to 95% cells of higher viability, at a fraction of the time. Specifically, LW HT1000 enables synchronized and automated washing of 96 samples eliminating the needs for centrifugation, which is often a source of error and a limiting factor in sample quality. Furthermore, the LW AUTO system offers a powerful yet affordable platform for fully automated sample preparation, further simplifying and expediting cell processing with enhanced consistency and reproducibility. Here we report that the LW method improved cell retention and viability compared to the conventional centrifuge method while lowering the background and achieving better separation of signals in a fraction of time. Our results show reduced clumping and better identification of TILs from dissociated tumor samples as well as a significantly higher percentage of lentivirus-transduced cells and efficient debris removal for vector generation. Also, LW reduced background even in the absence of pronase treatment in a B cell flow crossmatching assay. Notably, we also discuss how the LW AUTO system can help improve the overall workflow and the data quality in the gene therapy samples using fetal haemoglobin (HbF)-expressing human erythrocytes. In conclusion, we demonstrated that the Laminar Wash technology offers major benefits to sample preparation allowing more consistent, reproducible, and rapid data acquisition. The LW AUTO platform offers a completely automated sample processing solution, simplifying and expediting cell preparation, thus improving the overall quality of data in gene and cell therapy pipeline.

481. Robust Lentivirus and Adeno-Associated Virus Production Through the Optimization of Key Transient Transfection Experimental Variables

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The promise of mainstream utility for cell and gene therapies is reliant upon robust and reproducible manufacture of higher titer lentivirus (LV) and adeno-associated virus (AAV)- the upstream tools used for gene delivery to target cells and tissues. Transient transfection of plasmids encoding essential virus components into 293-derived cell types is the most broadly used approach for generating recombinant LV and AAV; however, manufacture of these viruses is often hindered by low transfection efficiency and inadequate functional virus production. To address this need, Mirus Bio developed a new platform technology that includes a novel lipid and polymer formulated transfection reagent, *Trans*IT-VirusGEN®, as well as specific LV and AAV enhancers to boost functional titers. In the pursuit of developing the *Trans*IT-VirusGEN® AAV and LV platforms for increasing LV and AAV titers, Mirus Bio performed comprehensive testing of other key variables for virus manufacture including DNA dose, cell density at the time of transfection, cell type, and cell growth medium. Surprisingly, in suspension adapted 293 cells, the importance of these individual factors frequently differed for recombinant LV versus AAV production. Through independent optimization of these parameters in the context of the *Trans*IT-VirusGEN® Transfection Reagent and viral vector-specific enhancers, two distinct sets of conditions emerged for increasing LV and AAV yields. These two sets of optimized parameters serve as the basis for robust upstream manufacturing processes and reduce costs by increasing the efficiency through which high functional titers are obtained. To support large-scale manufacturing during preclinical and early clinical gene therapy trials, Mirus Bio introduced *Trans*IT-VirusGEN® SELECT, a higher-grade formulation of its research-grade reagent, which includes release criteria for performance, identity, sterility, endotoxin and mycoplasma. Mirus Bio is producing a GMP-compliant *Trans*IT-VirusGEN® Transfection Reagent and GMP-compliant enhancers that have been specifically engineered for either AAV or LV production in order to better support customers who require the most highly qualified raw materials.

CAR T-Cell Therapies II

482. CD19-Positive Brain Pericytes as Targets of Immunotherapy-Associated Neurotoxicity

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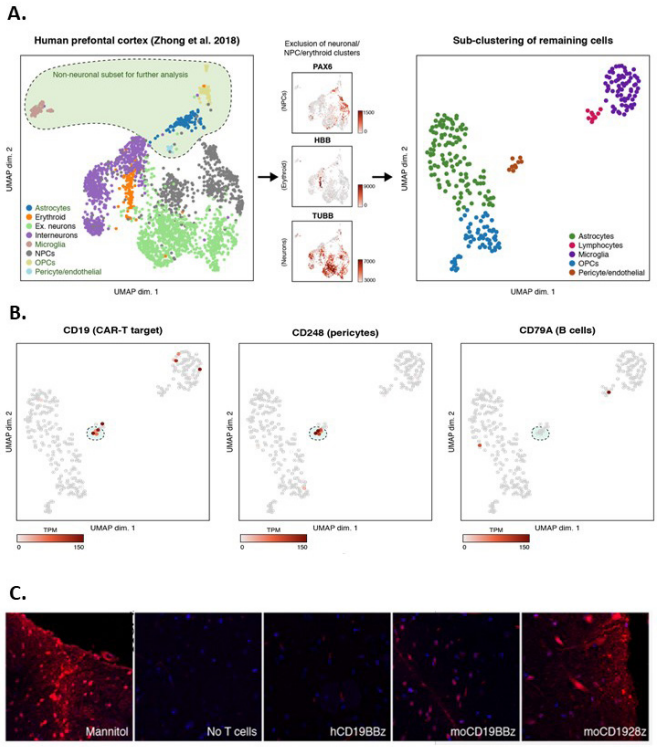
CD19-directed immunotherapies are clinically effective for treating B-cell lymphomas but also display a high incidence of associated neurotoxicity. Subsets of patients treated with CD19-directed chimeric antigen receptor (CAR) T cells or CD19/CD3 bispecific T-cell engager (BiTE) antibodies display severe neurotoxicity, including cases of fatal cerebral edema associated with T cell infiltration into the brain. The pathophysiology of this neurotoxicity remains unclear yet its understanding is critical for the development of safer CAR and BiTE therapies. Here we report that a small population of pericytes in human brain expresses *CD19* and contributes to CD19-specific CAR-T-induced neurotoxicity. Pericytes are mural cells that wrap endothelial cells and are critical for maintaining blood-brain-barrier (BBB) integrity. Using single-cell RNA-sequencing (scRNA-seq) datasets, we identify *CD19* expression in human brain pericytes that also express *CD248*, among other pericyte markers (**Figure 1A-B.**). To assess the expression of CD19 protein in human pericytes, we performed immunohistochemistry on several regions of the human brain using a clinically-validated anti-human CD19 antibody on samples from healthy deceased subjects. We found CD19 expression on cells present adjacent to the vessel basement membrane walls in perivascular areas. We also performed flow cytometric analysis on C57BL/6J mice and human normal brain samples, and were able to identify CD19 positive cells in the pericyte population. We further interrogate the contribution of CD19+ pericytes as targets for anti-CD19 CAR-T related neurotoxicity using immunodeficient NSG mouse models. Following mouse CD19-specific CAR-T cell infusion, CAR-T cells disrupt the BBB through a B cell-independent mechanism as measured through Evans Blue Dye (EBD) extravasation in brain parenchyma. Mice treated with human CD19-specific CAR-T cells or untreated mice did not demonstrate BBB permeability. Moreover, this disruption is more evident in CD28-based CAR-T cells compared to 4-1BB-based CAR-T cells, in agreement with clinical observations of greater neurotoxicity with CD28-based CAR-T cells (**Figure 1C**). This finding was confirmed using C57BL/6J mice treated with syngeneic anti-CD19 CAR-T cells and using 9.4 Tesla high-resolution magnetic resonance imaging (MRI) machine (not shown). In summary, we discovered occult CD19 expression on brain pericyte cell surface and characterized anti-CD19 CAR-T cell-induced BBB disruption. The knowledge of this novel neurotoxicity mechanism provides a rationale to enhance the safety of the current standard of care 4-1BB-based Tisagenlecleucel (Kymriah®) and especially the CD28-based axicabtagene ciloleucel

(Yescarta®) by developing "logic-gated" CAR constructs capable of detecting a specific pattern of antigenic expression. Furthermore, these data provide insights into the underlying mechanism of neurotoxicity in CD19-directed therapies and demonstrate the value of a single-cell human atlas for cell therapy design.



483. Cellular Delivery of Oncolytic Adenoviruses Utilizing Mesenchymal Stromal Cells (MSCs) in Combination with CAR-T Cell Therapy

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Intratumor administration of oncolytic virotherapy has been used successfully to infect, replicate, and lyse primary tumor cells but shows limited efficacy in controlling metastases. Intravenous delivery of oncolytic virotherapy is even less effective as many patients have pre-existing anti-viral immunity and the virus is cleared before it reaches the site of the tumor. To overcome this limitation, cellular delivery systems have been utilized to mask the virus until it reaches the tumor site. We have utilized mesenchymal stromal cells (MSCs) to deliver gene therapies and oncolytic viruses to primary tumors as they have the ability to home to sites of inflammation and solid tumors while preventing viral clearance from neutralizing antibodies. Studies utilizing a novel combinatorial adenovirus (Ad) vector with an oncolytic Ad and helper dependent Ad that can express IL-12 and anti-PD-L1 (CAd12_PD-L1) have shown strong anti-tumor activity in vitro while producing functional transgene products. We hypothesize that infected MSCs can systemically deliver and produce functional virus that can target and lyse lung tumor cells and stimulate anti-tumor activty in combination with HER.2 specific CAR-T cells.MSCs infected with CAd12_PD-L1 produced functional cytolytic virus and transgene products measured through tumor cell apoptosis and IL-12 secretion detected by ELISA. Tri-culture of MSCs, tumor cells and T cells increased tumor cell death in conditions with CAd-infected MSCs compared to uninfected MSCs and T cells alone ($p=0.007$, $n = 5$), suggesting an improved anti-tumor activity with viral oncolysis and immunostimulatory IL-12 and anti-PD-L1 components. To further mimic the solid tumor setting, we also tested CAd-infected MSCs in combination with CAR-T cells in three dimensional tumor spheroid models. We confirmed MSC delivery of CAd12_PD-L1 tagged with RFP in 3D tumor spheroids to determine virus spread over time. Spheroid death measured by annexin V staining was increased with combination of CAd12_PD-L1 and HER.2 CAR T cells compared to CAR-T cell only with varying effector to target ratios. These results were replicated in two orthotopic lung tumor xenograft models in which animals treated with both adenovirus infected MSCs and HER.2 specific CAR-T cells had increased tumor control compared to either component alone. A combination of oncolytic virotherapy providing immunostimulatory components to enhance CAR-T cell activity provides a greater antitumor effect compared to each therapy alone. We have confirmed that MSCs travel to the lungs and release oncolytic virus that infects tumor cells locally, providing an efficient means to prevent virus inactivation by pre-existing immunity. Anti-tumor activity of HER.2 specific CAR-T cells can be further enhanced by IL-12 and anti-PD-L1 encoded by the virus to potentiate a Th1 effector response after initial disruption of the tumor and its microenvironment through oncolysis. Interestingly, we are able to observe an increase in effector cytokine secretion (Granzyme, IFNg) from non-transduced cells in combination of CAd MSCs suggesting an increase in polyclonal T cell response and promotion of epitope spreading. These studies combine the benefits produced by CAR-T cells (predictable targeting, specific activity) with the advantages of oncolytic virotherapy (cancer cell lysis, disruption of the tumor microenvironment, and immune stimulation) to provide overall enhanced anti-tumor activity and an alternative therapy for solid tumor malignancies.

484. Enhancing Tumor Directed T Cells with a Costimulatory CAR

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The ability of the T cell receptor (TCR) to target both intracellular and extracellular cancer antigens presented as peptides in the context of the major histocompatibility complex allows for a large number of potential tumor targets, providing an advantage over T cell therapies with chimeric antigen receptors (CARs) which are limited to surface proteins. Signaling through the TCR requires both signal 1 (activation) and signal 2 (costimulation) to sustain proliferation and persistence; however, most cancer cells lack expression of costimulatory molecules, resulting in incomplete T cell activation and only transient anti-tumor effect. In order to overcome the absence of signal 2 and improve anti-tumor efficacy, we genetically modified T cells that recognize the tumor antigen through a native or transgenic TCR with a costimulatory CAR (CoCAR) targeting CD19. Like classical CARs, CoCARs combine the antigen binding domain of an antibody with

costimulatory endodomains that trigger proliferation of the host T cell. However, a CoCAR is distinguished from a traditional CAR through its lack of cytotoxic ζ chain. Hence, the CoCAR can provide signal 2 upon encountering its antigen but lacks innate toxicity. The CoCAR was first tested in combination with an HLA-A*0201 restricted survivin-specific transgenic TCR (sTCR). In a sequential co-culture stress-test with HLA-A*0201+ BV173 tumor cells that co-express survivin (signal 1) and CD19 (signal 2), T cells co-transduced with the CoCAR and sTCR maintained their cytotoxic function for a median of seven rounds [Interquartile range (IQR): 4-7] of tumor challenge vs. a median of 3 rounds (IQR: 2-3) in the sTCR only condition. The improvement in sequential killing correlated with an average 5-fold increase in IFN-γ (5.0 ± 1.7; p=0.0003) and 3.2-fold increase in perforin secretion (3.2 ± 1.2; p=0.0205) for each donor tested. The CoCAR modification also produced enhanced sequential killing in co-cultures against tumors with heterogeneous expression of CD19 (signal 2 not expressed on the majority of target cells), but not in the complete absence of CoCAR antigen. T cells modified with sTCR with or without CoCAR had no effect against CD19-positive BV173 cells without survivin epitope presentation (ß2m knockout BV173 cells), confirming that tumor killing is only occurring through the sTCR and not the CoCAR. In a BV173 leukemia mouse xenograft model, sTCR+CoCAR+ T cells produced better leukemia control compared to sTCR+ T cells. At day 42 post treatment, tumor signal was 95% lower than in mice treated with sTCR+ T cells (2.9e7 ± 1.6e7 vs. 5.8e8 ± 5.3e8 p/s; n=5) and animals treated with sTCR+ CoCAR+ T cells survived longer (p=0.001) compared to mice treated with sTCR+ T cells. To evaluate whether transgenic CoCAR expression also exerts its co-stimulatory effects in the context of native TCRs, we generated CoCAR+ Epstein-Barr virus specific T cells (EBVSTs) directed against EBV-positive malignancies. In vitro, CoCAR+ EBVSTs produced a mean 2.4 fold (±1.9, n=6) increase in the frequency of IFN-γ secreting cells in response to EBV peptides compared to non-transduced EBVSTs. In an EBV lymphoblastoid cell line mouse xenograft model, EBVST derived bioluminescent signal at one week was 7.3 times higher in CoCAR+EBVST treated mice compared to EBVST treated mice (1.7e9 ± 1.1e9 vs. 1.2e10 ± 1.6e9 p/s; n=4; p<0.0001). The T cell expansion was accompanied by a reduction in flank tumor size by 77.1% in CoCAR+ EBVST treated mice on day 13 (0.81 ± 0.25 vs. .13 ± 0.12 cm^3; p=0.005). In summary, CoCAR-engineered T cells receive co-stimulation that leads to increased cytokine production and improved anti-tumor efficacy regardless of whether the cells exert their effect through a native or a transgenic TCR. Our data also show that it is not necessary for the CoCAR antigen to be expressed on every tumor cell to achieve enhanced anti-tumor effect.

485. Multiplexed Cytidine Deamination Enables Generation of Fratricide-Resistant 'Universal T v T' Chimeric Antigen Receptor Cell Therapy

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Targeting T cell malignancies using chimeric antigen receptor (CAR) T cells is hindered by 'T v T' fratricide against shared antigens such as CD3 and CD7. Genome-editing can overcome such hurdles through targeted knockout of problematic shared antigens as well as addressing barriers to using HLA mismatched cells by disrupting αβ T cell receptor expression , HLA molecules or CD52 (the target antigen for Alemtuzumab). Existing approaches relying on nuclease mediated DNA breakage and repair are associated with predictable and unpredictable chromosomal translocations. Targeted base editing using CRISPR guided cytidine deamination mediates highly precise seamless C→U→T conversion, which can be precisely directed to create stop codons or to disrupt splice sites, including in a multiplexed fashion for simultaneous disruption of multiple genes. A CRISPR-CAR lentiviral configuration was designed to couple anti-CD7 CAR (CAR7) expression with a sgRNA guide specific for TCR β chains (TRBC1/2) and achieved high level base conversion and efficient disruption of TCRαβ expression following electroporation of codon-optimized base editor (coBE) mRNA. Co-delivery of additional, uncoupled sgRNAs mediated up to 92% triple knockout of TCRab/CD7/CD52 in CAR7 expressing cells, and following magnetic bead mediated depletion of TCRαβ T cells resulted in <1% residual TCRαβ T cells. Generation of anti-CD3 CAR (3CAR) T cells from the same donor was achieved by disruption of TCRab/CD3 expression, and resistance to fratricidal effects was conferred by simultaneous targeting of CD7. This allowed co-culture and expansion of CAR7 and 3CAR T cells. When T cells generated using either spCas9 for cleavage mediated knockout or base editing were compared, similar phenotypes were observed and equivocal cytotoxic responses were detected against leukaemia targets in functional assays. However, while Cas9 mediated genomic disruption of both TRBC and CD7 resulted in readily detectable low level (1-3%) translocations, such predictable events were virtually undetectable in base edited cells. In vivo anti-leukemic function of base edited CAR T cells was investigated in NOD/SCID/γc mice engrafted with luciferase+eGFP+ Jurkat T cells pre-enriched to express CD3 and CD7, alone or in combination. Infusion of anti-CD3 and anti-CD7 effector CAR co-cultured T cells mediated clearance of luciferase expressing cells and inhibited leukaemic progression in keeping with the antigens expressed. Ongoing characterisation of wider RNA and DNA effects of cytidine deamination in edited T cells is underway, and a time-limited therapeutic application of 'universal' anti-T CAR T cells to secure enhanced molecular remission as a bridge to allogeneic haematopoietic stem cell transplantation is envisaged.

486. A Phase 1 Clinical Trial Using Armored GPC3-Car T Cells for Children with Relapsed/ Refractory Liver Tumors

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Background: CAR T therapies have benefited only a handful of patients with solid cancers and novel therapies are needed. Glypican 3 (GPC3) is an attractive immunotherapeutic target due to its preferential overexpression on multiple pediatric and adult solid cancers. We have systematically tested a set of GPC3-CAR containing the CD3ζ domain alone, with the CD28, 4-1BB costimulatory endodomains or their combination. These receptors were expressed in T cells and evaluated in multiple preclinical models of solid tumors. We found that the inclusion of the 4-1BB and CD3ζ endodomains enabled GPC3-CAR T cells to obtain a TH1-polarized effector cytokine profile, expand and persist the most and induce robust antitumor responses *in vitro and in vivo*. We are now examining T cells expressing this GPC3-CAR for the first time in children with relapsed/refractory liver tumors including hepatoblastoma (HB) and hepatocellular carcinoma (HCC; GAP, NCT02932956). **Methods:** The objective of this Phase 1 trial is i) to define the safety and establish the Recommended Phase 2 Dose of GPC3-CAR T cells, ii) to determine the persistence and anti-tumor activity of GPC3-CAR T cells and iii) to examine changes in gene and protein expression in CAR T cells and cancer and non-neoplastic tumor cells within the tumor microenvironment to define potential immune escape mechanisms. Eligibility for the trial includes: age greater than 1 and less than 18 years of age; histology proven, GPC3-positive tumor; life expectancy > 12 weeks and acceptable organ function. Toxicity is monitored using the CTCAE v5 and the RP2D will be determined by 3+3 dose escalation. Persistence is quantified using RT-PCR and flow cytometry. Antitumor activity of CAR T cells is defined at week 4-6 post-CAR T cell infusion by standard imaging using RECIST 1.1 criteria and the immune-related response criteria. **Results**: To date, three children with relapsed/refractory liver tumors have been treated with GPC3-CAR T cells on dose level 1 (CAR+ T cells dose: 1 x $10E^7/m^2$). Two patients completed full safety and response assessment. No dose limiting toxicities have been detected and there have been no infusion-related or serious adverse events. CAR T cells were detected in all three patients in peripheral blood and two patients had a greater than 40-fold CAR T cell expansion by week 2 post-infusion by RT-PCR. The circulating CAR T cells persisted for at least 4 weeks. The two evaluable patients had greater than 60% decrease in serum AFP levels. While patient 1 had progressive disease at week 5 post-infusion, the size of the patient's primary lesion remained stable, and multiple metastatic lung lesions decreased in size or disappeared altogether. Patient 2 had stable disease of her single, large mediastinal mass based on imaging 5 weeks post-infusion. Patient 3 imaging is pending. **Conclusions:** The initial results from this clinical trial show that GPC3-CAR T cells are well tolerated and have anti-tumor activity in children with liver tumors.

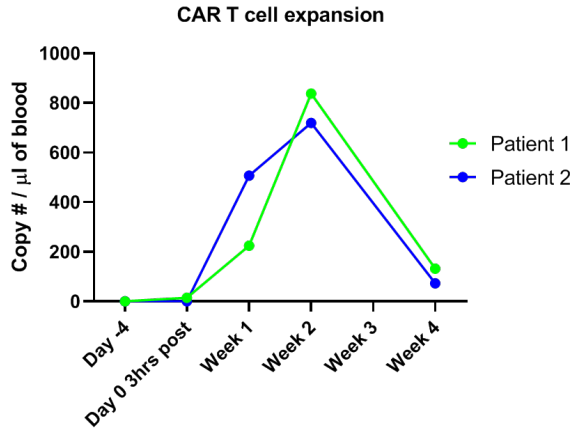

Figure 1: GPC3-CAR T cell expand in the peripheral blood of patients post-infusion. Quantitative PCR was used to evaluate the number of CAR transgene copies in each patient's peripheral blood at indicated timepoints.

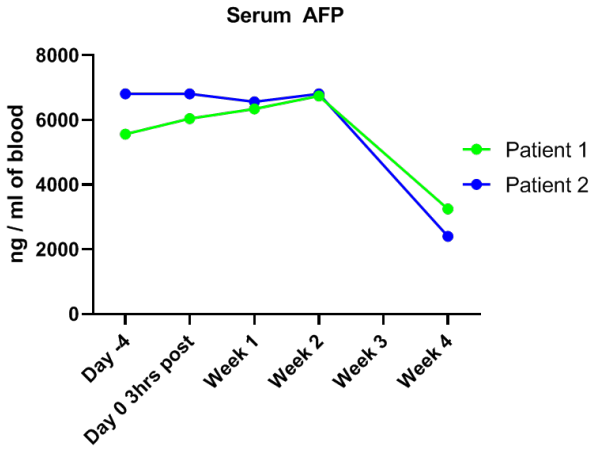

Figure 2: Alfa-feto protein (AFP) tumor marker levels significantly decrease after GPC3-CAR T infusions. AFP levels of patients measured by clinical laboratory test shown at indicated timepoints.

487. Automatic Neutralization of IL6 Storm and Blockade of IL1 Signaling During CART Therapy to Reduce Cytokine Toxicity and Minimize Neurotoxicity

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The revolutionary Chimeric antigen receptor (CAR) - T cell therapy still faces severe cytokine toxicity and neurotoxicity. CAR-T cells effectively eradicate cancer cells and secrete first wave of cytokines (e.g. GM-CSF, IFNG, and TNFA), triggering the host immune system to release a second wave of inflammatory cytokines (e.g. IL6 and IL1 mainly by monocytes). While recent studies suggested that IL6 and IL1 contribute to severe cytokine release syndrome (CRS) and neurotoxicity, GM-CSF might also involve in CRS for its role in activating monocytes. Therefore, we ablated the GM-CSF (and TCR) genes using CRISPR-mediated knock-out (KO) in CAR-T cells, which

are engineered with a 3rd generation lentivector for simultaneous co-expression of a 2nd generation anti-CD19 or anti-BCMA CAR (41BBζ) together with anti-IL6 scFv and IL1RA - designated as CD19 or BCMA CART/IL6/IL1. Xenograft studies demonstrated CD19 CART/IL6/IL1 GM-CSF/TCR KO effectively eradicated Nalm6 leukemia cells to achieve long term survival in 5 out of 6 (83%) treated NSG mice 10 months after CAR-T infusion. Also, TCR/GM-CSF KO CAR-T cells decreased gradually after tumor cells were undetectable, supporting the notion that GM-CSF KO CAR-T cells are safe at least in mouse model. Here we present initial results of 11 patients treated with CD19 or BCMA CART/IL6/IL1 (Table 1): GM-CSF/TCR KO for patients #2, 4 and 7, and wildtype (WT) for the rest 8 patients. After infusion, patients were monitored for clinical signs of cytokine toxicity, while IFNG was chosen as a major indicator of CART mediated cytotoxicity against cancer cells and IL6 as a major indicator of CRS. We noticed differential levels of IFNG peaks during therapy, but consistently low levels of IL6 at the time of peaked IFNG in all patients. Further analysis revealed that a high-level of IL1RA was correlated with a high-level of IFNG, suggesting CART could effectively produce anti-IL6 scFv and IL1 blocker while eliminating tumor cells. In regard to patient #5, fever was only observed from day 1 to 6, without hypoxia or hypotension or neurotoxicity, during which IL6 level was maintained at a low level. Interestingly, IL6 increased dramatically at day 9 and maintained at high levels from day 9 to 23, probably due to decrease of CAR-T produced anti-IL6 scFv after tumor cells were eradicated and renal dysfunction in the patient. Mild neurotoxicity was only observed during day 13 to 17, but without fever, hypoxia or hypotension. Analysis of cytokines revealed low levels of IFNG, IL1B, IL2, IL4, IL10, IL17A, TNFA and GM-CSF from D13 to D17, suggesting high level of IL6 alone might cause neurotoxicity. In summary, we reported an optimized CAR-T cells co-expressing IL6 and IL1 blockers is able to automatically neutralize IL6 storm and serve a promising platform for reducing cytokine toxicity and minimizing neurotoxicity. Our results indicated that proliferation and therapeutic efficacy of above CAR-T cells targeting CD19 or BCMA were not compromised. Analysis of patients treated with GM-CSF/TCR KO CART revealed high frequency of CD3- CAR+ T cells and no unexpected CART expansion, supporting the safety of CRISPR edited T cells in clinical applications. Further studies are ongoing to explore the effect of GM-CSF KO and IL1 signaling blockade on reducing cytokine toxicity and neurotoxicity.

Table 1. Characteristics of the treated patients. * details provided in abstract.

Pt #	Type	GM-CSF/TCR	IFNG(IL6) (pg/mL)	Re-sponse	CRS grade	Neurotox-icity	Tocili-zumab
1	DCB-CL	WT	2.63(UD)	NR	None	None	No
2	MM	KO	3.3(1.5)	CR	None	None	No
3	ALL	WT	5.48(3.15)	CR	None	None	No
4	NHL	KO	20.21(6.43)	CR	None	None	No
5	MM	WT	50.85(97.41)	CR	1	None*	No
6	ALL	WT	124.89(2.93)	CR	2	None	No
7	MM	KO	1343.21(4.76)	CR	2	None	No
8	CLL	WT	2014.2(49.85)	CR	3	None	No
9	ALL	WT	2979.29(16.34)	CR	3	None	No
10	ALL	WT	3812.54(8.56)	CR	3	None	No
11	ALL	WT	4118.03(16.36)	CR	2	None	No

488. Iberdomide Increases the Potency of the Anti-BCMA CAR T Cell Product Orvacabtagene Autoleucel (orva-cel)

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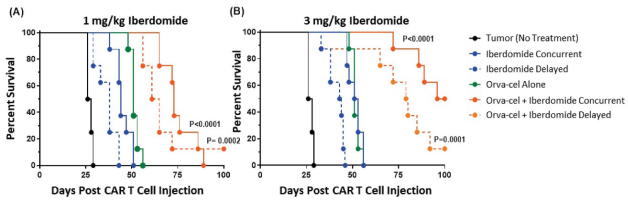
Background. Anti-B-cell maturation antigen (BCMA) chimeric antigen receptor (CAR) T cell therapies have shown promise in patients with heavily pretreated relapsed/refractory multiple myeloma (MM). However, a subset of patients progress within 1 year of treatment, and durability of response remains poor. Combinatorial approaches that can achieve orthogonal MM tumoricidal activity alongside CAR T cells and/or enhance CAR T cell potency may extend the duration of the high response rates already achieved. Iberdomide is a potent cereblon E3 ligase modulator (CELMoD) compound under clinical investigation in patients with relapsed/refractory MM that is capable of co-opting cereblon, modulating its activity, and redirecting the protein degradation machinery of the cell toward the elimination of target proteins (Ikaros and Aiolos), resulting in therapeutic effects. **Methods.** The immunomodulatory effects of iberdomide on orva-cel CAR T cells were assessed in acute and long-term chronic stimulation assays using T cells derived from both healthy subjects and MM patient donors. Recombinant BCMA-coated beads provided a 7-day chronic stimulus, and CAR T cell function was subsequently analyzed in rechallenge assays with BCMA+ MM tumor cells by measuring cytolytic activity and cytokine production. A cereblon-mutant MM cell line (DF15R) was cultured with orva-cel in a long-term serial stimulation assay to assess the effect of the drug solely on the intrinsic performance of orva-cel as measured by CAR T cell expansion over time. In vivo studies in NOD scid gamma (NSG) mice evaluated response against a disseminated, iberdomide-sensitive MM tumor using daily dosing of iberdomide in combination with a single infusion of a subtherapeutic dose of orva-cel from a patient donor. Two dosing schedules explored both concurrent (Day 0) and delayed dosing (Day 12) of iberdomide after CAR T cell administration. **Results.** Iberdomide increased orva-cel CAR+ T cell counts, cytolytic activity, and cytokine production

across acute and chronic stimulation assays. Additionally, use of iberdomide extended anti-BCMA CAR T cell expansion in serial stimulation assays and increased effector function as measured by increased cytokine production at multiple time points through the course of the assay. In vivo, both concurrent and delayed iberdomide dosing schedules enhanced the function of subcurative doses of orva-cel from a patient donor who was refractory to lenalidomide. The combination led to decreased tumor burden and increased survival as compared to the antitumor activity of iberdomide or a subcurative dose of orva-cel alone (**Figure**). **Conclusions.** In MM, iberdomide treatment, when used in combination with orva-cel, has the potential to not only eliminate malignant plasma cells but also improve the pharmacological performance of the CAR T cells. Iberdomide resulted in potent antimyeloma activity and activated CAR T cells at a much lower concentration than currently available immunomodulatory drugs. Such a combinatorial approach could be considered to improve both the depth and duration of response in patients with relapsed/refractory MM.

Figure: Administration of Iberdomide in Combination With orva-cel Significantly Enhanced Survival for Both Delayed and Concurrent Dosing of the Drug in the OPM2 Disseminated Xenograft Tumor Model



NSG mice were implanted with 2 million OPM2 cells, which were allowed to engraft. One day before orva-cel administration (0.5 million CAR T cells), iberdomide treatment at (**A**) 1 mg/kg and (**B**) 3 mg/kg was given daily for 32 days to the mice in the concurrent group. Twelve days after CAR T cell administration, the delayed dosing group initiated daily iberdomide dosing for 21 days.

Preclinical Large Animal Studies for Neurologic Diseases

489. Combination Hematopoietic Stem Cell Transplantation and Intravenous AAV Gene Therapy Corrects Neurologic Phenotype in Canine Krabbe Disease

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Globoid cell leukodystrophy (GLD, Krabbe disease) is a progressive, incurable neurodegenerative disease caused by deficient activity of the hydrolytic enzyme galactosylceramidase (GALC). The ensuing cytotoxic accumulation of psychosine results in widespread central and peripheral nervous system (CNS, PNS) demyelination. Hematopoietic stem cell transplantation (HSCT) is the only treatment available for presymptomatic infantile GLD. However, correction of PNS disease is not complete. Herein we evaluated HSCT, intravenous (IV) AAVrh10, and combination HSCT + IV AAVrh10 in the canine model of GLD. While HSCT alone in 3 GLD dogs resulted in no increase in survival (11.43 ± 1.8 weeks of age) as compared to untreated GLD dogs (15.7 ± 4.8 weeks of age), combination HSCT + IV AAVrh10 in two dogs resulted in increased survival to 55 and 82 weeks of age. A 5-fold increase in the AAVrh10 dose, in combination with HSCT, further improved efficacy and normalized neurologic disease up to 2 years of age, the predetermined endpoint. In dogs treated with IV AAVrh10 alone (1x dose), without prior transplantation, GLD dogs reached endpoint at an average of 41.2 weeks of age (range 23.4 - 55.4). Intravenous delivery of AAVrh10-cGALC alone or in addition to HSCT normalized nerve conduction velocity in the tibial, ulnar, and radial nerves, indicating attenuation of demyelination in the PNS. However, in the CNS, combination HSCT + IV AAVrh10 provided a greater correction to brain myelination than IV AAVrh10 alone. Biochemically, intravenous AAVrh10 alone or in combination with HSCT was able to normalize GALC enzyme activity and psychosine levels in the PNS. However, only combination therapy at the increased 5x dose was able to restore levels of GALC enzyme activity and psychosine to within normal range in the CNS. The current standard of care, HSCT, provides incomplete correction of PNS disease in children. These data suggest that systemic AAV gene therapy can normalize nerve conduction velocity, GALC enzyme activity, and psychosine levels in the PNS. However, a high dose of IV AAV is necessary for correction of CNS disease. These data should guide clinical translation of systemic AAV gene therapy as an addition to HSCT.

490. Method to Prevent AAV-Induced Dorsal Root Ganglia Toxicity & Axonopathy in Nonhuman Primates, and Insights into the Pathophysiology

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Delivering adeno-associated virus (AAV) vectors into the central nervous systems (CNS) of nonhuman primates (NHP) via the blood or cerebral spinal fluid can lead to dorsal root ganglia (DRG) toxicity. This toxicity comprises neuronal degeneration, mononuclear cell infiltration, and secondary axonopathy of central and peripheral axons of the dorsal spinal cord and peripheral nerves, respectively. We observed this toxicity with a variety of capsids and transgenes, with vectors purified by gradient or column chromatography, and in animals receiving different immunosuppressive regimens. Toxicity was usually minimal to moderate and subclinical. However, some cases exhibited clinical signs of proprioceptive defects and ataxia. We hypothesized that a high transduction rate leads to cellular stress from overproduction of the transgene product, which ultimately results

in DRG toxicity. To test this hypothesis and potentially develop an approach for eliminating the toxicity, we exploited expression of a sensory neuron-specific miRNA family that is largely restricted to DRG neurons and some sensory organs. We introduced sequence targets for those miRNA into the vector genome within the 3' untranslated region of the corresponding transgene mRNA. After screening several miRNA targets in mice, we selected one with very specific silencing activity in DRG neurons with preserved or enhanced expression in other parts of the CNS and peripheral organs. We injected vectors with our top candidate miRNA targets into the cisterna magna of NHPs in the context of two different transgenes. Inclusion of miRNA targets in the vectors reduced transgene expression in, and toxicity of, neurons within DRGs without affecting transduction elsewhere in the brain. This approach should help reduce DRG toxicity of any CNS-directed AAV gene therapy.

491. One-Time Intrathecal Administration of AAV5-miATXN3 in Non-Human Primates

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uniQure, Amsterdam, Netherlands

Spinocerebellar ataxia type 3 (SCA3), or Machado-Joseph disease (MJD), is a fatal neurodegenerative disorder characterized by spinopontine and cerebellar atrophy. Clinical manifestations predominantly include progressive gait ataxia with the involvement of cranial nerves. The cause of the disease is an expansion of a CAG trinucleotide repeat in the ataxin-3 gene (*ATXN3*), which results in the accumulation of aberrant, toxic ataxin-3 protein in brain regions located in the posterior fossa. Lowering the expression of the causative *ATXN3* gene should result in alleviation of mutant protein toxicity. Using the proprietary, next-generation miQURE™ technology, an artificial therapeutic microRNA was engineered to target the *ATXN3* gene and packaged into adeno-associated viral (AAV) vector type 5 (AAV5-miATXN3). Previously, we have shown that AAV5-miATXN3 significantly lowers ATXN3 in human induced pluripotent stem cell (iPSC)-derived neurons and that intracisternal administration resulted in strong mutant ATXN3 protein knockdown in the primary sites of SCA3 neuropathology in SCA3 mice. One of the challenges of mice as a model of neurodegenerative diseases is their small brain size and short spinal cord, making successful translation to the SCA3 patient difficult. Therefore, we have further investigated the distribution, expression, and tolerability of AAV5-miATXN3 in non-human primates (NHPs). Six NHP (*Macaca fascicularis*) were injected intrathecally in the cisterna magna and lumbar region with AAV5-miATXN3 (n=3) and control AAV5-miCTRL (n=3). At necropsy, 8 weeks after dosing, samples were taken from different parts of the brain and spinal cord to measure vector genome copies and ATXN3-microRNA expression. Intrathecal administration of AAV5-miATXN3 resulted in widespread transduction of brain and spinal cord, with the highest genome copies found in the posterior fossa and cortical regions. The processed ATXN3-microRNA was expressed in all brain regions and correlated with vector genome copies. Using in-situ hybridization we could confirm the presence of high quantities of mature miATXN3 microRNA molecules in the NHP brain. Currently, we are developing assays to measure wild-type ataxin-3 RNA and protein in order to assess ataxin-3 lowering in NHPs. The combination of widespread vector distribution and ATXN3-microRNA expression after one-time intrathecal administration of AAV5-miATXN3 in non-human primates supports the continuation of the development of the miQURE™-based ataxin-3-lowering gene therapy for SCA3 and translation into the clinic.

492. Abstract Withdrawn

493. Intravenous AAV Gene Therapy Improves Lifespan and Clinical Metrics in Feline Sandhoff Disease

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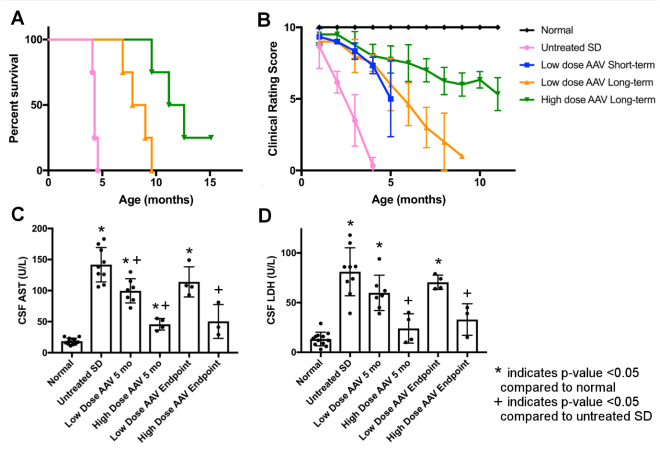
Sandhoff Disease (SD) is a fatal lysosomal storage disease that causes progressive neurodegeneration and death of severely affected children before 5 years of age. There are currently no FDA-approved treatment options, and the only available therapies are palliative. This autosomal recessive disease, a type of GM2 gangliosidosis, results in the dysfunction of the enzyme ß-hexosaminidase (Hex) and the subsequent accumulation of GM2 ganglioside in neuronal lysosomes. In previous studies in a feline model of infantile SD, intracranial administration of adeno-associated viral (AAV) vector quadrupled lifespan and increased quality of life. This success, in part, led to AAV treatment of 2 children with GM2 gangliosidosis as part of an expanded access clinical trial at the University of Massachusetts Medical School. In an attempt to reduce the risk of intracranial surgery and achieve greater systemic vector distribution, we treated 11 SD cats intravenously (IV) at one month of age with a bicistronic AAV9 vector expressing both subunits of Hex. These cats were divided into three groups: low dose (5e13/kg) short-term (n=3), low dose long-term (n=4), and high dose (2e14/kg) long-term (n=4). Animals in the short-term group were euthanized 16 weeks post-treatment, while cats in the long-term group were followed to a predetermined humane endpoint (inability to stand). While untreated SD cats live to 4.3±0.2 months, cats treated with the low and high doses lived to 8.3±1.2 and 11.1±1.5 months, respectively, with one cat in the high dose group currently alive at 15.1 months (Fig 1A). In-life assessments revealed clear clinical benefit of AAV treatment, with the most dramatic improvement seen in the reduction of tremors, the most debilitating feature of feline SD. While untreated SD cats quickly develop overt full-body tremors that interfere with their ability to stand, most AAV-treated cats only displayed fine resting tremors, if any, that stabilized early in disease progression. Fine tremors progressed to full-body tremors by the humane endpoint in 3 of 4 cats treated with the low dose but none of the cats treated with the high dose. Treated animals generally reached the predetermined humane endpoint through severe limb paresis due to spinal cord compression by proliferative tissue within the vertebral column. Bimonthly neurological examinations demonstrated markedly improved quality of life in AAV-treated animals, as indicated by scores on a clinical rating scale (Fig 1B). Cerebrospinal fluid (CSF) levels of aspartate aminotransferase (AST)

and lactate dehydrogenase (LDH) were decreased both at 16 weeks post-treatment and at long-term endpoints (Fig 1C & D), indicating a reduction of cell damage within the central nervous system. These results support the efficacy of IV delivery of a bicistronic AAV vector, especially at a high dose, for improving lifespan and quality of life in a feline model of SD.



494. Translating Gene Therapy for Spinocerebellar Ataxia Type 1: Unforeseen Consequences in Higher Mammals

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Spinocerebellar ataxia type 1 (SCA1) is one of several polyglutamine expansion diseases and is characterized by cerebellar ataxia and neuronal degeneration in the cerebellum and brainstem. Currently, there are no effective treatments. Previously, we showed that RNA interference (RNAi)-mediated silencing of *ataxin-1* mRNA (miS1) delivered by adeno-associated virus 1 (AAV1) provides therapeutic benefit in SCA1 mouse models, preventing or reversing motor phenotypes, neuropathology, and transcriptional changes in pre-symptomatic or symptomatic animals, respectively. Testing this approach in non-human primates is the next step toward the clinic. We used mixed gender rhesus macaque monkeys, randomized to receive buffer-control or AAV1.miS1 at escalating doses. To simulate a clinical setting, ClearPoint®, a real-time MRI compatible neurosurgical interface was used for all surgical interventions. Investigators were blinded to treatments throughout the study. Animals were monitored daily for signs of neurological changes or other abnormal behavior. Physical examinations, weights, and neurological rating examinations were given at regular intervals throughout the GLP study. Surgical intervention was refined and optimized to deliver AAV to the deep cerebellar nuclei of NHPs. Animals injected with all doses of AAV1.miS1, but not buffer-control, showed unexpected neurological symptoms initiating at 30 to 100 days post-intervention. Presentation included tremor, dysmetria, head-tilt, and ataxia. Prior to euthanasia, MRI analyses demonstrated significant changes in cerebella of animals that received AAV1.miS1, consistent with toxicity. Post-mortem analyses including immunohistopathology, QRTPCR, and NOVAseq were performed to assess the mechanism of toxicity. Preliminary results show cerebellar neuronal loss and increased microgliosis and astrogliosis following the pattern of vector distribution. This unforeseen toxicity of a therapeutic agent that was beneficial in mouse models of SCA1 at comparable doses indicates the presence of important differences in potential toxicity between species, highlighting the importance of using higher mammals for IND-enabling safety studies for this therapeutic approach. Further studies are underway to identify the underlying mechanism of toxicity, and modifications needed to optimize the safety profile of RNAi for SCA therapy.

495. Effect of Administration Route and AAV Serotype for Treatment of Feline GM1 Gangliosidosis

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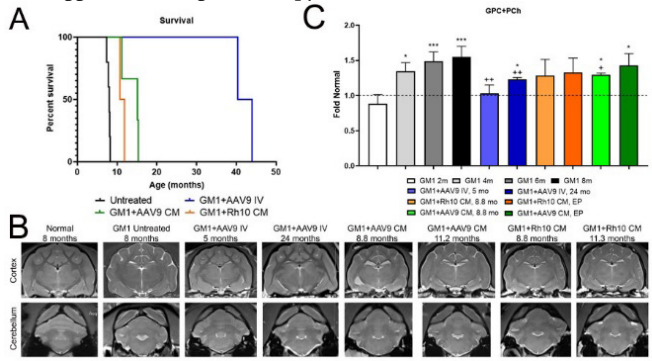
GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (βgal). GM1 animals are effective models for studying gene therapy since the therapeutic vector can also act as a reporter construct. Cerebrospinal fluid (CSF) administration of adeno-associated viral (AAV) therapy is hypothesized to be an effective method for treating neurodegenerative diseases. In this study, we evaluated two serotypes (AAV9 and AAVrh10) using CSF delivery via the cisterna magna (CM). Additionally, we compared these results to intravenous administration of AAV9. All treatment cohorts received 1.5e13 vector genomes/kg body weight at 1.8 ± 0.5 months of age. Untreated GM1 animals survived 8.0 ± 0.6 months while treated animals lived significantly longer (Figure 1A). GM1+AAV9 CM (N=3) animals survived 13.9 ± 1.8 months, GM1+AAVrh10 CM (N=2) animals survived 11.3 ± 0.5 months, and GM1+AAV9 IV (N=2) animals survived 42.1 ± 1.8 months. Clinical assessments included neurological exams, CSF biomarkers, and 7T magnetic resonance imaging (MRI) and spectroscopy (MRS). Postmortem analysis included βgal and virus distribution. Neurological abnormalities, which in untreated GM1 animals lead to an inability to stand by 8 months of age, were delayed but not halted in both CM treated cohorts and all animals became blind as their disease progressed. The IV treated cohort had mild neurological symptoms, similar to those seen early in disease stages, but no further impairments. Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), biomarkers of central nervous system damage when measured in the CSF, were both reduced in all treatment cohorts. MRI revealed delayed progression of neurodegeneration in both CM cohorts and preservation of brain architecture in the GM1+AAV9 IV

cohort (Figure 1B). Glycerophosphocholine and phosphoscholine (GPC+PCh), a MRS biomarker that increases with loss of myelin integrity, showed no correction in the GM1+AAVrh10 cohort, correction only in the cerebellum of the GM1+AAV9 CM cohort, and correction in several brain locations of the IV cohort (Figure 1C). In the CM cohorts, βgal activity was restored in the cerebellum and spinal cord but did not penetrate deep brain structures (such as thalamus). The GM1+AAV9 IV cohorts had increased βgal activity throughout the CNS. All cohorts had some degree of βgal restoration in the heart, liver, and skeletal muscle, and the IV cohort also had normalized levels in the sciatic nerve. Using a similar vector backbone and same total dose, this study demonstrates AAV efficacy in all treatment cohorts and suggests that IV gene therapy is most effective in the feline model.



Musculo-skeletal Diseases I

496. Progress Toward Translating AAV. CRISPR-Cas13 Therapy for FSHD

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Facioscapulohumeral Muscular Dystrophy (FSHD) is among the most prevalent muscular dystrophies, ranging from 1 in 8,333 to 1 in 20,000. FSHD is typically characterized by slowly progressive muscle wasting involving the face, shoulder girdle, and upper arms. In some FSHD cases, involvement of other muscles, like those of the lower limb girdle and abdominals, can lead to wheelchair dependence and reliance on caregiver assistance. Despite significant efforts to develop therapies for this disease, there is currently no treatment that alters the course of FSHD, and therapy development is still an unmet need in the field. *DUX4* de-repression is the main culprit of FSHD. Since DUX4 protein is toxic to muscle, any treatment that could reduce *DUX4* gene expression would likely prevent disease progression and ameliorate disease symptoms. Toward this goal, we developed an AAV6.CRISPR-Cas13 therapy for silencing toxic *DUX4* expression in muscles of our FSHD mouse model. Unlike the more broadly utilized Cas9 protein, the Cas13 enzyme does not have DNase activity, but instead cleaves RNA when directed to a target transcript by a sequence-specific guide RNA (gRNA). As a result, this system avoids potential risks of permanent off-target genome editing inherent with DNA-targeting systems. Therefore, we hypothesized that CRISPR-Cas13 could be a powerful method for silencing DUX4 at the mRNA level. To demonstrate this, Cas13 and our lead-candidate gRNA, which markedly decreased *DUX4* expression in FSHD myoblasts/myotubes and prevented cell death *in vitro*, were packaged into separate AAV6 particles. After performing safety and dose escalation studies in C57BL/6 mouse muscles, two different doses, 5E10 vgc of each AAV vector or 3E10 vgc of AAV6.Cas13 and 7E10 vgc of scAAV6.U6-gRNA, were co-injected into adult and neonatal FSHD mice via intramuscular injection. RNAscope and qRT-PCR results to detect DUX4 mRNA demonstrated reduction in *DUX4* expression in treated mice, corresponding with improved histopathological outcomes. To investigate possible off-target effects of our CRISPR-Cas13 system, we performed RNA-seq analysis of treated versus untreated human myoblasts. As a next step, we are attempting to increase the *DUX4* silencing ability of our AAV.CRISPR-Cas13 system for *in vivo* applications by optimizing the gene expression cassette, the AAV serotype, and route of administration in our mouse model. This study provides proof-of-principle for using CRISPR-Cas13 gene therapy as a novel strategy to treat FSHD through DUX4 mRNA inhibition and set the stage to apply this approach to other genetic diseases.

497. CRISPR-Mediated Gene Correction in a Severe Humanized Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe, highly debilitating and incurable muscle disease. DMD is caused by an X-linked recessive mutation in the dystrophin gene, which encodes a vital structural protein in the muscle membrane. Loss of dystrophin causes muscle deterioration, weakness, and premature death. Previous gene replacement strategies utilizing miniaturized dystrophin transgenes delivered via recombinant adeno-associated viruses (rAAVs) have been successful in ameliorating dystrophic pathology to create a Becker muscular dystrophy (BMD)-like phenotype. Still, accelerated muscle turnover, an immune response to the foreign dystrophin protein, and packaging constraints of rAAVs are significant barriers to achieving maximal therapeutic efficacy for DMD. CRISPR/Cas9 is a promising strategy for genome editing and allows permanent, targeted excision of defective dystrophin exons. Previously, our laboratory has utilized CRISPR/Cas9 in DMD patient myoblasts and in dystrophic mice via AAV delivery to excise exons and produce a shorter, yet functional protein. CRISPR treatment also improved skeletal muscle function and respiratory pathology in *mdx* mice and our humanized mouse model (hDMDΔ52/*mdx*) of DMD, respectively. However, currently available mouse models do not adequately reflect the severe muscle pathology observed in the clinic that could impact the persistence of CRISPR-mediated correction. Furthermore, no attempts have been made to compare gene editing versus gene replacement approaches in the context of DMD, where constant muscle turnover can lead to loss of the episomal AAV genome. To explore the efficacy of these strategies in a preclinical model that better recapitulates human

genetics and pathology, we have developed a humanized mouse model (hDMDΔ52/*mdx*/Utrn KO mice), which demonstrates an exacerbated dystrophic phenotype, shortened lifespan, as well as significant motor and respiratory deficits compared to our previous model. Neonatal injection of hDMDΔ52/*mdx*/Utrn KO mice with AAV-CRISPR targeting exon 51 successfully restored dystrophin expression in skeletal and cardiac muscle, and motor function analyses are ongoing. We will also assess the maintenance of CRISPR-mediated correction versus microdystrophin overexpression after AAV delivery and evaluate functional benefit over time in a cohort of mice treated as adults. Overall, this study will provide insight into remaining concerns surrounding therapeutic efficacy and persistence of CRISPR-mediated approaches for treating DMD.

498. A New Laminin a2-Based Gene Therapy for Congenital Muscular Dystrophy 1A

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Although gene therapy using recombinant Adeno-Associated Virus (rAAV) has great potential to treat genetic disorders, the rAAV packaging limit prevents its use in delivering large genes such as *LAMA2*. The *LAMA2* gene encodes the laminin α2 protein, which in concert with laminin β1 and γ1, comprises laminin 211, the predominant laminin in the muscle extracellular matrix (ECM). Laminin 211 stabilizes skeletal muscle in part by binding membrane proteins, including integrin α7β1 and dystroglycan, via its laminin α2 G1-G5 domains. Mutations in *LAMA2* cause Congenital Muscular Dystrophy 1A (MDC1A), a severe muscle disease involving progressive muscle weakness, failure or loss of ambulation, and premature death due to respiratory weakness. There are currently no FDA-approved treatments for MDC1A that modify disease outcome. Our goal was to develop a novel gene therapy for MDC1A using the laminin α2 G1-G5 domains. We used rAAV9 and the constitutive cytomegalovirus (CMV) promoter to express two secreted (s) forms of recombinant laminin α2 G1-G5: G1-G5 (sLAMA2 G) and G1-G5 fused to a heparin-binding (HB) domain (sHB-LAMA2 G). We derived the HB domain from HB-EGF, a trophic factor known to tightly bind skeletal muscle ECM. We hypothesized that rAAV-mediated expression of sHB-LAMA2 G, with its enhanced ECM binding, would be more effective than sLAMA2 G in restoring myofiber-ECM connections, thereby improving muscle strength and reducing muscle pathology in the laminin α2-deficient dy^W mouse model of MDC1A. We injected equivalent doses of rAAV9 containing sLAMA2 G or sHB-LAMA2 G intravenously into newborn dy^W mice and assessed muscle function and muscle pathology at 3 or 4 months of age. While dy^W mice expressing sLAMA2 G had no improvement in body mass-normalized forelimb grip-strength (2.8 ± 0.1 g/g, n=7), dy^W mice expressing sHB-LAMA2 G had significantly greater grip-strength (3.4 ± 0.2 g/g, n=6 mice) relative to untreated dy^W mice (2.7 ± 0.1 g/g, n=12 mice, p = 0.025). This improvement led to grip strength that was comparable to wild-type mice (3.5 ± 0.1 g/g, n=12 mice) at 3 months of age. In dy^W triceps muscles, sLAMA2 G was only expressed in 10.6 ± 3.2% of myofibers, while sHB-LAMA2 G was expressed in 36.3 ± 3.8% of myofibers (n=9 muscles/group). This difference occurred despite there being roughly equivalent levels of AAV viral genomes and transgene expression in muscles of both groups. In dy^W triceps muscles, sHB-LAMA2 G-expressing myofiber diameters averaged 41.6 ± 1.3μm, while non-expressing myofibers averaged 30.0 ± 2.1 μm (n=9 muscles/group, $p < 0.0001$). The percentage of centrally nucleated myofibers, a marker of muscle degeneration/regeneration, was lower in sHB-LAMA2 G-expressing myofibers (21.4 ± 2.1%) than in non-expressing myofibers (36.82 ± 9.25%, n=9 muscles/group, p = 0.0008). In triceps muscles of mice expressing sLAMA2 G, average myofiber diameter was also significantly greater in expressing myofibers than in non-expressing myofibers (43.8 ± 3.2 μm vs. 31.5 ± 2.7μm, n=9 muscles/group, p = 0.0116), and the percentage of central nucleated myofibers (15.3 ± 2.3%) was significantly less than in non-expressing myofibers (24.8 ± 1.6%, n=9, p = 0.0049). In general, there were not significant improvements in overall muscle pathology between dy^W mice receiving either treatment unless expressing fibers were considered as a separate group due to relatively low and variable protein expression levels. These studies suggest that one can express recombinant G1-G5 functional domains of laminin α2 using AAV in muscle in a manner that may impact muscle disease, and that this approach may be improved by engineering in ECM-binding moieties into the recombinant laminin protein.

499. AAV.CAPN3 Systemic Gene Therapy Improves the Phenotype in the Calpain3-Null Mouse Model for LGMD2A

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Mutations in calpain3 gene (*CAPN3*) lead to one of the most common limb-girdle muscular dystrophies, type 2A (LGMD2A). There is no treatment for this inherited disease. Evidence indicates CAPN3 loss of function is pathogenic and can be repaired by CAPN3 gene transfer. In this study rAAVrh74, with a tMCK promoter restricting expression of CAPN3 to skeletal muscle was used for systemic delivery. Vector was delivered to 21-23 week-old CAPN3 KO mice via tail vein at low dose (LD; 3E12 vg, n=12) and high dose (HD; 6E12 vg, n=12). Treatment efficacy was tested at 20 weeks post-gene delivery using functional, physiological and histopathological outcomes. Age-matched (n=15) CAPN3 KO mice injected with Ringer's lactate served as controls. Gene Therapy at LD and HD improved treadmill compared to control group with no gender difference ($p<0.00001$). There was no statistical difference between the treated cohorts, although females (n=5) at HD performed significantly better than males (n=7; p=0.006). Using succinic dehydrogenase stain for fiber typing, females in both cohorts showed a switch to fatigue resistant oxidative fiber types; a percent increase of slow twitch oxidative (STO) and fast twitch oxidative (FTO) fibers were seen while fast twitch glycolytic (FTG) fibers significantly decreased (p=0.003, LD; 0.014, HD). Fiber diameters increased in all fiber types in both treatment groups for both genders, however the

total fiber size increase at HD significantly favored males, which was most prominent for STO (29.4% vs. 8.8%), followed by FTO (24.5% vs. 7.6%) fibers. Varying degrees of heel cord contractures were seen as part of the aged CAPN3 KO phenotype in the untreated and LD treatment cohorts, only in the males; HD treatment rescued this phenotype. Taking this variability into consideration, we compared the combined treatment groups to the untreated cohort for the analysis of *in vivo* muscle contractility data which is obtained by stimulating the tibialis nerve and recording from the gastrocnemius. Both Twitch Max ($p= 0.0010$) and Tetanic Max responses ($p= 0.047$) improved significantly in the treated mice. Vector copy numbers and CAPN3 expression levels showed variability in various muscles in a dose dependent manner. In western blots, 94 kDa CAPN3 protein bands in the quadriceps and gastroc muscles with highest CAPN3 expression levels were also variable and did not correlate with RNA levels. The total RNA levels from all 4 muscles showed a linear regression with run to exhaustion test in the LD ($r^2=0.52$, $p= 0.0082$) while in the HD cohort no correlation was seen, and the slopes were significantly different between two groups ($p=0.02$). The mice with high performance in the LD overlapped with best-performers of the HD suggesting that mRNA levels exceeding a threshold might not further improve the performance. In-house toxicology studies in the HD cohort revealed no organ tissue abnormalities; specifically, there was no histopathological evidence of cardiotoxicity. Our studies show that systemic administration of AAVrh74.tMCK.hCAPN3 in the LGMD2A mouse model significantly improved the functional, physiological and histopathological parameters in LD and HD without cardiotoxicity. The improvements in the treadmill test correlated with remodeling of muscle, a switch to fatigue resistant oxidative fibers in females along with fiber size increase and a significant increase in fiber diameter, was most prominent in STO fibers in males. These translational studies are relevant for consideration of viral dose in clinical trials; gender differences may also have implications for LGMD2A.

500. SGT-001 Microdystrophin Gene Therapy for Duchenne Muscular Dystrophy

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SGT-001 is an adeno-associated virus (AAV) microdystrophin gene therapy in development for the treatment of Duchenne muscular dystrophy (DMD). It is intended to deliver a functional surrogate for the protein absent in DMD, dystrophin, in order to restore muscle stability, associated molecular signaling pathways, and overall function. SGT-001 delivers a five-repeat microdystrophin transgene, termed μDys5, which includes the classically described essential domains of dystrophin and also uniquely includes the more recently identified critical R16/R17 neuronal nitric oxide synthase (nNOS) binding domain. Expression of the nNOS binding domain has been shown to be required for localization of the nNOS protein to the muscle membrane where it is able to properly function to regulate local vasodilation by the production of the paracrine signaling molecule nitric oxide (NO). As a result, the inclusion of the nNOS binding domain in SGT-001 microdystrophin may potentially provide important additional functional benefits such as diminished muscle fatigue and protection against ischemic muscle damage. SGT-001 has been evaluated extensively in pre-clinical studies and shown to result in dose-dependent increases in microdystrophin protein levels in muscle tissues and proper molecular function of the protein in localizing to the muscle membrane and recruiting critical dystrophin associated proteins such as β-sarcoglycan and nNOS. These molecular functional improvements correspond with dose-dependent improvements in muscle function, including increased muscle force and endurance. Clinical evaluation of SGT-001 in the IGNITE DMD trial at 5E13 vg/kg and 2E14 vg/kg dose levels has demonstrated microdystrophin expression in a dose-responsive manner, with associated stabilization and co-localization of dystrophin associated proteins such as β-sarcoglycan and nNOS at the muscle membrane. The levels of serum creatine kinase (CK), a biochemical marker of muscle damage, declined from baseline in patients in the 2E14 vg/kg cohort. Together, these data provide evidence supporting the biological activity of SGT-001 and continued clinical evaluation for the treatment of DMD patients. Recently announced biomarker data will be discussed in this presentation.

501. AAV PGC1-Alpha Gene Therapy Improves the Cardiac Function in a Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked disease due to mutations in the dystrophin gene. Absence of dystrophin results in loss of ambulation and death from respiratory and/or heart failure. Dystrophin deficiency increases the susceptibility of the sarcolemma to contraction-induced damage, leading to an influx of ions and disruption of cellular organelles like sarcoplasmic reticulum and mitochondria. Mitochondrial dysfunction in DMD contributes to reduced energy metabolism in DMD. PGC1-alpha is a major regulator of mitochondrial biogenesis and oxidative metabolism in muscle. We hypothesize that overexpression of PGC1-alpha in DMD heart by gene therapy can improve mitochondrial biogenesis and oxidative metabolism in cardiomyocytes, hence, attenuate heart disease in DMD. To test our hypothesis, we delivered 1.85E13 viral genome particles of PGC1-alpha adeno-associated virus (AAV) vector intravenously to 12-m-old female mdx mice, an authentic DMD cardiomyopathy model. Four months after injection, we detected significant improvement of left ventricular hemodynamic function in treated, but not untreated mice. AAV PGC1-alpha therapy also increased the expression of PGC1-alpha target proteins that are involved in oxidative metabolism, mitochondrial biogenesis, fatty acid oxidation, and mitochondrial homeostasis. These findings confirmed our initial hypothesis and suggest that AAV PGC1-alpha gene therapy may hold promise to treat DMD heart disease (Supported by Jackson Freel DMD Research Fund).

502. Assessment of Immunomodulatory Regimens that Allow Systemic Redosing of an AAV-CRISPR/Cas9 Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a fatal muscle wasting disease typically caused by out-of-frame mutations in *DMD,* which leads to lack of the dystrophin protein. However, in-frame *DMD* mutations typically lead to the milder Becker muscular dystrophy (BMD) where a partially functional dystrophin protein is produced. Due to this association, the idea to reframe the *DMD* gene, thus converting a Duchenne mutation into a Becker mutation, is a therapeutic strategy for DMD. One way in which the *DMD* gene could be reframed is by employing the CRISPR/Cas gene editing system. We and others have demonstrated effective restoration of dystrophin after delivery of CRISPR to muscle using adeno-associated virus (AAV) in preclinical models of DMD. However, without efficient muscle stem cell targeting, muscle turnover and growth may dilute out the reframed nuclei and thus, being able to redose AAV-CRISPR or other gene therapies would be highly advantageous. Repeat dosing of AAV is impossible without additional procedures such as immune suppression because exposure to AAV can lead to both humoral and cellular immune responses against the capsid. Previous studies have demonstrated that repeat delivery of AAV in mice, primates, and now a human clinical trial can be accomplished if immunosuppression is used. However, those studies did not systematically determine the essential immunosuppression components and whether those same regimens would be successful using CRISPR as the AAV cargo. Lastly, the human trial has only used intramuscular and not systemic administration of AAV. Here we describe a pilot study to optimize an immunosuppression regimen for systemic repeat dosing of AAV-CRISPR. To determine the essential immunomodulatory components required for AAV redosing, we systematically removed one immunosuppressant drug at a time and assessed the expression of two different AAV injections in our humanized hDMD del45 mdx mouse model. We anticipate this will lead to improved efficacy and safety for use with AAV-CRISPR in DMD.

AAV Vectors - Clinical Studies

503. Systemic Gene Transfer with rAAVrh74.MHCK7.SGCB Increased Beta-Sarcoglycan Expression in Patients with LGMD Type 2E

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Background: Limb girdle muscular dystrophies (LGMD) usually manifest with progressive hip/shoulder muscle weakness extending to other muscles. LGMD2E (due to β-sarcoglycan [SGCB] deficiency) includes cardiac involvement and elevated creatine kinase (CK). We present initial findings of an ongoing, phase 1 multiple ascending-dose clinical gene transfer trial of ≤9 patients with LGMD2E who received rAAVrh74.MHCK7.SGCB (NCT03652259). **Methods:** Participants were patients 4-15 y with confirmed SGCB mutation (both alleles), negative for antibodies against rAAVrh74, and >40%n on 100-meter timed test. Patients received single IV infusion of $5x10^{13}$ vg/kg rAAVrh74.MHCK7.SGCB. Another cohort of patients in the study have been dosed at a higher dose. Prednisone 1 mg/kg/day was initiated 1 day before gene delivery, tapering after 30 days. Primary endpoints were ≥20% SGCB-positive fibers (Day 60 muscle biopsy) and safety (Day 270). Secondary endpoints were CK decrease and functional endpoints (Day 270). **Results:** For the first 3 patients enrolled (age 13, n=2; age 4; n=1), robust SGCB expression was observed by immunohistochemistry (IHC), with a mean of 51% SGCB positive fibers (range 42-63%) expressing a mean 47% intensity (range 38-57%). Co-localization of α-sarcoglycan was observed by IHC. Western blot showed a mean 36.1% SGCB expression vs normal (range 34-39%). Mean CK levels were reduced by 82%, suggesting slowed muscle destruction. Two patients had elevated liver enzymes (1 serious) and 1 had elevated bilirubin following oral steroid taper, which subsequently returned to baseline. Two patients had transient mild nausea, corresponding with increased steroid dosing. No other clinically significant lab findings. **Conclusion:** Gene transfer in patients with LGMD2E following an infusion of rAAVrh74.MHCK7.SGCB was positive for the defined endpoints. This is the second gene therapy inducing protein production post transgene delivery with rAAVrh74 vector and MHCK7 promoter, demonstrating potential benefits of a rationally designed delivery system.

504. ADVM-022 Intravitreal Gene Therapy for Neovascular AMD - Results from the Phase 1 OPTIC Study

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Introduction: In neovascular AMD (nAMD), intravitreal gene therapy has the potential to markedly reduce the burden of anti-vascular endothelial growth factor (VEGF) treatment and improve vision in patients with the condition without surgery. OPTIC (NCT03748784) is an ongoing, Phase 1 open-label, multicenter, dose-ranging study in treatment-experienced patients with a confirmed response to anti-VEGF therapy. The study is assessing safety, tolerability and efficacy of a single intravitreal injection of ADVM-022, an AAV.7m8-aflibercept gene therapy, in patients with nAMD. The results presented here are from Cohort 1 of 4. **Methods:** Patients were administered a single intravitreal injection of ADVM-022 at 6x10^11vg/eye for Cohort 1 (n=6) and at 2x10^11vg/eye for Cohort 2 (n=6). Incidence and severity of adverse events, change in visual acuity (BCVA), anatomical outcomes on OCT, and number of aflibercept rescue injections were evaluated. **Results:** Immediately prior to ADVM-022, patients in Cohort 1

were maintaining good levels of BCVA (mean 65.8 ETDRS letters), but required frequent anti-VEGF injections to achieve this result (mean 9.2 injections over 12 months). Administration of a single intravitreal injection of ADVM-022 was followed by a median of 44 weeks follow-up (range 40, 52 weeks), during which there were no serious adverse events, no dose limiting toxicities and no non-ocular adverse events related to study drug. Ocular inflammation occurred chiefly in the anterior segment, and was both mild and manageable with topical steroids. Mean change in BCVA from baseline was maintained at -1.0 ETDRS letters (range -7,+7). OCT revealed improvements in retinal anatomy with signs of either resolved or stabilized exudation in all patients. No rescue injections were required for any patient. New data from the ongoing, open-label OPTIC study will be presented, including 52-week data from Cohort 1. **Conclusions:** Through a median of follow-up of 44 weeks, patients with nAMD treated with a single injection of ADVM-022 were able to maintain BCVA and improve retinal morphology without the need for any anti-VEGF rescue injections.

505. AAV8 Gene Therapy as a Potential Treatment in Adults with Late-Onset OTC Deficiency: Results from a Phase 1/2 Clinical Trial

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Introduction: Ornithine transcarbamylase (OTC) deficiency is an X-linked disorder resulting in impaired ammonia flux through the urea cycle and subsequent hyperammonemia. The current standard of care includes a proteinrestricted diet and nitrogen-scavenging agents, but there remains a high risk of hyperammonemic crises with irreversible neurocognitive damage. DTX301, an AAV8 vector containing the *OTC* transgene, is currently under investigation for OTC deficiency. **Methods:** CAPtivate is a global, multicenter, open-label phase 1/2 dose-escalation trial evaluating the safety and preliminary efficacy of DTX301 in adults with late-onset OTC deficiency. The primary endpoint is incidence of adverse events (AEs). Secondary endpoints are changes in the rate of ureagenesis and 24-hour plasma ammonia levels. **Results:** DTX301 dosing of three subjects in each cohort 1 (2 x 10^{12} Genome Copies [GC]/kg), cohort 2 (6 x 10^{12} GC/kg), and cohort 3 (1 x 10^{13} GC/kg) is complete. No infusion-related or treatment-related serious AEs were reported; all AEs were grade 1 or 2. Mild, transient, asymptomatic ALT increases in six subjects resolved with a per protocol tapering course of oral corticosteroids. After DTX301 administration, three subjects (one per cohort) had a complete response (CR) with a sustained improvement in the rate of ureagenesis and improvement in 24-hour ammonia levels; these subjects discontinued nitrogen-scavenging agents and liberalized dietary protein restrictions. In cohort 2, another subject began responding at week 52, that was confirmed at week 78; this subject is tapering nitrogen-scavenging agents and liberalizing dietary protein restrictions after a 218% increase in the rate of ureagenesis and a 74% decrease in ammonia levels. In cohort 3, a second subject is considered a responder after a 90% decrease in 24-hour ammonia levels through week 24; this subject has yet to cease nitrogen-scavenging agents and liberalize dietary protein restrictions. In cohort 3, a third subject is a potential responder through week 12, with a 123% increase in the rate of ureagenesis; more time is needed to further evaluate this subject. **Conclusions:** Data from CAPtivate indicate that DTX301 has an acceptable safety profile and may be a potential new therapy for patients with OTC deficiency. Followup of all subjects is ongoing and enrollment in cohort 4 (1 x 10^{13} GC/kg and prophylactic steroids) is planned.

506. Optogenetics in the Clinic: PIONEER, a Phase 1/2a Gene Therapy Program for Non-Syndromic Retinitis Pigmentosa

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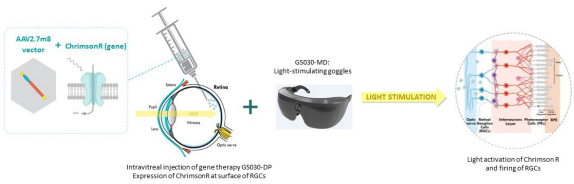
Background: Retinitis pigmentosa (RP) is an inherited retinal disease with over 100 known mutations. In late-stage RP, cone photoreceptors degenerate, causing loss of central visual acuity and eventually blindness. GS030 is an optogenetic treatment using a drug product (GS030-DP) with a medical device (GS030-MD) in clinical development to potentially restore vision function in RP patients independent of their underlying mutation. **Methods:** GS030-DP is an optogenetic gene therapy targeting retinal ganglion cells that encodes a lightsensitive channelrhodopsin, *ChrimsonRtdTomato (ChR-tdT),* delivered by an AAV2.7m8 vector and administered via intravitreal injection (IVT). GS030-DP are visual interface stimulating goggles that encode images of the visual world and modulate an amplifying light source projected onto the genetically engineered retina. PIONEER is a Phase 1/2a open-label study to evaluate the safety and tolerability of GS030 in subjects with end-stage non-syndromic RP. Three dose-escalation cohorts (5E10, 1.5E11, 5E11 viral genomes [vg]/eye) will include 3 subjects each, and an extension cohort will be treated at the highest tolerated dose. **Results:** Inclusion in the first cohort started in Q4 2018. Up to 1 year after treatment administration, there were no serious adverse events (SAEs) nor study discontinuations. The most common adverse events (AEs) were mild anterior chamber inflammation responsive to corticosteroid treatment, and mild sensitivity to light starting after drug administration but before light stimulation by the googles. The use of GS030MD to stimulate ChR-tdT was initiated two months after administration of gene therapy and showed no safety concerns, before or after IVT with GS030-DP. Two subjects of the second cohort were injected with 1.5E11 vg/eye in Q3 2019. No serious AEs and no unexpected AEs have been reported. **Conclusion:** PIONEER is the first clinical trial for RP combining the simultaneous action of a gene therapy and a medical device, a therapeutic approach independent of

underlying genetic defects. Treatment was well tolerated in the first cohort, and completion of the second cohort with an intermediate dose is currently ongoing. Treatment of the third cohort at the highest dose is planned in Q1 2020.

IMAGE - OPTOGENETICS THERAPY



507. Improving the Quantification of rSIV.F/HN Vectors for Cystic Fibrosis Gene Therapy Using Droplet Digital PCR

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We are developing viral and non-viral vectors for the treatment of a range of acquired and inherited respiratory disorders. We have previously demonstrated that a minimal, self-inactivating, replication-defective simian immunodeficiency virus (rSIV) vector, pseudotyped with the F and HN glycoproteins from Sendai virus (the murine form of human parainfluenza virus I) has high tropism for multiple cell types found within both the conducting airway epithelium and the gas exchanging alveolar regions of the mouse, sheep and human lung. To facilitate clinical development, we routinely produce a large number (multiple Lots/week; target >1e10 TU) of highly purified (anion-exchange/TFF), high-concentration (target >1e9 TU/mL) research-grade preparations of rSIV.F/HN vectors. Our animal-free process is centred on a rocking (WAVE, GE) bioreactor upstream production workflow that utilises suspension-adapted HEK293T cells. We typically use two titre assays to assess vector Lots and to normalise vector dose between studies: (i) a viral particle assay (reported as VP/mL) is derived from the concentration of viral RNA found within vector Lots and is evaluated using real-time qRT-PCR, (ii) a transducing assay (reported as TU/mL) is derived from the concentration of vector-derived DNA within cells transduced with dilutions of viral Lots and is evaluated using real-time qPCR. While both approaches are widely used, and accepted by multiple competent authorities, they can suffer from significant inter- and intra- assay variation. Furthermore, they are dependent on either standard curves of *in vitro* transcribed RNA or linearised DNA molecules, both of which (for optimal assay performance) need to be prepared in a matrix that mimics the ultimate analytes. Interestingly, droplet digital PCR (ddPCR) methods offer the opportunity to quantify nucleic acid targets using the robust three-primer chemistry utilised in the real-time qPCR approach without the need to prepare, qualify and maintain absolute standard curves. After defining optimal primer, target and amplification conditions, the detection range and lower limit of quantification (LLOQ) of our ddPCR assay was determined using a plasmid construct containing one copy of the *WPRE* target sequence. Under our assay conditions, we found the relationship between the amount of template and number of copies of the target gene was linear between 41 and 2897 copies (linear regression r^2 0.998, $p<0.001$) while the LLOQ was determined to be 31 copies. We subsequently compared the use of qPCR and ddPCR approaches as the final quantification step in our TU/mL assay. A dilution series of a laboratory reference rSIV.F/HN vector Lot was used to transduce suspension-adapted Gibco™ Viral Production Cells. Seventy two hours post-transduction, cellular DNA was extracted using our standard column-based approach (Qiagen 96 well DNeasy kit) and quantified for levels of both WPRE (rSIV.F/HN vector target) and CFTR (single-copy cellular refence gene) using both qPCR and ddPCR. The qPCR method indicated the LV product contained 7.29e8 TU/mL with a relative standard deviation (RSD) of 59% whereas the ddPCR method produced a more robust and repeatable value of 2.28e8 TU/mL (21% RSD). We are now extending the use of ddPCR to our VP/mL assay where the use of transcribed RNA standard for our qPCR approach is particularly time-consuming. Additionally, ddPCR appears to be more robust to variations in matrix composition allowing in-process samples to be analysed without extensive sample preparation - a potentially significant saving in time, effort and cost.

508. Engineering Viral Protein Ratios to Impact AAV Potency

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The baculovirus/insect cell system has proven to be a robust production method for the manufacture of adeno-associated virus (AAV) for gene therapy. Although the current baculovirus process is adequate for drug manufacturing, it has seen little optimization since it was first introduced to produce recombinant proteins. Recently there has also been evidence of decreased vector potency for some AAV serotypes. This decrease has been attributed to a non-wild type like ratio of viral proteins VP1:VP2:VP3 resulting in the reduction of virus infectivity. Our lab sought to assess the impact of VP stoichiometry on *in vivo* potency and *in vitro* expression of the transgene. Using a battery of molecular biology approaches that modify VP1 expression our lab has consistently shown detectable changes in VP stoichiometry. VP Ratios were quantified by CE-SDS, transduction efficiency was quantified by TCID50, and an Expression ELISA assay was used to monitor total amount of protein expressed by Lec2 cells transduced with sf9/bac material *in vitro*. *In vivo* potency and biodistribution were also conducted in mouse studies. Results demonstrated a clear correlation in VP expression and potency. It has provided insight into methods of engineering the AAV capsid for an improved product quality profile.

509. Phase 1 Study of Gene Therapy in Late-Onset Pompe Disease: Analyses of Safety and Secondary Endpoints

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Gene therapy could eliminate the need for enzyme replacement therapy (ERT) in Pompe disease by creating a liver depot for acid alpha-glucosidase (GAA) production, as demonstrated with an adeno-associated virus (AAV) serotype 8 vector containing a liver-specific promoter to drive human GAA expression (AAV8-LSPhGAA) that corrected GAA deficiency and cleared accumulated glycogen in the muscle of mice with Pompe disease. We report the initial analyses of safety and bioactivity through Week 26 for the first cohort (n=3) in an open-label, single-dose, dose-escalation 52-week study (NCT03533673) in patients with late-onset Pompe disease (LOPD) following a single intravenous infusion of AAV8-LSPhGAA. The primary study objective is to evaluate the safety of AAV8-LSPhGAA in adult subjects during concurrent treatment with ERT, as assessed by the incidence of adverse events (AEs), serious AEs (SAEs), and clinical laboratory abnormalities. Secondary objectives include evaluating the six minute walk test (6MWT), forced vital capacity (FVC), and muscle biopsy testing. Subjects had confirmed LOPD, no detectable anti-AAV8 neutralizing antibodies, >102 weeks treatment with ERT at a stable dose, and the ability to walk at least 100 meters on the 6MWT. The first cohort received a low dose of the vector and continued bi-weekly ERT, until withdrawal at Week 26. Withdrawal of ERT was based on the detection of sustained, quantifiable serum GAA activity from AAV8-LSPhGAA, and the absence of clinically significant declines in FVC or 6MWT performance. All three subjects enrolled in Cohort 1 met criteria for ERT withdrawal at 26 weeks. As of Week 26, there were no treatment-related SAEs, and laboratory assessments suggested acceptable safety of AAV8-LSPhGAA. A single subject experienced two mild to moderate severity treatment-related AEs consisting of headaches that resolved. Pulmonary function testing revealed that 2 of 3 subjects had increased FVC between screening and Week 24 (+4% predicted for each). The 6WMT demonstrated increased distance for 2 of 3 (+25 and +9 meters). Two validated endpoints for LOPD, the Quick Motor Function Test and Gait, Stairs, Gower, Chair Test, remained unchanged from the baseline visit at Week 24. Serum alanine aminotransferase was not elevated during the course of the study, suggesting a lack of T cell responses against the AAV8 capsid protein. Overall, these initial data support the safety, bioactivity, and continued clinical development of AAV8-LSPhGAA therapy in LOPD.

AAV Vectors - Virology and Vectorology II

510. A Reversible RNA On-Switch That Controls Expression of Aav-Delivered Transgenes In Vivo

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Widespread use of gene therapy technologies is limited in part by the lack of small genetic switches with wide dynamic ranges that control transgene expression without the requirement of additional protein component. Self-cleaving ribozyme-based RNA switches, with their small size and lack of dependence on exogenous proteins, make ideal regulators of therapeutic transgenes. In this study, by converting a previously optimized type I *Schistosoma mansoni* hammerhead ribozyme to a type III ribozyme, and sequentially optimizing the ribozyme's stem III, stem I and loop I sequences, we engineered a class of hammerhead ribozymes that are highly efficient in *cis*-cleaving mammalian mRNAs. We then developed efficient RNA on-switches by using octaguanidine dendrimer-conjugated *phosphorodiamidate morpholino oligonucleotide,* a steric-blocking antisense oligonucleotide (ASO), to tightly regulate these ribozyme's self-cleavage activity. This RNA switch system enables efficient and reversible regulation of adeno-associated virus (AAV)-delivered transgenes in vivo, allowing dose-dependent and up to 200-fold regulation of protein expression over at least 43 weeks in mice. To test the potential of these RNA switches in gene-therapy applications, we demonstrated regulated expression of physiological levels of erythropoietin (Epo) with a well-tolerated dose of the inducer oligonucleotide. These ASO-regulated, small, modular and efficient RNA switches have the potential to improve the safety and efficacy of gene therapies and broaden their use. A figure (Figure 1) is provided to serve as a graphical abstract for a better illustration of the study. A manuscript related to this study has been accepted for publication by *Nature Biotechnology* (Zhong G*, Wang H, He W, Li Y, Mou H, Tickner Z, Tran M, Ou T, Yin Y, Diao H, Farzan M*. A reversible RNA on-switch that controls gene expression of AAV-delivered therapeutics in vivo. *Nature Biotechnology*. 2019.).

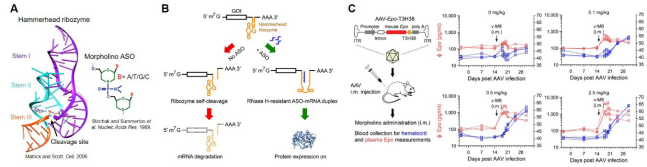

Figure 1. ASO-ribozyme switch-regulated expression of AAV transgenes in vivo. *(A) Structures of a Schistosoma mansoni hammerhead ribozyme and a phosphorodiamidate morpholino oligonucleotide. (B) Working model of the ASO-regulated RNA switch system. (C) Morpholino ASO dose-dependent regulation of AAV-expressed Epo in vivo.*

511. AAV Capsid-Promoter Interactions Determine CNS Cell Selective Gene Expression *In Vivo*

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Cell selective gene expression comprises a critical element of many AAV vector based gene therapies, and to date achieving this goal has focused upon AAV capsid engineering, cell specific promoters or cell specific enhancers. Recently, we discovered that the capsid of AAV9 exerts a differential influence on constitutive promoters of sufficient magnitude to alter cell type gene expression in the rat CNS. For AAV9 vectors CBA promoter driven gene expression exhibited a dominant neuronal gene expression in the rat striatum. Surprisingly, for otherwise identical AAV9 vectors the truncated CBh promoter shifted gene expression towards striatal oligodendrocytes. In contrast AAV2 vector gene expression was restricted to striatal neurons, regardless of the constitutive promoter used. Furthermore, a six glutamate residue insertion immediately after the VP2 start residue shifted CBA driven cellular gene expression from neurons to oligodendrocytes. Conversely, a six alanine insertion in the same AAV9 capsid region reversed the CBh mediated oligodendrocyte expression back to neurons without changing AAV9 capsid access to oligodendrocytes. Given the preponderance of AAV9 in ongoing clinical trials and AAV capsid engineering, this AAV9 capsid-promoter interaction reveals a previously unknown novel contribution to cell selective AAV mediated gene expression in the CNS.

512. The Genomic Architecture of a Genotoxic Recombinant AAV Integration in a Murine Hepatocellular Carcinoma

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There is a growing body of observational and experimental data suggesting that AAV integration, both after a wildtype AAV infection in humans, and following rAAV gene therapy in mice, can be associated with an increased risk to develop hepatocellular carcinoma (HCC). The earliest observations of HCC formation after rAAV gene therapy in mice by Donsante and Sands (PMID: 17656716) was confounded by the lack of detection of the rAAV transgene in the HCC, which made the claim of causation by rAAV controversial. Many subsequent studies, using various mouse models, AAV serotypes and vector transgenes, have continued to document HCC formation in mice after AAV exposure. In addition, there have also been several recent reports showing that sequences from wildtype AAV can participate in the formation of HCCs in humans when integrated into tumor suppressor genes or near genes involved in cellular growth. The combined observations from experimental models and human patient observations add urgency to understand the mechanism(s) by which AAV might exert genotoxic sequelae, given the widely accepted claim that AAVs are intrinsically nonpathogenic. Previous surveys of AAV integrations in HCCs, used either inverse PCR or a modified LAM-PCR, and were unable to recover both sides of clonal integrations, which has suggests these pathogenic events exhibit genomic complexity. In this study, we used whole genome sequencing (WGS) to interrogate the genomic structure of a genotoxic AAV integration in a mouse HCC that appeared after treatment with a canonical AAV8 vector designed to express the *MMut* gene under the control of the liver specific TBG promoter. After purification of high molecular weight DNA from the HCC and control tissue, WGS was accomplished using the 10X Genomics platform, with an alignment focused on reads that contained parts of the vector sequence to build a contig encompassing the entire integration event. Nested PCR with Sanger sequencing was used to confirm the WGS results. In the middle of a quadranucleotide repeat in *MiR341*, a complex and rearranged AAV derived structure 1042 base pairs (bp) in length, which lacked the therapeutic transgene, was present. At one end, a truncated 62bp ITR comprised of the D A' RBE sequences was juxtaposed to a cryptic AAV hepatic enhancer, the polyA sequence of the vector, and joined tail to head with another rearranged 95 bp ITR containing the D A'RBE C C' B RBE' sequences abutting by another copy of cryptic AAV hepatic enhancer, and followed by 624 bp of the TBG enhancer/promoter, which then rejoined the *MiR341* locus. The sequence of this vector derived insertion likely derived from a head-to-tail vector concatemer, that subsequently underwent multiple recombination events either during or after integration. Furthermore, the ITR sequences are unlikely to have allowed packaging, which supports the genesis of the rearrangement as an *in vivo* event. This AAV product appears to act as an enhancer of *Rtl1* and *MiRs*, which are proximal to the AAV integration, and promotes HCC formation by transactivation. WGS of AAV integrations in HCCs may reveal shared sequences which might be used to develop assays to detect formation of vector derived recombination products in model systems, or *in vivo*, to predict possible vector genotoxicity.

513. PESCA: A Scalable Platform for Regulatory Element Engineering of Viral Gene Therapy Vectors

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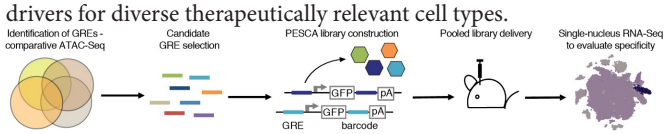
While recently coming of age with the first regulatory approvals, current generation virus-based gene therapeutics still largely lack specificity for the desired target organs and cell types, with payload expression often falling below therapeutically useful ranges. In this regard, the majority of such therapeutics currently under development employ one of a few historically chosen promoter elements to drive payload expression. It is now appreciated that short (300-500 bp) distal enhancer elements, not promoters, serve as the primary determinants of cell-type-specific gene expression. Moreover, recent advances in epigenomic profiling allow for the genome-wide identification of candidate enhancer elements in diverse cell types. To date, however, our ability to exploit these findings has been severely limited by current approaches for testing the expression of candidate cell-type-specific vectors, which remain laborious, expensive, and low-throughput, often relying on the production of individual viral vectors and the

assessment of expression across a limited number of cell types using in situ hybridization- or immunofluorescence-based methods. To address these obstacles, we recently developed the Paralleled Enhancer Single-Cell Assay (PESCA), a scalable strategy for the generation of cell-type-restricted viral drivers. In this method, comparative epigenomic profiling between cell types in the target tissue is used to generate a library of gene regulatory elements (GREs) enriched for highly cell-type-restricted enhancers. Following viral packaging, the resulting barcoded GRE library is screened *en masse* for cell-type-specific activity *in vivo* via single-cell RNA sequencing, with the expression of each constituent GRE evaluated through the use of an orthogonal cell-indexed system of transcript barcoding across tens of thousands of individual cells in the target tissue. The resulting datasets thus reveal the specificity of enhancer-driven payload expression across the full complement of cell types present in the target tissue. Having previously validated the approach by identifying GREs that confine AAV payload expression to somatostatin (SST)-expressing interneurons in the highly heterogeneous murine primary visual cortex, we are currently employing PESCA to restrict viral payload expression to a series of therapeutically relevant target cell populations, including multiple neuronal and non-neuronal cell types in the central nervous system and dorsal root ganglia. The resulting vectors have potential applications in the treatment of Spinal muscular atrophy (SMA) and other neurodegenerative conditions, chronic pain, and congenital hearing loss, and should further establish PESCA as a broadly useful platform for the generation of recombinant cell-type-specific viral drivers for diverse therapeutically relevant cell types.



514. Tailored AAV-Based Transgene Expression in the Inner Ear with Cell Type-Specific Promoters

Adam Palermo[1], Martin Schwander[1], Xudong Wu[1], Shu-Lin Liu[1], Sarah Cancelarich[2], Xichun Zhang[1], Gabriela Pregernig[1], Kathryn Ellis[1], Fuxin Shi[1], Kathy So[1], Leah Sabin[2], Meghan Drummond[2], Ning Pan[1], Joseph Burns[1], Jonathon Whitton[1]

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Expression of transgenes at the appropriate place and time can be important for the safety and efficacy of gene therapies. Unfortunately, few cochlear cell type-specific promoters have been reported that are small enough to fit within the limited packaging capacity of adeno-associated virus (AAV). In this study, we used single-cell RNAseq and single-cell ATACseq data to identify promoters that are both specific to cochlear cells and small enough to fit into AAVs. We first identified a promoter capable of driving expression of GFP in both inner and outer hair cells. This expression pattern was consistent when AAV was delivered to cochlear explants, mouse cochleae in vivo, and primate cochleae. In the context of gene therapy with TMC1, this hair cell-specific promoter was able to support better recovery than ubiquitous promoters. Similarly, when used to drive gene therapy with OTOF, more durable phenotypic rescue was observed with the hair cell-specific promoter. We next designed promoters targeting transgene expression to either inner hair cells or outer hair cells. Here, we combined sequence-driven regulatory region analysis, single-cell RNA sequencing, and single-cell ATACseq data to identify putative expression control elements. With this approach, we were able to identify several outer hair-cell specific promoters and one inner hair cell-specific promoter

515. Structure-Guided Rational Design of Adeno-Associated Viral Capsids with Expanded Sizes

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The application of recombinant Adeno-Associated Virus (rAAV) vectors as gene delivery vehicles has been limited by the modest packaging capacity (5.2kb) of rAAV. This limitation is imposed by the capsid size of 25nm diameter, which is determined by the capsid's T=1 icosahedral geometry. Current methods for rAAV delivery of oversized cargo, typically involving co-delivery of dual vectors, suffer from challenges such as low efficiency in co-infection and reassembly of full-length products, indefinite ratios of the two vectors in individual cells, and necessity of re-design and re-validation for every new cargo. Inspired by the size polymorphism observed in natural viruses, we hypothesized that the T=1 icosahedral geometry of AAV capsids could be changed so that each capsid is built from more than 60 subunits. Here we present two rational design strategies that lead to eXtra Large AAV capsids (XL-AAVs). These strategies involve modifying the inter-subunit interactions and the assembly pathway of AAV capsids. Capsids engineered through both strategies form heterogeneous, 35nm-70nm spherical particles (Figure 1). The XL-AAV capsids can still package rAAV genomes, and the same protein design principles can be applied across multiple serotypes we tested (including AAV9, AAV2, AAV5, and AAVDJ). Ongoing work seeks to determine whether the XL-AAV capsids can fully protect and deliver conventionally oversized rAAV genomes. These design principles and the emerging XL-AAV capsids represent the first steps towards creating rAAV vectors with larger clone capacities, opening a path towards the delivery of disease-related genes and genetically-encoded tools with long coding sequences in single rAAV vectors.

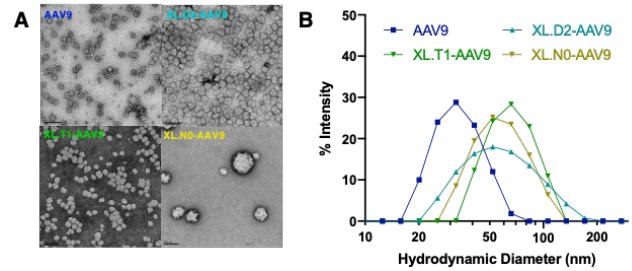

Figure 1. XL-AAVs show expanded capsid sizes. (**A**) AAV9 and XL-AAV9 variants imaged with negative staining transmission electron microscopy (TEM). Scale bar = 100nm. AAV9 forms homogeneous 25nm particles, while XL-AAV variants form heterogeneous particles with the majority sized between 35-70nm. (**B**) Hydrodynamic

diameters of AAV9 and XL-AAV9 variants measured with dynamic light scattering (DLS). Note that the hydrodynamic diameter measured with DLS is larger than the diameter measured with TEM as the former is influenced by the solvent layer associated with the capsid.

516. Engineering CpG-Free ITR for AAV Gene Therapy

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Recombinant adeno-associated virus (AAV) holds great promise as a clinical gene therapy vector due to its lack of pathogenicity and ability to efficiently deliver therapeutic genes to the target tissues in animal models and human patients. However, the immune response remains a major concern in AAV gene therapy, especially when a large dose of the AAV vector is administered systemically. The AAV vector genome is composed of an expression cassette and two flanking inverted terminal repeats (ITRs). ITRs are the only wild type (wt) AAV sequences in the AAV vector. ITRs are essential for the replication of the vector genome, generation of the single-stranded progeny genome, and encapsidation of the progeny genome into preassembled capsids. The CpG motif is a well-recognized immunogenic component. Removing CpG motifs from the expression cassette has been shown to attenuate the AAV immune response. Besides CpG motifs in the expression cassette, the ITRs also contain 32 CpG motifs. Since the immune response can be induced with even a single CpG motif, elimination CpG motifs in the ITR may further reduce AAV immunogenicity. Here we report the engineering of the first-ever CpG-free ITR and characterization of the CpG-free ITR AAV vector. Specifically, C and/or G in the CpG motif were substituted with A and/or T without altering the T-shaped hairpin structure of the ITR. Two cis-plasmids were engineered to carry an identical micro-dystrophin expression cassette, with one using the wt ITR and the other using the CpG-free ITR. The integrity of the ITR was confirmed by sequencing. AAV9 vectors were generated using the transient transfection method in 293 cells and purified through two rounds of $CsCl_2$ ultracentrifugation. The yields of the wt ITR vector and the CpG-free ITR vector were (1.18±0.08) x10e5 vg/cell and (3.03±0.32) x10e4 vg/cell, respectively. By electron microscopy, empty particles accounted for ~12% of the vector preparation irrespective of the ITR CpG content. The biological potency was determined by injecting 2.8 x 10e11 vg particles of the vector to the tibialis anterior muscle of the same mdx mouse (n=6), one side with the wt ITR vector and the contralateral side with the CpG-free ITR vector. Mice were harvested at 4 months after injection. The wt ITR vector and the CpG-free ITR vector showed similar levels of micro-dystrophin expression, muscle weight, cross-sectional area, absolute and specific twitch and tetanic forces, and eccentric contraction profile. Our results suggest that complete depletion of the CpG motif in the ITR does not compromise biological activity of the AAV vector (Supported by NIH, DoD, Jackson Freel DMD Research Fund).

Evaluating Genome Editing Activity and Precision

517. Large-Scale CRISPR-Cas Genome-Wide Activity Profiling in Human Primary T-Cells Reveals Genetic and Epigenetic Determinants of Genome-Wide Nuclease Activity

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CRISPR-Cas9 nucleases are a transformative genome editing technology because of the simplicity with which new sites can be targeted, but fundamental principles that govern Cas9 cellular specificity remain unknown. Here we describe **CHANGE-seq** (Circularization for High-throughput Analysis of Nuclease Genome-wide Effects by Sequencing), a fast, streamlined, Tn5 tagmentation-based assay for measuring the genome-wide activity of Cas9 *in vitro* that is easily scalable to many targets and samples, and overcomes limitations of CIRCLE-seq. To directly compare CIRCLE-seq to CHANGE-seq, we generated CHANGE-seq profiles at 10 sites previously characterized with CIRCLE-seq. We found that CHANGE-seq and CIRCLE-seq read counts were significantly correlated, and the overlap in sites detected by both CHANGE-seq and CIRCLE-seq is comparable or better than CIRCLE-seq technical replicates (>90%). To systematically evaluate Cas9 genome-wide activity, we performed CHANGE-seq on 110 targets across 13 therapeutically-relevant loci in human primary T-cells. Analysis of the 201,934 off-target sites identified by CHANGE-seq revealed that G-base frequency and nucleotide diversity in the target site itself are strongly associated with specificity. The large-scale CHANGE-seq dataset we generated also enabled the training of a highly accurate machine learning model to predict off-target activity. Next, we performed GUIDE-seq on two sets of sites previously evaluated by CHANGE-seq, a set chosen without *a priori* knowledge of specificity, and a set based on specificity as defined by CHANGE-seq. The number of sites detected by CHANGE-seq and GUIDE-seq were strongly correlated (R^2=0.85), indicating that CHANGE-seq can identify highly-specific target sites. To validate the sensitivity of CHANGE-seq for identifying sites of *bona fide* cellular off-target mutations, we selected 648 off-target sites from six sgRNAs target sites, for analysis by targeted tag sequencing. Of the 648 CHANGE-seq detected sites we examined, we confirmed 278 (42.5%) by targeted tag sequencing, including an average of 98.3% of the sites detected by both CHANGE-

seq and GUIDE-seq. Notably, we confirmed 84 of 427 (18.3%) off-target sites detected exclusively by CHANGE-seq and not GUIDE-seq that we analyzed by targeted tag sequencing. Our results demonstrate that CHANGE-seq genome-wide activity profiles are strong predictors of cellular activity and that CHANGE-seq is more sensitive than GUIDE-seq for identifying *bona fide* sites of low-frequency cellular off-target activity. Additionally, we sought to evaluate the impact of chromatin accessibility on cellular off-target activity by comparing CHANGE-seq with matched GUIDE-seq cellular off-target, chromatin, and transcriptional profiles generated from the same human primary T-cells. We found cellular off-target activity was focused near active promoters, enhancers, and transcribed regions. Finally, CHANGE-seq profiling of 6 targets across 8 individual genomes revealed that ~15.2% of human single-nucleotide variants analyzed had significant effects on nuclease activity. CHANGE-seq is, to our knowledge, the first high-throughput method for defining CRISPR-Cas9 genome-wide activity *in vitro* and establishes a simplified, sensitive, and scalable approach to understanding this important class of genome editors for research and therapeutics.

518. Evaluation of the Long-term Effects of AAV-Meganuclease Genome Editing of PCSK9 in Macaque Liver

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When proprotein convertase subtilisin/kexin 9 (PCSK9), a serine protease, binds to hepatic low-density lipoprotein (LDL) receptors, it can accelerate the degradation of LDL receptors and increase LDL cholesterol levels. Genetic studies show that individuals carrying PCSK9 loss-of-function alleles have lower LDL levels and a reduced risk of myocardial infarction. Monoclonal antibodies or RNAi can successfully reduce PCSK9 levels in humans. *In vivo* genome editing that targets the PCSK9 gene represents a novel approach for treating hypercholesterolemia and preventing cardiovascular events. Our team previously demonstrated that a single infusion of an AAV8 vector expressing an engineered meganuclease targeting PCSK9 in non-human primates (NHPs) resulted in a dose-dependent disruption of PCSK9 in liver, as well as a stable reduction in circulating PCSK9 and serum LDL levels for up to 11 months. Here, we followed treated NHPs for up to three years after a single intravenous administration of an AAV8 vector expressing an engineered meganuclease targeting PCSK9. We found sustained on-target genome editing and a long-term reduction of PCSK9 and LDL levels. We performed consecutive liver biopsies on these animals to evaluate the stability of on-target editing and residual vector expression. We also monitored the long-term safety of *in vivo* genome editing by conducting clinical pathology tests, evaluating T-cell responses to AAV capsid and meganuclease expression in peripheral blood mononuclear cells, carrying out liver histopathology, and analyzing off-target editing in consecutive liver biopsy samples. We also evaluated two additional treatment groups; one that received the same AAV8 vector in combination with prednisolone treatment to reduce the magnitude of serum transaminase elevation and another which received AAV3B vector expressing the meganuclease. This long-term evaluation of NHPs demonstrates highly efficient, stable, and physiologically relevant *in vivo* genome editing. Furthermore, these results illuminate the safety issues that require careful consideration during translation to the clinic.

519. Unintended Consequences of CRISPR/Cas9-Mediated Genome Editing for Treating Sickle Cell Disease

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There are several possible gene editing strategies to ameliorate sickle cell disease (SCD) among which correction of the SCD mutation on beta-globin gene (*HBB*) or producing high enough levels of fetal hemoglobin (HbF) could be curative. We and others recently demonstrated therapeutic potential of genome engineering approaches using CRISPR/Cas9 in patient $CD34^+$ hematopoietic stem and progenitor cells (HSPCs); (i) delivery of CRISPR gRNA/Cas9 ribonucleoproteins (RNPs) together with single-stranded oligonucleotide (ssODN) donor or (ii) rAAV6 vector packaging a corrective template resulted in high level correction of the mutant HBB alleles and (iii) recapitulation of naturally occurring 13-bp hereditary persistence of fetal hemoglobin (HPFH) deletion on gamma-globin gene (*HBG*) or (iv) disruption of the GATA1 binding site at the B-cell lymphoma/leukemia 11A (*BCL11A*) erythroid enhancer induced HbF. Bolstered by these recent findings, CRISPR-Cas9 based genome editing is moving to the clinical trials. For therapeutic genome editing, potential nuclease-induced off-target effects need to be carefully analyzed, and significant challenges exist in both accurately predicting potential off-target sites and in performing genome-wide unbiased searches. In addition, the issue of on-target mutagenesis has become increasingly important in light of the recent finding that on-target DNA double strand break (DSB) could induce large deletions and complex genomic rearrangements, which may have pathogenic consequences. The current gold standard for quantifying Cas9 activity is short-range PCR amplification followed by next generation sequencing (NGS). This method relies on PCR amplification of short sequences around the cut site, which significantly limits the accuracy of assessment, results in missing the quantification of edited alleles with large deletion, chromosomal rearrangement or loss. Therefore, for high throughput discovery and quantification of such large modifications, we developed LongAmp-Seq (Long-range PCR Amplification based Sequencing) assay to capture both small and large repair outcomes at the Cas9 cut-site. In SCD patient HSPCs treated with CRISPR-Cas9 genome editing approaches using gRNAs targeting *HBB*, *HBG* and *BCL11A*, we found diverse large deletions of up to several kilobases, insertions and complex rearrangement events. Our findings indicate that current assessments using typical short-read NGS miss a substantial proportion of Cas9-induced mutations in HSPCs, some of which may have pathogenic consequences. In the future, the frequency and distribution of large modifications in engrafting hematopoietic stem cells (HSCs) as well as potential *in vivo* clonal expansion of the cells carrying unintended changes need to be studied.

520. Muscle-Specific Editing in a Therapeutic Target of Duchenne Muscular Dystrophy Using Cas9 and miRNA-Repressible Anti-CRISPR Proteins

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CRISPR-Cas systems are bacterial and archaeal adaptive immune systems that have revolutionized biotechnology and biomedical applications. Despite the potential for human therapeutic development, there are many hurdles that must be overcome before Cas9 can be implemented in clinical settings. Some clinical safety concerns arise from editing activity in unintended cell types or tissues upon *in vivo* delivery [e.g., by adeno-associated virus (AAV) vectors]. Prolonged Cas9 expression and activity may increase the likelihood of off-target editing. Although tissue-specific promoters and AAV serotypes with tissue tropisms can be used, suitable compact promoters are not always available for desired cell types, and AAV tissue tropism specificities are not absolute. To reinforce tissue-specific editing, we exploited anti-CRISPR proteins (Acrs) that have evolved as natural countermeasures against CRISPR immunity. To inhibit Cas9 in all ancillary tissues without compromising editing in the target tissue, we established a flexible platform in which an *Acr* transgene is repressed by endogenous, tissue-specific microRNAs (miRNAs). We previously demonstrated that endogenous miRNAs regulate the expression of an *Acr* transgene bearing miRNA response elements (MREs) in its 3'-UTR and control subsequent genome editing outcomes in a liver-specific manner upon *in vivo* delivery of AAV9 expressing Nme2Cas9, as well as AcrIIC3 that is targeted for repression by liver-specific miR-122 (Lee *et al.*, 2019). We extend this strategy to achieve muscle-specific editing in a therapeutic target of Duchenne Muscular Dystrophy (DMD), a severe muscle degenerative disorder caused by loss-of-function mutations in the *DMD* gene. Delivery of SpyCas9 and sgRNA targeting exon 51 that promotes exon skipping restores dystrophin proteins in large animal models of DMD, suggesting a promising new approach for treatment of DMD using CRISPR-Cas9 (Amoasii *et al.,* 2018; Moretti *et al.,* 2020). To maximize therapeutic benefit, editing in skeletal muscles will require systemic delivery of AAV, necessitating further confinement of Cas9 activity *in vivo*. We designed AcrIIC3 constructs with MREs regulated by muscle-specific miRNAs ("myomiRs," e.g. miR-1 and miR-208) and validated Nme2Cas9 sgRNA targeting exon 51. This platform in which Cas9 editing is regulated by miRNA-repressible anti-CRISPR proteins provides another layer of safeguard for therapeutic development. Moreover, we report on immunogenicity of anti-CRISPR proteins *in vivo* to shed light on the impact of using anti-CRISPRs for Cas9 regulation in clinical settings.

521. Base Editing of Splice Sites Mediates High Efficiency Multiplex Disruption and Modulation of Genes in Primary Human T Cells and NK Cells

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CRISPR systems have allowed for an unprecedented ability to produce targeted gene editing. These applications are of particular interest in the fields of cell based immunotherapies, where the multiplexed knock-out of immunosuppressive genes such as PDCD1, CTLA4, and TGFBR2, in tandem with the knock-in of chimeric antigen receptors specific to tumor antigens yields promise in the development of therapies to recalcitrant malignancies. While CRISPR-Cas9 nucleases work exceptionally well for single gene editing, multiple concerns have been raised about double stranded break (DSB) induction, including large scale genomic rearrangements, reduced pluripotency, and oncogenesis, all of which are exacerbated in a multiplex setting. An alternative tool to edit genes without the need for DSBs are CRISPR-Cas9 base editors. Despite the array of papers demonstrating the various ways base editors can be used to knockout genes and modulate splicing (BE-splice approach), there are two main gaps in the field, namely 1) a comprehensive tool for designing both cytidine base editors (CBEs) and adenine base editors (ABEs) sgRNAs to target both splice donor (SD) and splice acceptor (SA) sites, and 2) a head-to-head comparison of the methods of base editing mediated gene knockout encompassed across, ABEs, CBEs, BE-splice methods, and pmSTOP induction. Here we present an easy-to-use webtool SpliceR (z.umn.edu/splicer), for the design of base editing sgRNAs to target splice-sites of any ensembl annotated organism. Analysis of the human genome showed that 95.86% of all protein coding gene transcripts, and 99.85% of all transcripts from non-single-exon-single-isoform genes are targetable. We assessed these predictions, and compared BE-splice to the induction of premature stop codons in a mid-throughput screen of sgRNAs targeting genes in the TCR-CD3 MHC Class I immune synapse. From this screen we discovered three main trends; 1) CBE mediated splice-site knockout was higher and more reliable than ABE mediated knockout, 2) Across CBE and ABE, splice-donor sgRNAs produced more robust knockouts, and 3) among the genes in our screen BE-splice sgRNAs produced more frequent knockouts than pmSTOPs sgRNAs. Additionally, we also found that targeting middle exons tends to produce more reliable knockouts across all approaches, than targeting either first or later exons. Interestingly, our data also suggests that the intracellular domains of several TCR-CD3 complex genes are non-essential for assembly and trafficking of the complex to the cell surface. Finally, here we demonstrate to the first of our knowledge, multiplex base editing of NK cells. Collectively these results show that multiplex base editing of splice sites is a robust, accessible, strategy for disrupting and modulating genes in primary lymphocytes that has application across a variety of cell based immunotherapies.

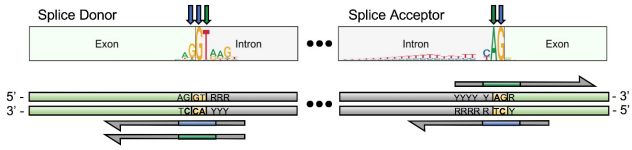
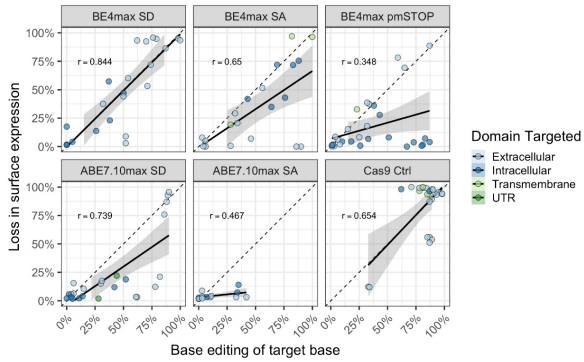


Diagram of BE4max and ABE7.10max base editor and splice0site motifs. Splice-site motif logo diagrams generated from analysis of all human protein coding genes. BE4max can target C:G base pairs, while ABE7.10max can target A:T within the conserved motifs.



Relationship between observed knockout efficiency and rate of base editing. Observed knockout measured via flow cytometry of surface expression. Data points colored by region within the gene targeted by the sgRNA, as determined by the TCR-CD3 crystal structure. BE4max SD, ABE7.10max SD, and BE4max SA sgRNAs yield proportional KOs relative to Cas9.

522. High-Throughput Screen to Optimize gRNA Pairs for CRISPR-Cas9-Mediated Exon Deletion

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Exon skipping or deletion has proven to be a powerful strategy for the correction of genetic diseases, where removal of an exon can correct reading-frames distorted by aberrant splicing or other mutations. For example, Duchene muscular dystrophy (DMD), often caused by the deletion of one or more exons in the DMD gene, has a number of promising exon-skipping strategies. Antisense oligonucleotides have been used to force exon skipping during RNA splicing, but the effects are transient and require re-administration. CRISPR-Cas9 can also be directed to cleave the intronic regions on either side of an out-of-frame exon, enabling the NHEJ repair process to permanently remove the exon from the genome. This strategy of delivering *S. aureus* Cas9 (SaCas9) and two gRNAs with AAV has shown promise *in vivo*, with exon deletion efficiencies ranging from 2-4% in the heart and skeletal muscle. These DNA editing rates have restored dystrophin protein to almost 10% of wild-type levels in skeletal muscle, however, it is estimated that higher protein expression is required for an asymptomatic phenotype. Here we use high-throughput CRISPR screens to discover gRNA pairs that mediate high efficiency exon deletion. We focus on optimizing gRNA design because there is a wide range of on-target activity between different genomic target sites, and the large size of DMDintrons (tens to hundreds of kb) provide ample space for finding gRNAs with desirable on- and off-target editing profiles. Previous studies have not taken advantage of this targeting range or sequence diversity, only testing tens of gRNAs. Moreover, these studies have used high individual gRNA activity to predict optimal gRNA pairs, even though it is established that the context of the gRNA pair is an important parameter in determining genomic deletion efficiency. In our approach, cells stably expressing SaCas9 are transduced with lentiviral libraries of gRNA pairs at low MOI. However, the accurate measurement of relative deletion efficiencies is difficult: the low, cell type-specific expression of dystrophin precludes the use of a genetic reporter and PCR-based assays would be heavily biased by the wide range of deletion sizes. Thus, in order to quantitatively readout deletion efficiency in this screen, we focus on detecting the mutant junctions that are created after a deletion event. Since each gRNA pair yields a unique junction, the frequency of each junction is a direct measure of the deletion efficiency for a gRNA pair. After treating a cell population with the library of gRNA pairs, junctions are enriched through the use of biotinylated probes and detected with Illumina-based sequencing. We designed a library of 2,080 pairs of gRNAs for deletion of human DMD exon 51 in HEK293T cells (52 gRNAs in intron 50 x 40 in intron 51). After performing the screen, we ranked the individual gRNAs and pairs based on the frequency with which they were identified, normalizing for initial gRNA abundance and bias introduced by probe hybridization. The screen identified novel high-efficiency gRNA pairs, many of which performed better than the previous standard, with the top two new pairs at ~5x more relative counts than the standard pair. Validation of these hits is ongoing, but initial results confirm that the new hits efficiently delete exon 51 when tested as individual pairs. Future work will apply variations of this method to quantify the level of exon deletion made by each gRNA pair in HEK293Ts, patient myoblasts, and in the hDMDΔ52/*mdx* mouse. Since this screening method relies only on genomic DNA as an output, it does not require a reporter and can be applied on any locus in any cell type. Thus, it can be easily adapted to optimize gRNA pairs for any genetic disease where a targeted deletion is a viable therapeutic strategy.

523. A Dual AAV Vector System to Visualize Prime Editing in Mammalian Cells

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Prime editing permits targeted re-writing of the genome and offers incredible promise for precision medicine, but its potential *in vivo* activity is unknown. Prime editing uses a SpCas9-nickase fused to a reverse transcriptase in combination with a prime-editor guide RNA (pegRNA). The pegRNA specifies both the site of targeting and the desired editing outcome. The range of edits thus far described include targeted insertions or deletions up to several dozen base pairs. Despite its demonstrated versatility *in vitro*, its *in vivo* utility is untested. As a first step to advancing *in vivo* application, we set up a method to visualize prime editing outcomes. Additionally, because prime editing machinery exceeds the packaging capacity of adeno-associated virus (AAV), we sought to determine the efficiency of a split vector approach. To visualize prime editing, we designed a 31bp sequence, including a nuclear localization signal, that can be inserted into the N-terminus of tdTomato in the Ai14 transgenic reporter allele in Ai14/Ai6 compound heterozygous mouse embryonic fibroblasts (MEFs). Transfection of the

prime-editor plasmids permits editing of the Ai14 allele. Subsequent transfection with Cre plasmid activates both reporter alleles. Prime editing is visualized by an accumulation of tdTomato in the nucleus with no impact on the localization of ZsGreen (from the non-targeted, Ai6 reporter allele). The editing-induced switch of cytoplasmic-to-nuclear localization of tdTomato noted via fluorescence microscopy was validated by PCR amplification and sequencing of genomic DNA from prime-editor transfected Ai14/Ai6 MEFs. To deliver the prime editing machinery with dual AAV vectors, we split the SpCas9-nickase and reverse transcriptase (RT). Although the linkage of the RT to the SpCas9-nickase likely improves efficiency, we reasoned that it is not essential for prime editing. One AAV vector construct contained the SpCas9-nickase and the other contained the RT and pegRNA expression cassettes. Using the same fluorescent reporter approach as above, we demonstrate that prime editing activity is retained following co-transfection of the dual AAV vector plasmids into Ai14/Ai6 MEFs. Work is now underway to test prime editing *in vivo* using our dual AAV vector system. The insights gained from these studies will guide future work to apply prime editing to animal models of genetic disease.

Clinical Gene Therapies for Inborn Errors of Metabolism

524. Transpher A, an Open-Label, Multicenter, Single-Dose, Dose-Escalation, Phase 1/2 Clinical Trial of Gene Transfer of ABO-102 in Sanfilippo Syndrome Type A (Mucopolysaccharidosis IIIA): Safety, Tolerability, Biopotency and Neurocognitive Data

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Background: Sanfilippo syndrome type A (Mucopolysaccharidosis type IIIA; MPS IIIA) is a lysosomal storage disorder caused by mutations in the *SGSH* gene that result in intracellular heparin sulfate accumulation, cellular dysfunction, and cell death. A multi-system disorder, it manifests in early childhood with relentless neurodegeneration, organomegaly and culminating in premature death. **Methods:** ABO-102 is a self-complementary AAV9-based gene vector containing the human *SGSH* gene under control of the U1a promoter. The Transpher A study is a Phase 1/2 clinical trial assessing safety and efficacy of ABO-102 in MPS IIIA patients enrolled in three dose cohorts testing; 0.5 X 10^{13} vg/kg (Cohort 1, n=3), 1 X 10^{13} vg/kg (Cohort 2, n=3) and 3 X 10^{13} vg/kg (Cohort 3, n=8). Evaluations included serial measures of general safety, neurocognitive development, biomarkers in the CNS, plasma and urine and liver and brain volumes. **Results:** Fourteen patients have been enrolled. Cohorts 1 and 2 have completed the 24 months follow-up, as well as 4 patients in Cohort 3 while the remaining 4 patients have been followed to a mean 19.4 months (range 15.4-22.4 months) at time of reporting. Intravenous administration of ABO-102 proved well tolerated, with no infusion or serious drug-related adverse events. Drug-related adverse events have been Grade 1 or 2 only and are all resolved. Mild transient cellular immune responses have been detected in 8 out of 14 patients as assessed by ELISpot (interferon-gamma). A rapid and sustained dose-dependent reduction in CSF heparin sulfate (HS) was observed in all patients treated: In Cohort 3, CSF HS levels decreased 65% from pre-treatment levels at day 30 (n=8), 78% at Month 6 (n=8), 71% at Month 12 (n=7) and 78% at Month 24 (n=2), reaching the lower level of quantitation of the assay. Abdominal MRI showed a rapid decrease in liver volume in all patients as early as 30 days post administration of ABO-102, with several patients normalizing liver volume by Month 6, though some rebound was observed at month 24. While one patient aged 57 months at the time of treatment showed some signs of cognitive stabilization, it is notable that patients younger than 30 months of age (n=3) at the time of treatment have demonstrated development progress consistent with normal population variance at 18 and 24 months follow-up - deviating from the natural course in untreated disease. **Conclusion:** Intravenous administration of ABO-102 in children with MPS IIIA showed a favorable long-term safety and tolerability profile two years after treatment and led to durable and dose-dependent improvement in disease biomarkers and liver volume. Patients treated before 30 months of age show development tracking in the normal range, providing evidence of neurological benefit when treatment is initiated early, before neurodegeneration is advanced.

525. Preliminary Outcomes of Haematopoietic Stem Cell Gene Therapy in a Patient with Mucopolysaccharidosis IIIA

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Mucopolysaccharidosis Type IIIA (MPSIIIA) is a paediatric, autosomal recessive lysosomal storage disease (LSD) caused by deficiency of sulfamidase (SGSH), an enzyme involved in the degradation of heparan sulfate (HS). Absence of SGSH leads to the accumulation of

partially degraded HS glycosaminoglycans in lysosomes, giving rise to cellular dysfunction with devastating clinical consequences. Affected individuals exhibit severe central nervous system degeneration with progressive cognitive impairment and behavioural abnormalities, alongside more attenuated somatic symptoms. Intravenous enzyme replacement therapy is ineffective, as the SGSH enzyme cannot cross the blood brain barrier and access the central nervous system. We previously described an *ex vivo* autologous haematopoietic stem cell gene therapy (HSCGT) approach, synthesising a high-titre lentiviral vector driving the expression of SGSH under the control of the myeloid-specific CD11b promoter (LV. CD11b.SGSH). We demonstrated preclinical proof of concept in MPSIIIA mice, achieving 5-fold normal SGSH levels in bone marrow and 10% of normal SGSH activity in brain, as well as demonstrating safety and efficacy of the investigative drug product. Here, we describe preliminary outcomes in the first MPS-IIIA patient treated using gene modified autologous haematopoietic stem cells engineered to produce the deficient enzyme. Therapy was provided under the UK special license arrangements after institutional ethical review. Haematopoietic stem cells were mobilised using G-CSF and plerixafor, and 13.42 x 10^6 $CD34^+$ cells transduced with LV.CD11b. SGSH at a mean vector copy number (VCN) of 3.79 were delivered to the patient, following myeloablative busulfan conditioning. Successful multilineage engraftment of gene-modified cells was achieved within 39 days, and sustained vector copy number at a similar range to input was achieved. Leukocyte SGSH enzyme activity was 22 fold of normal levels at 9 months, with rapid decreases in urine, plasma and CSF Heparan Sulphate levels by 3 months post-transplant. These data support the safety and feasibility of HSCGT in patients with MPSIIIA. A subsequent phase I/II proof-of-concept clinical trial to evaluate HSCGT in patients with Mucopolysaccharidosis IIIA has been initiated in Manchester in December 2019.

526. Transpher B, an Open-Label, Multicenter, Single-Dose, Dose-Escalation, Phase 1/2 Clinical Trial of Gene Transfer of ABO-101 in Sanfilippo Syndrome Type B (Mucopolysaccharidosis IIIB)

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Background: Sanfilippo syndrome type B (Mucopolysaccharidosis type IIIB MPS IIIB) is a lysosomal storage disorder caused by mutations in the *NAGLU* gene that result in intracellular glycosaminoglycan accumulation, cellular dysfunction, and cell death. It manifests in early childhood as a multi-system disease, including severe neurodegeneration, organomegaly and premature death. **Methods:** ABO-101 is a single stranded AAV9-based gene vector containing the human *NAGLU* gene under control of the CMV promoter. The Transpher B study is a Phase 1/2 clinical trial assessing safety and efficacy of ABO-101 in MPS IIIB patients enrolled in three dose cohorts testing 1 X 10^{13} vg/kg, 5 X 10^{13} vg/kg and 1 X 10^{14} vg/kg. Evaluations include measures of general safety, neurodevelopment, biomarkers and liver and brain volumes. **Results:** 8 patients have been treated, including two pair of siblings (one sibling was diagnosed soon after birth and treated at the age of 4 months under a protocol waiver): 2 patients treated in Cohort 1 (follow-up 13-26 months), 5 in Cohort 2 (follow-up 2.3-9 months) and 1 patient treated in Cohort 3 (just dosed). Intravenous administration of ABO-101 has been well tolerated, with no infusion adverse events and no serious drug-related adverse events. Non-serious drug-related adverse events include subclinical, transient ALT/AST elevations without changes in GGT or bilirubin. ELISpot to AAV9 capsid peptides pools have been negative in all subjects except one transient positive response at Month 12 in one subject (Cohort 1) that became negative again at Month 18. *NAGLU* plasma enzyme activity showed a dose-dependent normalization, lasting up to 3 months in Cohort 2. A rapid reduction in CSF heparin sulfate (HS) was observed in all patients treated, up to 79% decrease at Month 12 (longest follow-up, Cohort 1, n=1). Abdominal MRI showed a rapid decrease in liver volume, sustained at months 6 (n=2) and 12 (n=1) in Cohort 1. Limited follow-up duration to date preclude adequate assessment of neurological outcomes. **Conclusion:** Intravenous administration of ABO-101 in children with MPS IIIB was well tolerated with no treatment-related SAEs. ABO-101 showed a clear biologic effect,

including a dose-dependent normalization of enzyme activity in plasma up to month 3, decreased CSF HS levels (maintained up to Month 12 in Cohort 1) and diminished liver volume. A longer follow-up is needed to evaluate neurodevelopmental changes.

527. Hematopoietic Stem Cell Gene Therapy for Cystinosis: Initial Results from a Phase I/II Clinical Trial

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Cystinosis is a lysosomal storage disorder characterized by the accumulation of cystine within the lysosomes of all organs and caused by mutations in the *CTNS* gene encoding the transmembrane lysosomal cystine transporter, cystinosin. Major complications of cystinosis include early renal Fanconi syndrome, kidney failure requiring transplantation, eye defects that can lead to blindness, hypothyroidism, and neuromuscular problem causing swallowing dysfunction and fatal pulmonary aspiration. Cysteamine, the only FDA-approved drug to treat cystinosis, only delay the progression of the disease. Preclinical studies showed that transplantation of wild-type hematopoietic stem and progenitor cells (HSPCs) in $Ctns^{-/-}$ mice leads to cystine content decrease in every tissue tested, as well as functional and structural improvement in kidney, eye and thyroid. The mechanism of action involves lysosomal cross-correction via tunneling nanotubes from graft-derived macrophages. Given the risks of mortality and morbidity associated with allogeneic HSPC transplantation, we developed an autologous transplantation approach of HSPCs modified ex vivo using a Self-Inactivated-lentiviral vector to introduce a functional version of the *CTNS* cDNA, pCCL-CTNS (drug product name: CTNS-RD-04). We performed the pharmacology and toxicology studies, and manufacturing development for this strategy, and received FDA-clearance to initiate a phase I/II clinical trial in December 19, 2018. We have now started the clinical trial at UC San Diego to evaluate the safety and efficacy of CTNS-RD-04 that is expected to include six patients. The manufacturing of the products is performed in the UCLA GMP facility by Dr. Donald Kohn. Following G-CSF/plerixafor stem cell mobilization, leukapheresis and cell manufacturing, subjects receive busulfan myeloablation before HSPC transplantation. Subjects undergo comprehensive assessments before and after treatment at 3, 6, 12, 18 and 24 months to evaluate the impact of CTNS-RD-04 on the clinical outcomes and cystine levels in blood and tissues. The first patient, a 20-year old male with no kidney transplant, was included and transplanted. At the time of this report, this patient just completed the 3-month follow-up evaluation. Peripheral blood vector copy number (VCN) was around 2 integration per cell, and intracellular granulocyte cystine (while off cysteamine therapy) went from 7.77 nmol half-cystine/mg protein at Baseline to 1.53 at 3-month post-transplant (80% decrease, with the conventional target for cysteamine therapy being <1.95 nmol/mg). Analysis of the tissue cystine crystal data and clinical outcomes is ongoing. The second patient on study has now completed Baseline assessments.

528. Pathophysiological Mechanisms of Bone Defects and Impact of Ex Vivo Hematopoietic Stem Cell Gene Therapy in Mucopolysaccharidosis Type I Hurler

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Mucopolysaccharidosis type I Hurler (MPSIH), an inherited autosomal recessive metabolic disorder, is caused by deficiency of alpha-L-iduronidase (IDUA), involved in the catabolism of the heparan and dermatan sulphate glycosaminoglycans (GAGs). Accumulation of GAGs in tissues results in progressive multi-organ dysfunction and a wide range of skeletal manifestations, known as *dysostosis multiplex*. Despite the clinical success of allogeneic hematopoietic stem cell transplantation (HSCT) in ameliorating the majority of clinical symptoms, skeletal abnormalities remain and severely affect patient's quality of life, likely due to limited penetration of the (normal levels) donor enzyme into poorly vascularized tissues and/or to the irreversibility of the skeletal damage at the time of transplantation. Preliminary data of the phase I/II clinical trial of *ex-vivo* hematopoietic stem cell gene therapy (GT) in MPSIH patients, ongoing at SR-Tiget, show supraphysiologic IDUA activity in the blood and early normalization of excretion of urinary glycosaminoglycans, suggestive of metabolic correction. This was accompanied in the first 2 patients treated (1.5 and 1 year post-GT) by improved growth velocity and joint mobility. As the underlying mechanism of bone defects in MPSIH is not completely understood, we investigated the biological and functional characteristics of mesenchymal stromal cells (MSCs) and osteoclasts (OCs) of MPSIH patients pre- and post-GT and their interplay in bone after GT. *Ex-vivo* differentiation and bone resorption activity were not affected in patient-derived OCs pre-GT, as well as isolation and *ex-vivo* differentiation towards osteoblasts and adipocytes of patient-derived MSCs pre-GT. As expected, IDUA activity was absent in MPSIH-MSCs. We hypothesized that OCs derived from genetically modified hematopoietic stem cells could deliver IDUA enzyme in the bone microenvironment, thus cross-correcting other resident bone cells, including MSCs and their progeny. In order to better elucidate the cross-correction mechanisms, we investigated the expression of receptors capable to mediate IDUA uptake. We found a robust expression of LAMP-1 and sortilin-1 and an efficient uptake of IDUA by MPSIH-MSCs exposed for 16h to conditioned medium derived from OCs generated from MPS IH patients after GT. Indeed, post-GT OCs displayed supraphysiological enzyme activity levels.

Since *in vitro* culture of MPSIH-MSCs does not recapitulate the *in vivo* GAGs accumulation, we exposed MPSIH-MSCs to heparan sulphate *in vitro* to mimic the pathologic condition of patients. We observed an impaired capacity to degrade GAGs compared to healthy donor MSCs. We will further investigate the *in vitro* impact of GAGs accumulation on MPSIH-MSC functions and biology and we will confirm the detoxification of bone cells after GT by a 3D *in vitro* bone model. Moreover, we will analyse bone biopsies obtained from MPSIH patients pre- and post-GT by transmission electron microscopy and immunohistochemistry.

529. Gb3 Substrate in Endothelial Cells of Renal Peritubular Capillaries Was Reduced in a Previously Untreated Classic Fabry Male Patient Treated with AVR-RD-01 Investigational Lentiviral Gene Therapy

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Lysosomal disorders are attractive candidates for *ex vivo* gene therapy based on the potential to transform a patient's own cells into a drug product to deliver sustained functional protein/enzyme after a single treatment. Fabry disease (FD) is caused by mutations in the *GLA* gene that result in functional deficiency of the lysosomal enzyme, alpha-galactosidase A (AGA), which leads to pathological accumulation of substrates and metabolites, including globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3). Significant morbidity and early mortality result from damage to kidneys, heart, and brain. AVR-RD-01 is an investigational autologous *ex vivo* gene therapy that involves transplantation of autologous stem cells genetically modified with a lentiviral vector encoding the human AGA complementary deoxyribonucleic acid (cDNA) sequence. Initial results on 5 previously ERT-treated patients in a Phase 1 trial of AVR-RD-01 showed increases in plasma and leukocyte AGA activity and decreases in substrate (Gb3) and metabolite (lyso-Gb3) in plasma, sustained in up to more than two years after gene therapy treatment. A Phase 2 clinical trial in 8-12 treatment-naïve males (16-50 years) with classic FD investigates the safety, tolerability, and efficacy of AVR-RD-01, including its effect on substrate accumulation in the kidney after 48 weeks. Kidney biopsy results for the first patient demonstrated reduction in renal peritubular capillary (PTC) Gb3 inclusions, quantitatively assessed by the BLISS methodology. At 48 weeks, Gb3 inclusions were reduced from an average of 3.55 to 0.47 per PTC corresponding to an 87% reduction versus baseline (BL). Leukocyte and plasma AGA activity increased, associated with declines in plasma and urine Gb3 and lyso-Gb3, including an 87% reduction in plasma lyso-Gb3 at 48 weeks versus BL. Treatment-emergent adverse events were as expected with conditioning, underlying FD and pre-existing conditions, with no serious adverse events related to AVR-RD-01. Low-titer anti-AGA antibodies were transiently detected at week 24. Data from additional three treatment naïve patients is emerging, and the study is actively recruiting.

530. Late Phases of Hematopoietic Reconstitution in Metachromatic Leukodystrophy Gene Therapy Patients Are Characterized by Lineage Commitment of Individual HSC Clones

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In the self-inactivating lentiviral vector-based (LV) hematopoietic stem and progenitor cell gene therapy (HSPC-GT) clinical trial for metachromatic leukodystrophy (MLD) conducted at SR-Tiget, we characterized the hematopoietic reconstitution at single clonal level by integration site (IS) analysis in 29 patients (up to 8 years follow-up). We retrieved integration sites (IS) from $CD34^+$, myeloid, and lymphoid cells purified at different time points after HSPC-GT (1, 3, 6, 9, 12 months post-treatment, and every 6 months thereafter) from bone marrow and/or peripheral blood. We retrieved an average of >48,000 IS per patient, many of which persisted long-term with multi-lineage potential. By applying a new statistical method for data integration, allowing normalizing and harmonizing IS data from different sequencing platforms and experimental methodologies, we did not observe any clonal dominance events, or any bias toward integration near cancer genes in any patients. These data confirmed the long-term safety of the HSPC-GT treatment with highly diversified polyclonal and multilineage reconstitution of hematopoiesis without signs of genotoxicity or any report of malignancy or adverse events indicative of oncogenic transformation. Detailed analysis of the output of different hematopoietic cell lineages and their contribution to hematopoietic reconstitution over time showed three main phases: 1) a first phase up to 12 months after transplant where short-lived myeloid progenitors sustained the initial waves of hematopoietic reconstitution and progressively exhausted over time (results confirmed also in the LV HSPC-GT clinical trial for Wiskott-Aldrich syndrome, WAS). During this initial phase, circulating lymphoid cells were instead more oligoclonal. 2) a second phase, starting from 9 up to 24 months after transplant, was characterized by an intense HSC multilineage output; 3) a third phase where the multilineage HSC output turns progressively into a lineage-committed hematopoiesis. Overall, our results indicate that in MLD patients treated with HSPC-GT, where the hematopoietic reconstitution after transplantation occurs without selective advantage, late phases of hematopoietic reconstitution are characterized by a lineage-committed output of HSCs towards the myeloid lineage, in line with a recently described model of homeostatic maintenance of normal hematopoiesis by committed progenitors.

Applications of Physical, Chemical and Exosomal Delivery

531. Induction of Long-Term Tolerance to a Specific Antigen Using Anti-CD3 Lipid Nanoparticles Following Gene Therapy

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Hemophilia A is a serious bleeding disorder resulting from a deficiency of coagulation Factor VIII (FVIII). The current treatment for hemophilia A patients is replacement therapy of FVIII protein. However, some hemophilia A patients develop FVIII inhibitors that neutralize coagulation function of infused FVIII, leading to treatment complications that significantly increased morbidity and mortality in patients. Humanized anti-CD3 monoclonal antibody has been used to treat immune-mediated disease. Anti-CD3 treatment depletes both CD4+ and CD8+ effector T cells to very low levels however the population of regulatory T cells (Tregs) was affected to a less extent, leading to a more immunotolerant environment. In clinical trials, anti-CD3 therapy can effectively modulate the immune response of allograft rejection or autoimmune diseases without eliciting major adverse effects. Delivery of mRNAs encapsulated lipid nanoparticles (LNPs) can achieve high expression levels of transgenes without risk of insertion or oncogenic mutation compared to viruses carrying genetic materials. In order to overcome anti-FVIII immune response, we performed anti-CD3 therapy as a transient immunosuppressive strategy that can prevent the formation of anti-FVIII inhibitor and induce long-term tolerance to FVIII. We first investigated the efficiency of depleting T lymphocytes using various LNPs encapsulated mRNA encoding anti-CD3 monoclonal antibody (anti-CD3 LNPs). Four different mRNAs encoding anti-CD3 antibodies, including two single-chain antibodies (mCD3_Fc-scFv2.co and mCD3_Fc-scFv2.se) and double-chain (heavy chain and light chain) antibodies (mCD3_mAb_LC+HC and mCD3_(Fab')2_LC+HC) were designed and packaged into LNPs. We have injected these into four separate groups of mice and found that both single-chain antibodies depleted T cells efficiently, whereas the double-chain antibodies cannot. The cell percentages of CD3+, CD4+ and CD8+ cells in all anti-CD3 LNPs treated mice returned to normal cell numbers two months after injection. Next, we investigated the application of anti-CD3 LNPs to modulate immune responses against FVIII. Hemophilia A mice were treated with selected anti-CD3 LNPs prior to induction of FVIII expression by hydrodynamic injection of a liver-specific FVIII plasmid. After anti-CD3 LNPs treatment, hemophilia A mice displayed significantly higher percentage of Tregs (CD4+CD25+Foxp3+) population in CD4+ T cells compared to control group one day after injection. The peak level of Tregs maintained about two weeks and slowly declined to comparable cell numbers to control group within two months, leading to contribution of immune tolerance to FVIII. Furthermore, anti-CD3 LNPs treated mice showed persistent FVIII gene expression for at least half a year (duration of the experiments) without induction of anti-FVIII antibodies, whereas FVIII gene expression levels fell to low to undetachable levels within one month following gene therapy in association with the formation of high-titer anti-FVIII antibodies. These results indicate that treatment of anti-CD3 LNPs induced long-term tolerance to FVIII. In conclusion, our study established a potential new strategy of immunomodulation therapies for hemophilia A.

532. Fusion Gene-Based Therapy for Prevention and Treatment of Obesity and Obesity-Associated Metabolic Diseases

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Gene therapy has been considered a promising approach for prevention and treatment of obesity and metabolic diseases. Using a method of hydrodynamic gene transfer, we have previously demonstrated anti-obesity activities of fibroblast growth factor 21 (FGF21) and superoxide dismutase 3 (SOD3) in mice. A fusion gene approach was taken in the current study to assess the effects of combination of selected genes on blocking high-fat diet-induced obesity, insulin resistance, and fatty liver development in mice. pLIVE vector with an albumin promoter was used to carry two genes connected by a linker sequence to preserve the reading frame. Fusion genes made include exendin 4 (EX4, analog of glucagon-like peptide) fused with FGF21 (pEX4-FGF21); exendin 4 fused with human alpha 1 antitrypsin (hAAT) (pEX4-hAAT); exendin 4 fused with SOD3 (pEX4-SOD3); hAAT fused with insulin receptor antagonist S961 (phAAT-S961); FGF21 fused with hAAT (pFGF21-hAAT); and single chain insulin analog (SCI) fused with hAAT (pSCI-hAAT). Exendin 4 was selected to suppress food intake; hAAT was used for its anti-inflammatory activity; and S961 and SCI were selected for targeting the insulin receptor. DNA sequencing was performed for each of the 6 plasmids to confirm fusion gene sequence. A single hydrodynamic injection of each plasmid (20 μg/mouse) was performed at the beginning of the experiment on C57BL/6 mice (male, 23-25 g) fed a high-fat diet (60% calorie from fat, 20% from carbohydrate, and 20% from protein). Control animals received empty vector. Body weights of animals were determined twice a week along with the food intake for 9 weeks. Body composition was determined at the beginning and the end of the experiments. Comparing to control mice injected with empty plasmid, mice injected with phAAT-S961, pSCI-hAAT and pEX4-SOD3 showed no difference in body weight. pEX4-hAAT had the best activity with 100% blockade of high-fat diet-induced weight gain, followed by pEX4-FGF21 with 50%, and pFGF21-hAAT with 30%. Lower body weight is coincided with lower fat mass, lower food intake, and decreased inflammation level in animals. Improved liver function and glucose homeostasis were also observed in mice treated with the pEX4-hAAT. pEX4-hAAT plasmid was further studied for its activity in blocking weight gain in *ob/ob* mice and for inducing weight loss in obese mice. Results show that pEX4-hAAT transfer decreases weight gain and improves metabolic homeostasis of *ob/ob* mice. Hydrodynamic delivery of pEX4-hAAT plasmid resulted in dose dependent and sustained fusion gene expression, induced weight loss, restored insulin sensitivity, and reduced hepatic fat accumulation in obese mice. Mechanistic study show that the beneficial effects obtained are mainly through suppressing energy intake and transcription of pivotal genes involving in inflammatory response and lipogenesis in the

white adipose tissue and liver. Results from these studies demonstrate that pEX4-hAAT is highly effective in treating high-fat diet-induced obesity and restoring metabolic homeostasis. More importantly, these findings demonstrate the feasibility of fusion gene-based therapy for prevention and treatment of obesity and metabolic diseases.

533. A Single Dose of Fast Half-Life CD117 Antibody Drug Conjugate Enables Hematopoietic Stem Cell Based Gene Therapy in Nonhuman Primates

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Autologous hematopoietic stem cell transplantation (Auto-HSCT) with gene-modification represents a potential cure for multiple genetic diseases, but its broad curative potential, is limited because of morbidity/mortality from cytotoxic chemotherapy-based conditioning. To overcome these limitations, we developed antibody drug conjugates (ADC) targeting CD117 (C-KIT) to specifically deplete the hematopoietic stem and progenitor cells (HSPC). To validate CD117 ADC-mediated depletion prior to HSCT preclinically, we developed an optimized non-human primate (NHP) fast half-life anti-CD117 ADC and evaluated it in an auto-gene modified HSCT in a well-established rhesus model. The CD117-ADC has potent depletion of human and NHP CD34+ cells *in vitro* (Figure 1A). Humanized NSG mice treated with a single dose had full depletion of human HSPCs in the bone marrow, while maintaining peripheral immune cells. In rhesus, a single administration achieved >99% HSPC depletion in bone marrow and was comparable to HSPC depletion observed following myeloablative busulfan conditioning (6 mg/kg/day x4). There was no effect of the ADC on the peripheral and bone marrow lymphocytes and the ADC was well tolerated compared to busulfan where multiple severe adverse events were seen. To facilitate use in the transplant setting, the CD117-ADC was engineered to have a fast clearance and the half-life was < 10 h in NHP. We next explored whether the tool CD117-ADC could enable auto gene modified HSCT in the rhesus model. Two rhesus NHP were mobilized with GCSF and plerixafor. The selected CD34+ cells were transduced with β-globin encoded lentivirus and cryopreserved. The tool CD117-ADC was dosed on day -6 and the CD34+ cells were infused on day 0. Bone marrow aspirates analyzed on the day of infusion (day 0) demonstrated >99% depletion of the HSPCs and maintenance of the bone marrow lymphocytes (Figure 1B). The primates engrafted neutrophils (day 8 and 10) and platelets (day 10 and 11), while peripheral lymphocytes were maintained throughout the transplant (Figure 1C-D). The gene marking in the granulocytes is stable for >180 days and comparable to busulfan conditioned animals previously reported (Tisdale, Molecular Therapy 2019). Longer follow up and data from additional animals with gene editing will be presented. In summary, we have developed a fast half-life CD117 ADC that shows potent activity on NHP CD34+ cells, achieved >99% HSPC depletion in vivo, has a favorable safety profile compared to busulfan, spares the immune system and is cleared rapidly. In a rhesus model of auto-gene modified HSCT, a single dose of the ADC enabled engraftment of gene modified HSCs without the need for busulfan. These proof of concept studies validate the use of CD117-ADC for targeted HSPC depletion prior to transplant and support its use as a new conditioning agent for auto-gene modified HSCT. This targeted approach for safer conditioning could improve the risk benefit profile for patients undergoing HSCT and enable more patients to benefit from these potentially curative therapies.

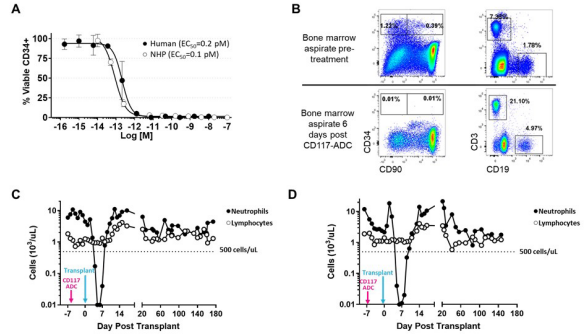

Figure 1: CD117 ADC is fully myeloablative and enables engraftment of autologous gene-modified hematopoietic stem cells (A) CD117-ADC was incubated with human and NHP bone marrow derived CD34+ cells for six days. Viable CD34+ cells were evaluated by flow cytometry. (B) Bone marrow aspirates from the NHP were taken pretreatment and 6 days following CD117-ADC administration. Bone marrow aspirates were evaluated for CD34+, CD34+CD90+, CD3+, and CD19+ cells by flow cytometry. (C-D) The CD34+ cells harvested from a rhesus NHP were transduced with lentiviral vector encoding β-globin. The transduced cells were transplanted into the same animal 6 days after a single dose of CD117 ADC. The neutrophils (closed circles) and lymphocyte (open circles) counts before and after transplantation of the transduced cells are shown for animal #1(C) and animal #2(D).

534. Developing Nonviral Gene Therapies for Inherited Blinding Disorders Using CRISPR/Cas9 Genomic Editing and Human Pluripotent Stem Cells

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Introduction: Inherited blinding disorders can be caused by multiple genetic variants that have very multiple mechanisms of action. To develop effective genetic therapies, several challenges need to be overcome, including gene editing approach, efficiency and delivery. Here we present a multi-faceted approach, around CRISPR/Cas9 genome editing, to tackle these challenges. Different gene-editing approaches for both autosomal dominant (knockout via allele specific editing) and autosomal recessive (via gene correction) mutations are used and we show nanoparticles comprising all the components required for gene correction, named 's1mplexes', that can increase correction efficiency of Cas9 genome editing. Patient derived stem cell platforms can be used to customize gene editing and viral gene therapeutic strategies. Finally, nonviral delivery "nanocapsules' are developed for subretinal delivery of genome editor.
Results and Discussion: Due to the differing mechanisms of action of different disease-causing mutations in the eye (e.g. CEP290, CRYGC, BEST1) different editing approaches should be used. We find that for

some autosomal dominant mutations, gene knockout may be more efficient while for some autosomal recessive mutations, gene correction is required. We show that s1mplexes, a hybrid nanoparticle comprising the Cas9 gene editing machinery attached to an ssODN template, increase gene correction frequencies compared to traditional methods. Furthermore, we show that nonviral polymeric carriers of protein based CRISPR machinery (e.g. ribonucleoprotein or s1mplexes) can be as efficient as commercial transfection reagents while being less toxic. We show that using s1mplexes, we are able to increase HDR correction efficiencies by up to 10-fold in human cells. Finally, we show the utility of stem cell models for testing of genome editing strategies. Nanocapsules are efficiently able to deliver genome editing machinery into the eye (See figure below). Our results show that by careful choice of genomic editing approach, tool and delivery method, and tailoring them to the specific mutation in a personalized fashion, it is possible to formulate very promising genome editing platforms for ocular therapies that overcome many of the challenges currently associated with them. This is demonstrated in model systems for both autosomal dominant and recessive mutations with nonviral gene editing strategies.

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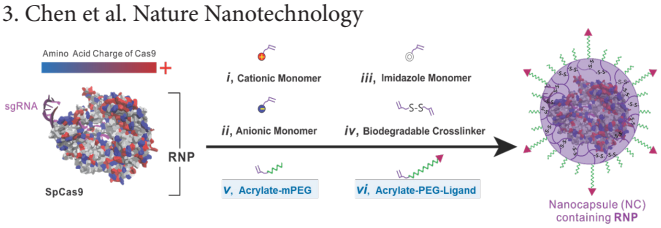

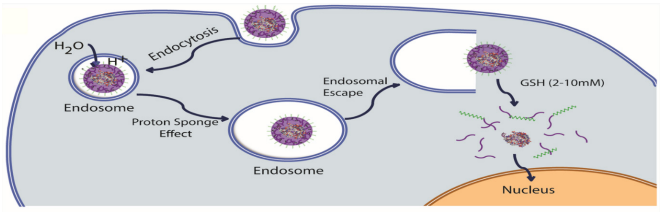

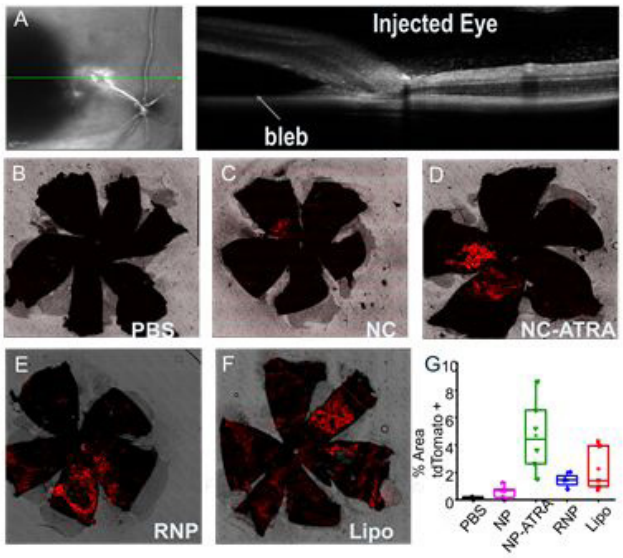

(A) OCT image demonstrates bleb formation for subretinal delivery. Flat mount RPE images showing tdTomato fluorescence after editing by PBS **(B)**, NC **(C)**, NC-tar **(D)**, RNP **(E)** and lipofectamine **(F)**. **(G)** Average % area of tdTomatto expression in injected eyes.

535. Building a Versatile and Clinically Translatable Platform for DNA-Based Antibody Therapeutics

Kevin Hollevoet[1], Elien De Smidt[2], Giles Vermeire[1], Liesl Jacobs[1], Stéphanie De Vleeschauwer[3], Nick Geukens[2], Paul Declerck[1]

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DNA-based antibody gene transfer aims to administer to patients the antibody-encoding nucleotides, rather than the antibody protein. This allows for a cost-effective and prolonged *in vivo* production, addressing some of the bottlenecks of conventional antibody therapy. This abstract outlines the progress our research group made towards building a versatile and clinically translatable DNA platform, using anti-cancer antibodies as a model. First, we optimized an intramuscular electroporation protocol for mice. Electroporation is the preclinical and clinical delivery approach of choice for plasmid DNA, allowing efficient and safe uptake into the target tissue. For each of the administered DNA-based antibodies, several months of *in vivo* expression was obtained. Attained antibody plasma concentrations were in the microgram per milliliter range and gave significant anti-tumor responses, demonstrating therapeutic relevance. Building on these promising results, we sought to expand the application of the DNA platform, both in terms of payload and administration route. In addition to full-length antibodies, we explored the *in vivo* expression of nanobodies, small and modular single-domain antibody

fragments that are typically cleared fast from circulation. Intramuscular electrotransfer of DNA-based multivalent and bispecific nanobodies in mice led to detectable expression for several months, enabling potent tumor regressions. Both in terms of pharmacokinetics and efficacy, DNA-based nanobodies outperformed their protein counterparts. Beside muscle, we explored the tumor as site of delivery, using DNA-based checkpoint inhibitors. When delivered systemically as proteins, these antibodies carry a high risk of side effects. Both intratumoral and intramuscular administration gave anti-tumor responses in a mouse model. Intratumoral administration, however, demonstrated a lower systemic antibody exposure, while conserving systemic efficacy. The resulting pharmacokinetic profile potentially provides a better safety profile. An additional focus of our research program is clinical translation, both exploring and improving the fit with human subjects. We thereto set up studies in 40-70 kg sheep, a relevant clinical model in terms of body weight, musculature and blood volume. Intramuscular administration of fully ovine DNA-based antibodies was done using a clinical-grade electroporation setup. Expressed antibodies were detected at microgram per milliliter plasma levels for several months, highlighting the clinical potential. The above results were all obtained with a conventional first-generation plasmid, which includes a bacterial backbone with antibiotic resistance gene and an expression cassette driven by a ubiquitous promoter. To align with regulatory recommendations and improve expression specificity, we evaluated minimal plasmids, which lack a bacterial backbone, and newly designed muscle-specific promoters, respectively. In mice, minimal plasmids gave an antibody expression similar to conventional plasmids, whereas the muscle-specific promoters were up to 50% more potent than ubiquitous promoters. These features were combined into a second-generation plasmid, resulting in improved antibody expression and biosafety. In conclusion, the presented results pave the way to a broadly and clinically applicable platform for DNA-based antibody therapeutics.

536. Novel Hybridization Approach for Loading of Extracellular Vesicles with Therapeutic RNA for Functional Delivery *In Vitro* and *In Vivo*

Olga Shatnyeva[1], Kristina P. Friis[2], Pinar Akcakaya[1], Michelle Porritt[1], Sasha Berdichevski[3], Elisa Lazaro-Ibáñez[1], Lavaniya Kunalingam[1], Xabier Osteikoetxea[1], Andreia Silva[1], Priya Sharma[1], Alexander Kapustin[2], Marcello Maresca[1], John Wiseman[1], Mohammad Bohlooly[1], Frank Seeliger[4], Camilla Johansson[5], Niek Dekker[1]

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RNA-based therapeutics have great potential to target undruggable genetic diseases, however drug delivery and RNA stability remain a challenge. Standard formulations for RNA therapeutics, such as lipid nanoparticles (LNP) and Adeno-associated virus -based vehicles are limited by the immune response in the host. Extracellular vesicles (EVs, aka. exosomes) have emerged as an attractive delivery vehicle for RNA therapeutics due to their non-toxic and non-immunogenic properties. Current approaches for RNA loading into EVs suffer from poor efficiency explaining the need for appropriate loading approaches. Here we demonstrate a novel and efficient approach for loading of RNA into EVs with examples of safe and efficacious delivery of mRNA and sgRNA. Fluorescently labelled RNA was introduced into bioengineered EVs using RNA-loaded LNP donor particles by pH-induced fusion. RNA encapsulation efficiency of resulted hybrid EVs (HEVs) was in the range of 20-40% which is a significant improvement in comparison with existing techniques. Functional delivery of Cre mRNA by HEVs using *in vitro* and *in vivo* assays was efficient in direct comparison to RNA delivery by LNPs. Intravenous delivery of HEVs (0.5 mg/kg) to Cre reporter mice showed efficient delivery to the liver without any measurable inflammatory response or tissue toxicity. Delivery of sgRNA formulated in HEVs to a transgenic mouse expressing human *PCSK9*. Cas9 was introduced separately by adenoviral delivery. Efficacy of gene editing was 15% *(*i.v. dose at 1.5 mg/kg without any significant safety responses. Overall, we have developed a novel approach for therapeutic RNA cargo loading into EVs. The method is efficient and safe for *in vivo* delivery. We envision that this approach will be useful for loading of RNA for a range of therapeutic applications.

537. Optimization of Lipid Nanoparticle Formulation Leads to Functional mRNA Delivery in Rat Lungs and New Opportunities for Gene Editing and Delivery

Kristina Friis[1], Lavaniya Kunalingam[1], Steve Oag[1], Sandra Gracin[1], John Wiseman[1], Alan Sabirsh[1], Alexandra Leijon[1], Elin Sand[1], Botilda Lindberg[1], Astrid Collin[1], Elisa Lázaro-Ibáñez[1], Kathryn Whitehead[2], Annette Bak[1]

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Gene therapy, based on delivering mRNA, sgRNA or siRNA for in situ protein expression, gene editing or RNA silencing, offers new hope for patients with unmet medical needs, especially for rare diseases with a genetic component. A major challenge when developing new nucleic acid-based drugs is the design of efficacious and safe delivery vehicles, with additional technical complexities and safety considerations for lung applications. To address these needs, we have synthesized a range of novel, biodegradable cationic lipids suitable for the delivery of inhaled nanomedicines and gene therapy. Lipid nanoparticles (LNPs) are used clinically as delivery vehicles because they protect the RNA cargo while facilitating cell uptake and endosomal escape. Typically, LNPs consist of a cationic lipid, which entrap the anionic RNA and enhance membrane transport, as well as several stabilizing lipids. The efficacy of this non-viral delivery approach depends on the ratios between the various lipids and the RNA cargo and how the particles interact with cells during uptake and intracellular processing. To date, an empirical, manual formulation development approach has been used to evaluate how novel lipids affect LNP performance. This work describes the optimization of novel nanomedicines using a high-

throughput, Design-Make-Test-Analyze (DMTA) cycle, an essential method for candidate drug refinement within the pharmaceutical industry. This miniaturized, automated, in vitro cell function testing ensures a fast and successful selection of optimal LNP formulations for novel lipid components, leading to 97-99% encapsulation and protection of the precious mRNA cargo and, in vitro transfection efficiencies of up to 100% as early as 6 hours post-dosing. In addition, many cell types can be quickly evaluated, and efficient mRNA delivery and protein expression is observed even in primary cells. Additional readouts, including particle uptake and cellular toxicity, can be easily added, creating a powerful, multivariate optimization system based on functional data. In vivo evaluation is ongoing and preliminary data indicates these new candidates for non-viral delivery perform well when compared with Adeno-associated virus delivery systems. Finally, this high throughput approach identified LNP particles with sufficient stability to be spray dried, creating a dry powder formulation suitable for inhalation devices, whilst preserving the functionality of the mRNA cargo. This allowed us to successfully administer the nucleic acid-loaded drug delivery system directly to the rat lung, with good protein translation and without inducing treatment-related inflammatory changes. In summary, this novel approach allows us to quickly and efficiently prepare and optimize new LNP delivery vehicles for nucleic acid-based therapeutics for in vivo testing within a few weeks from identifying a new target, and this independently of the delivery route (inhaled or systemic).

Vector and Cell Engineering, Production or Manufacturing III

538. Generation of Immune Resistant, Stem Cell Targeting Lentivirus for Cystic Fibrosis Airway Gene Therapy

Juliette Delhove[1,2], Martin Donnelley[1,2], David Parsons[1,2]

[1]Robinson Research Institute and Adelaide Medical School, University of Adelaide, Adelaide, Australia,[2]Respiratory and Sleep Medicine, Women's and Children's Hospital, Adelaide, Australia

Background: Cystic fibrosis (CF) is a recessive genetic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene and protein. CFTR is responsible for maintaining lung health by keeping the airways hydrated. Inadequate CFTR function results in lung surface dehydration leading to chronic infections, inflammation, and premature death, most commonly due to respiratory failure. Lentiviral (LV) gene therapy can be used to insert a correct copy of CFTR into the genome, with long-lasting expression resulting from transduction of basal stem cells. Current challenges that inhibit clinical translation of LV gene therapy for CF include vector detection and inactivation by the immune system, off-target transduction of pulmonary macrophages, and broad cell targeting leading to non-specific transduction of non-therapeutic cell types. Our aim was to implement multiple approaches to overcome each of these constraints. **Methods:** An immune resistant HEK293T vector production cell line was produced by CRISPR knockout of the immunogenic B2M locus followed by overexpression of the CD47 (anti-phagocytic) and CD55 (anti-complement mediated decay) receptors. To enable improved vector long-term therapeutic efficacy, a novel VSV-G envelope was generated through insertion of an arginine-glycine-aspartic acid (RGD) motif that has a high affinity for the receptors on basal stem cells. **Results:** LV particles generated from the HEK293T$^{B2m-/-, CD47High, CD55High}$ cell line demonstrated decreased immune inactivation and detection *in vitro*. The insertion of RGD did not interfere with viral production or transducibility (Fig 1).

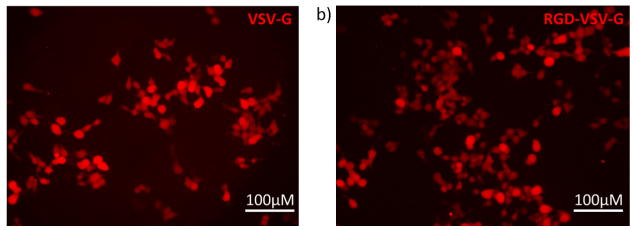

Fig 1. Expression of mScarlet following transduction using either a) unmodified VSV-G or b) VSV-G containing RGD. **Conclusions:** The HEK293T$^{B2m-/-, CD47High, CD55High}$ vector-producing cell line is effective *in vitro* at reducing immune cell uptake, detection, and inactivation and could provide improved transduction outcomes and targeting *in vivo*. Combating these critical obstacles should accelerate progress in gene therapy treatment for this life-limiting disease. **Acknowledgements:** Project funding provided to JD by the Women's and Children's Hospital Research Foundation.

539. HeLa 3.0: CRISPR Knockout of Genes Modulating Titer in Established rAAV-Producing Cell Lines

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Research, Ultragenyx Gene Therapy, Cambridge, MA

Utilizing an RNA-seq directed screening method, we have previously demonstrated that siRNA knockdown of specific cellular genes during production of rAAV in HeLa producer cell lines (PCLs) improved titer 2-4 fold. This approach, however, would be challenging to implement in an industrial manufacturing setting at the 2000L scale, which is a critical component of ensuring patient access as gene therapy programs continue to progress from Phase I/II to Phase III clinical trials and commercialization. To address this need, we have genetically modified two existing, highly optimized monoclonal HeLa PCLs generated for separate clinical programs. The KCNN2 gene (previously identified in our RNA-seq screen), which encodes a calcium-activated potassium channel protein, SK2, was knocked out *via* CRISPR/Cas9-mediated genome editing. Importantly, following SK2 knockout up to 3-fold increases in titer were observed in both of these already high titer producing HeLa PCLs. We next performed multi-combinatorial siRNA knockdowns of genes previously identified by our RNA-seq directed screening method to determine if targeting multiple genes simultaneously would produce an additive effect on titer. Increases in titer ranging from 3-11 fold were observed following double siRNA knockdowns, suggesting additional titer can be gained from established high rAAV-titer-producing monoclonal PCLs through direct targeting of multiple genomic regions.

540. A Complete "Self-Repressing" Adenovirus System Enables Efficient Manufacture of Adeno-Associated Viral Vectors without Contamination by Adenovirus or Small Drugs

Weiheng Su[1], Margaret Duffy[1], Len Seymour[1], Ryan Cawood[2]

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Adeno-associated virus (AAV) shows great promise as a gene therapy vector, however current manufacturing regimes cannot scale to meet increasing demands. Here we describe a new synthetic biology approach to regulate the 'helper' adenovirus replicative life cycle to allow the clean and efficient manufacture of AAV vectors. We achieved this by genetic manipulation of the adenovirus Major Late Promoter (MLP) *in situ* to enable temporal regulation of its late proteins using a tetracycline-controlled system. Modification of the adenovirus MLP *in situ* to provide inducible repression has not previously been demonstrated, primarily because the virus DNA polymerase coding sequence is in the opposing DNA strand. We show that strategic insertion of tetracycline repressor binding sites into the MLP and encoding the tetracycline repressor under its transcriptional control, allows for normal adenovirus replication in the presence of doxycycline but only enhanced genome amplification and early gene expression (important for the 'helper' functions) in its absence. Using this negative feedback self-repression system, we demonstrated delivery of adenoviral 'helper' functions, AAV Rep Cap genes, and the AAV transfer vector to yield >100-fold increases in AAV production and >6e5 AAV particles produced per cell. This complete transfection-free 'self-repressing' helper-vector system produces AAV particles with improve encapsulation and viral transduction, and a reduction in contaminating adenoviruses by up to 2.5 million-fold. We propose this as a high-yielding contaminant-free production system suitable for scalable AAV manufacture.

541. Massively Parallel Deep Diversification of AAV Capsid Proteins by Machine Learning

Drew Bryant[1], Ali Bashir[1], Sam Sinai[2,3,4], Nina Jain[4,5], Pierce Ogden[4,5], Patrick Riley[1], George M. Church[4,5], Lucy Colwell[1], Eric Kelsic[2,4]

[1]Google Research, Mountain View, CA,[2]Dyno Therapeutics, Cambridge, MA,[3]Harvard University, Cambridge, MA,[4]Wyss Institute for Biologically Inspired Engineering, Boston, MA,[5]Harvard Medical School, Boston, MA

Synthetic AAV vectors that retain the ability to package DNA payloads and transduce cells while evading the humoral immune system are urgently required. Natural AAV variants show significant diversity in their tissue tropism and sequence however their application as gene therapy vectors is hindered due to pre-existing immunity. Engineering synthetic AAV capsids that are significantly different from natural serotypes can remedy this problem by removing potential epitopes. Previous engineering strategies have had limited success at overcoming antibody neutralization because the resultant sequences remain quite similar to natural isolates. New technologies offer a qualitatively distinct opportunity: high-throughput DNA synthesis and sequencing technologies allow thousands of designed sequences to be assayed in parallel, enabling diversification guided by machine learning models that relate protein sequence to function without detailed biophysical or mechanistic modeling. Here, we applied deep learning to robustly design and synthesize a diverse set of novel adeno-associated virus (AAV) capsid proteins, far exceeding the diversity of the natural repertoire. Targeting a region of AAV2 encompassing buried, surface and interface regions, our iterative machine learning design approach was successful at generating diverse and viable sequence variants. While natural AAV serotypes differ from WT AAV2 at μ=12±6 positions, we generated 57,348 variants surpassing the average diversity of natural sequences with 12-29 mutations across the 28 amino acid target region. Among samples within 6 mutations from WT, our best models could predict with nearly 100% accuracy whether a randomly generated variant would package. While none of our tested randomly designed sequences were viable with >6 mutations, when machine learning models were used to propose samples with >12 mutations, 58.1% of these proposed sequences were viable, including variants with up to 19 substitutions or 15 insertions. Furthermore, our neural network models were 33 times more likely to be viable than sequences designed by simpler additive models at 18 mutations from WT, with even greater improvements at larger distances. Notably, while simple models like additive and logistic regression demonstrated high precision only for some training sets, the performance of neural network models were robust to variations in the amount and composition of training data. Additionally, neural network models were able to generate far more diversity than simpler models at a given level of precision, which is critical for AAV engineering since such diversity is key for potential gain of function mutations. By applying these techniques, we discovered hundreds of viable clusters of synthetic capsids distinct from any natural AAV. This work highlights the potential of deep learning approaches to utilize high-throughput data and search a broad space of putative AAV capsid sequences, enabling in-silico discovery of millions of viable synthetic capsids, which can then be further improved (through the same mechanisms) for delivery, safety and manufacturability. Furthermore, these results demonstrate the broad utility of machine learning models for genetic engineering, yielding general lessons that can directly applicable to optimizing any viral capsid or genetic payload.

542. Abstract Withdrawn

543. Characterization of Phenotypic and Genotypic Stability of rAAV Producing HeLa Cell Lines

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As rAAV gene therapy trials progress from Phase I/II to Phase III clinical trials and commercialization, it is essential to develop and establish industrial manufacturing processes that consistently produce large amounts of vector in a cost-effective manner to ensure affordable patient access. At Ultragenyx Gene Therapy, the HeLaS3 producer cell line (PCL) system has shown successful scalability up to 2000L with high yields and more favorable empty: full capsid ratios than

the traditional HEK293 triple transfection system. Interestingly, integration of the required rAAV components (vector genome, Rep and Cap) into the AAVS1 site typically yields the highest producing PCLs. HeLa-based manufacturing campaigns require that these PCLs maintain phenotypic and genotypic stability for at least 10 weeks, the time required to progress from cell thaw to outgrowth and rAAV production. To investigate PCL stability, research cell banks (RCBs) of monoclonal HeLa PCLs were thawed and passaged over a 16 week period to provide an additional 6 weeks of de-risking following the standard 10 week production time. Cells were sampled every 4 weeks and assessed *via* phenotypic characterization of cell growth, productivity, and viral rep/cap protein expression during production. Assessments also included genotypic characterization of integrated transgene and viral gene copy numbers. Minor variances in phenotypic stability were detected in certain PCLs, but were not representative of all producer cell lines. Importantly, no indication of genotypic instability was observed as integrated genome copies of both the transgene and viral genes remained constant at each time point. Targeted locus amplification (TLA) NGS analysis further confirmed genotypic stability of each PCL during the 16-week study period. Taken together, these data demonstrate that rAAV-producing HeLa cell lines maintain the phenotypic and genotypic stability required to facilitate and support industrial scale manufacture of gene therapy products.

544. Benchmarking of FLT210, A Potent Next Generation AAV-hFVIII Vector Candidate with Superior Quantity and Quality Characteristics, Against a Known FVIII-SQ Comparator

Andreas Schulze[1], Bettina Prieler[1], Martina Ohme[1], Britta Voland[1], Sandra Lange[1], Florian Sonntag[1], Nicole Spada[1], Marina Magerl[1], Felicia Thoennissen[1], Renée Kober[1], Samantha Correia[2], Azadeh Kia[2], Fabrizio Comper[2], Romuald Corbau[2], Markus Hörer[1]

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First-generation gene therapies for Haemophilia A exhibited large patient-to-patient variations in FVIII levels and a lack of durability that may at least in part be caused by the large size of the FVIII expression cassette, resulting in vector genome sizes exceeding that of the wild type AAV genome. FREELINE previously presented the design and characterization of FLT210, a next generation AAV-FVIII cassette, which we believe possesses the attributes required to become the best-in-class vector to treat Haemophilia A patients (ASH annual meeting, 2019). From the perspective of balancing size and potency, several methods were deployed - including in silico modelling, optimization of the coding sequence, implementation of a novel small liver-specific promoter, and sequence deletions - to increase the quality and activity of the resulting product and the predictability of FVIII expression. These efforts resulted in the design of our FVIII lead candidate FLT210 (4.71 kb), that demonstrated similar levels of FVIII expression relative to our non-optimized FVIII-SQ equivalent (4.85 kb) and a known comparator FVIII-SQ construct (4.97 kb) in wild type mice. Here we present data from an in vitro comparability study, in which FLT210 was analysed side by side to a known comparator FVIII-SQ construct using the FREELINE manufacturing platform and applying our proprietary toolbox for molecular characterization with a focus on different CMC-related quality attributes. We will provide data demonstrating the superiority of our lead candidate in terms of yields, DNA mispackaging levels and proportion of full-length vector genomes, the latter assessed by an alkaline gel electrophoresis assay. We think that obtained vector yields and vector genome to total particle ratios in combination with the improved vector quality attributes enable FREELINE to develop a state of the art Haemophilia A gene therapy program.

HSPC Gene Therapies for Hemoglobin Disorders

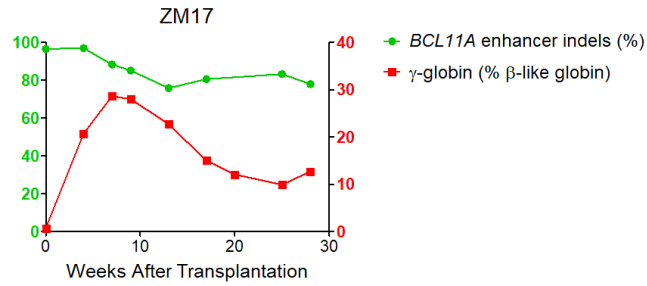
545. Durable Robust Fetal Globin Induction in Rhesus Monkeys Following *BCL11A* Erythroid Enhancer Edited Autologous Hematopoietic Stem Cell Transplant

Selami Demirci[1], Jing Zeng[2], Yuxuan Wu[2,3], Naoya Uchida[1], Jackson Gamer[1], Morgan Yapundich[1], Claire Drysdale[1], Anne H. Shen[2], Aylin C. Bonifacino[4], Allen E. Krouse[4], Nathaniel S. Linde[4], Theresa Engels[4], Robert E. Donahue[1], Juan J. Haro-Mora[1], Alexis Leonard[1], Tina Nassehi[1], Kevin Luk[5], Scot A. Wolfe[5], Cicera R. Lazzarotto[6], Shengdar Q. Tsai[6], Mitchell J. Weiss[6], Daniel E. Bauer[2], John F. Tisdale[1]

[1]CMTB/NHLBI, NIH, Bethesda, MD,[2]Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA,[3]Shanghai Key Laboratory of Regulatory Biology, ECNU, Shanghai, China,[4]TSCB/NHLBI, NIH, Bethesda, MD,[5]MCCB, UMass Medical School, Worcester, MA,[6]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN

Elevated fetal hemoglobin (HbF, $\alpha_2\gamma_2$) levels are beneficial for patients with β- hemoglobinopathies. Here, we evaluated engraftment and HbF induction potential of erythroid-specific *BCL11A* enhancer (+58 DHS) edited CD34+ HSPCs in a non-human primate transplantation model in which hemoglobin switching is conserved. Our initial pilot experiments resulted in modestly efficient gene editing at rates that stabilized 12 weeks after transplantation, persisting up to 101 weeks (~3-6% indels), suggesting no detrimental impact of *BCL11A* enhancer editing on hematopoietic fitness. With refined methods using 3xNLS-SpCas9 RNP electroporation providing >90% on-target indels, we evaluated engraftment and HbF induction potential of highly efficient *BCL11A* enhancer editing in two additional macaques (ZM26 and ZM17). ~95% indels and ~65-78% γ-globin protein production after ex vivo erythroid culture were noted in the infusion products. Animals (indels of 12-83% up to 46 weeks) displayed robust γ-globin induction early post-transplantation (8.1-28.6%) followed by a reduction and stabilization. There was a significant correlation between γ-globin level and indel frequency for all 4 transplanted animals ($R2=0.77$, $p<0.01$). In ZM17 (~80% indels and ~12% γ-globin at ~24 weeks), edits ranged from 78-81% across all PB/BM lineages (excluding CD3+ cells with 63% indels). All animals displayed normal blood parameters and had

a normal PB smear, indicating absence of erythroid toxicity. To test the correlation between stress erythropoiesis and *BCL11A* enhancer editing, we performed phlebotomy on two recipients (ZM26 and ZL25) in addition to four control animals. We observed partial anemia and elevated reticulocyte counts following phlebotomy, indicating accelerated erythropoiesis. ZM26, with ~6% indels mainly within biallelically edited clones, displayed significant γ-globin induction (from 0.6% to 4.4%) while ZL25, with ~3% indels mainly within monoallelically edited clones (from 0.4% to 1%), and control animals (from ~0.2-0.3% to ~0.5-0.7%) did not show substantial γ-globin induction, suggesting a positive interaction between reduced *BCL11A* expression and stress erythropoiesis with regard to γ-globin induction. Deep sequencing analysis of engrafting edited cells revealed that NHEJ repair alleles were preferentially retained and MMEJ alleles were lost in engrafting HSCs as compared to input cells. The NHEJ indel pattern was consistent with stable polyclonal hematopoietic engraftment. In summary, autologous *BCL11A* enhancer edited HSCs can persist long-term and provide potentially therapeutic levels of HbF without hematologic toxicity in rhesus macaques. The frequency of durable hematopoietic gene editing depends upon the efficiency of both the gene modification and autologous engraftment.



546. *In Vivo* HSC Gene Therapy for Hemoglobinopathies: A Proof of Concept Evaluation in Rhesus Macaques

Chang Li[1], Hongjie Wang[1], Audrey Germond[2], Alan Ung[2], Kate Guerriero[2], Jason Ogle[2], Veronica Nelson[3], Dean Jeffrey[2], Cari Coles[4], Aphrodite Georgakopoulou[1], Evangelia Yannaki[5], Thalia Papayannopoulou[1], Zsuzsanna Izsvak[6], Hans-Peter Kiem[3], Andre Lieber[1]

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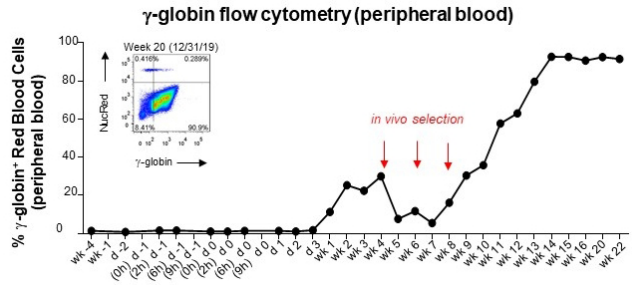
Current gene therapy or genome editing studies for hemoglobinopathies require highly sophisticated medical facilities to perform hematopoietic stem cell (HSC) collections/selections and genetic modifications. In addition, patients receive high-dose chemotherapy to facilitate engraftment of gene-modified cells. Thus, current gene therapy protocols will not be accessible to most patients suffering from hemoglobinopathies. Here we describe a highly portable and scalable approach using *in vivo* HSC gene therapy to potentially overcome these limitations. The central idea of our *in vivo* HSC gene therapy approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus HDAd5/35++ gene transfer vector system. Transduced cells return to the bone marrow where they persist long-term. Transgene integration is either achieved by a *Sleeping Beauty* transposase (SB100x) in a random pattern or by homology-directed-repair into a safe genomic harbor site. Currently, an *in vivo* selection system (involving the $mgmt^{P140K}$ gene/low-dose O^6BG/BCNU) is employed to achieve 80-100% marking levels in peripheral blood cells. We demonstrated safety and efficacy of our approach in mouse models for thalassemia intermedia and hemophilia A, where we achieved a phenotypic correction. We now present data in rhesus macaques. The first animal was followed for 22 weeks after *in vivo* HSC transduction with a human-γ-globin expressing HDAd5/35++ vector using SB100x for integration. Treatment with G-CSF/AMD3100 resulted in efficient HSC mobilization. Intravenous injection of the HDAd5/35++ vector system (total $1.5x10^{12}$ vp/kg, in two doses) into mobilized animals was well tolerated after pretreatment with steroids and the IL-6 receptor inhibitor, tocilizumab. After *in vivo* selection, gamma-globin marking in peripheral red blood cells rose to ~90% and was stable during the study duration (see Figure). Gamma-globin levels in red blood cells were ~18% of adult alpha1-globin (by HPLC). Enrichment of gene modified cells by in vivo selection was also reflected by an increase in mgtm and gamma-globin mRNA levels. There were no histological abnormalities at necropsy (week 22). Two more animals will be treated in February. Our data suggest that acute responses to the vector injection can be prevented with appropriate pretreatment. This is the first proof-of-concept study that *in vivo* HSC gene therapy could be feasible in humans and provide the necessary portability and accessibility to reach patients in places with limited medical resources. Ongoing studies involve the optimization of HSC mobilization, gene transfer vectors, and *in vivo* selection.



547. Base Editing of Gamma Globin Gene Promoters Generates Durable Expression of Fetal Hemoglobin for the Treatment of Sickle Cell Disease

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Sickle cell disease (SCD) is a monogenic disorder affecting beta globin function, leading to severe anemia and progressive multiple organ failure. A promising treatment for SCD is the re-expression of fetal hemoglobin (HbF), which occurs naturally in individuals with hereditary persistence of fetal hemoglobin (HPFH). SCD patients harboring natural genetic variations in the gamma globin gene promotors, *HBG1* and *HBG2* (*HBG1/2*), display elevated HbF levels and significantly fewer complications from SCD. Using adenine base editors (ABEs), we sought to generate single nucleotide polymorphisms in human CD34+ hematopoietic stem and progenitor cells (HSPCs) to recreate the HPFH phenotype as a treatment for SCD. We have previously demonstrated that ABEs could target the *HBG1/2* gene promoters in CD34+ cells with editing rates in excess of 90%. Here, we show that editing of the *HBG1/2* gene promoters was achieved in a dose-dependent manner following erythroid differentiation of CD34-derived cells electroporated with ABE mRNA and guide RNA (gRNA). Base editing occurred without compromising erythroid maturation and highly correlated with gamma globin production (R^2=0.99). These data suggest that >60% gamma globin induction could be achieved during optimal base editing *in vitro*. In addition, >80% base editing was observed in *in vitro* differentiated erythroid cells from a homozygous sickle cell donor, resulting in upregulation (>60%) of gamma globin with a concomitant decrease in sickle beta globin. To evaluate long-term engraftment potential, *ex vivo* edited human mobilized peripheral blood CD34+ HSPCs were transplanted into an immunocompromised mouse model (NBSGW). Both unedited and *HBG1/2* promoter-edited HSPCs achieved high human chimerism in the bone marrow (>90%). High levels of editing (>90%) occurred at the *HBG1/2* gene promoters and multi-lineage cell differentiation was observed at 16 weeks post-transplantation. These data suggest that edited hematopoietic stem cells were sustained *in vivo* and functionally contribute towards multi-lineage hematopoietic reconstitution. We sorted human erythroid cells from the bone marrow of 16 week post-transplanted mice and found that gamma globin levels were >65% compared to unedited cells (<1.5%). These findings indicate that precise editing in human CD34+ HSPCs produce durable, therapeutically relevant gamma globin protein expression in erythroid progeny generated *in vivo*. Similar results were achieved using human CD34+ HSPCs from a second mobilized peripheral blood donor at 18 weeks post-engraftment. Taken together, we have demonstrated that *ex vivo* delivery of ABE mRNA and gRNA achieves precise editing of nucleotide base residues within the *HBG1/2* gene promoters of human CD34+ HSPCs, which resulted in long-term engraftment and significant gamma globin protein expression *in vivo*. Our study serves as a proof-of-concept for a promising, novel strategy for the treatment of SCD.

548. Gene Correction Using CRISPR/Cas9: IND-Enabling Studies to Support a Clinical Trial of a CRISPR/Homology-Directed Repair Treatment for Sickle Cell Disease

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Sickle Cell Disease (SCD), one of the world's most common genetic disorders, causes anemia and progressive multiorgan damage that typically shortens lifespan by decades. SCD affects nearly 100,000 individuals in the United States, and many more worldwide. Allogeneic bone marrow transplant is currently the only cure, but access to transplant is limited by donor availability. A universal curative therapy for SCD would address a critical unmet medical need in the United States and worldwide. We have developed a CRISPR/Cas9-based strategy to correct the mutation in CD34+ HSPCs harvested from SCD patients following mobilization with plerixafor. This technique does not rely on viral vectors that can be challenging to manufacture, instead using synthetic reagents: a Cas9 ribonucleoprotein (RNP) targeting the sickle mutation, and a short single-stranded DNA to program gene correction via homology-directed repair (HDR). In a proof-of-concept study, plerixafor-mobilized CD34+ cells homozygous for the sickle mutation were corrected at an average of 33.6% of sickle alleles, declining to 23% in bone marrow after 4 months engraftment in immunodeficient mice (strain NBSGW). Observations of mixed-donor chimerism suggest that this level of correction may be beneficial in sickle cell disease patients. Analysis of indel allele diversity at *HBB* revealed polyclonal engraftment of edited cells. While ~30% of xenografted CD34+ cells contain at least one corrected, allele, 57% of cells contain two indel alleles. RNA-seq analysis suggest that indel/indel erythroblasts have a ß-thalassemia-like phenotype, and these cells may be eliminated *in vivo* due to the ineffective erythropoiesis that is a hallmark of ß-thalassemia. The investigational procedure used for proof-of-concept studies was adapted to clinical scale (~1 billion CD34+ cells) through a series of process development studies to optimize reagent dosing, electroporation conditions, and cellular density, before transfer to a cGMP manufacturing environment. cGMP development runs met predetermined release criteria, and the first run was used for a toxicology study in NBSGW mice. These products maintained gene editing and engraftment potential *in vivo* in mouse xenografts. Off-targets of the Cas9 RNP were identified using a combination of GUIDE-seq and bioinformatic search. GUIDE-seq

identified two off-target loci, both of them intergenic. These loci, along with 186 computationally predicted loci, were amplified using a combination of individual and pooled-primer PCR. Only the on-target site and one GUIDE-seq-identified off-target site had >0.2% indel alleles compared to untreated control cells. In sum, use of the high-fidelity Cas9 reduced off-target editing by 20-fold with no loss in on-target editing. These studies will be used to support an IND filing in 2020 to initiate a Phase I clinical study of this approach, the first of its kind using CRISPR/Cas9-mediated homology-directed repair in hematopoietic stem/progenitor cells.

549. Editing the LRF Repressor Binding Site in the γ-Globin Promoters Induces Therapeutically Relevant Fetal Hemoglobin Levels for the Treatment of β-Hemoglobinopathies

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β-hemoglobinopathies are genetic anemias caused by a reduced or abnormal synthesis of the adult β-globin chain. In β-thalassemia, the reduced (β+) or absent (β0) production of adult β-chains causes α-globin precipitation and death of red blood cell (RBC) precursors. In sickle cell disease (SCD), a single amino acid change ($\beta6^{Glu\rightarrow Val}$) in the adult hemoglobin (Hb) β^S-chain causes Hb polymerization with consequent red blood cell (RBC) sickling, vaso-occlusive crises, organ damage and reduced life expectancy. The co-inheritance of genetic mutations causing a sustained fetal γ-globin chain production in adult life (hereditary persistence of fetal hemoglobin, HPFH) reduces the clinical severity of β-hemoglobinopathies. HPFH mutations in the promoter of the two γ-globin genes, *HBG1* and *HBG2* disrupt the binding sites (BS) for transcriptional repressors (e.g., BCL11A and LRF). Recently, we demonstrated that CRISPR/Cas9-mediated disruption of the LRF BS in the *HBG* promoters via non-homologous end joining and microhomology-mediated end joining (MMEJ) repair mechanisms mimics the effect of HPFH mutations by impairing the LRF binding and re-activating the γ-globin expression (*Weber, Frati et al., Science Advances, in press*). Efficient editing of the LRF BS (≥ 3 γ-globin promoters in >70% of SCD hematopoietic stem/progenitor cells (HSPCs)) resulted in a robust HbF reactivation and a concomitant reduction in β^S-globin levels recapitulating the phenotype of asymptomatic SCD-HPFH patients. RBCs derived from edited HSPCs displayed HbF levels sufficient to correct the SCD cell phenotype. Similarly, LRF BS targeting in β0-thalassemic cells results in HbF reactivation potentially correcting the α/β-like globin imbalance. Xenotransplantation of human HSPCs edited using several gRNAs targeting the LRF BS showed a robust engraftment of edited cells that were capable to differentiate into multiple lineages. *HBG* editing in engrafted cells ranged from 26% to 76% with a decrease (-33%) of editing events compared to the input HSPCs, partially due to a reduced occurrence of MMEJ-mediated events in HSCs. Erythroid progenitors (*BFU-E*) obtained *ex vivo* from engrafted human cells, showed a relevant γ-globin expression (~40% of the total β-like chain) despite of the reduction in the number of edited promoters per BFU-E after transplantation. Moreover, mature RBCs *ex vivo* differentiated from edited human cells ensure therapeutically relevant HbF levels. Sequencing of top-scoring off-targets identified by GUIDE-seq showed a relatively high off-target activity within an intergenic site devoid of known regulatory elements both *in vitro* and *in vivo* in primary human cells treated with one of the gRNAs targeting the LRF BS. Although the occurrence of this off-target event in repopulating cells suggests that it has no detrimental effect on HSC engraftment and multilineage differentiation, we tested high fidelity Cas9 variants to reduce off-target activity in primary HSPCs. Finally, we used CAST-Seq assay to evaluate the potential chromosomal rearrangements in edited primary human cells *in vitro* and *in vivo*. This work identifies the LRF BS as a novel, effective and safe therapeutic target for the treatment of β-hemoglobinopathies. Preclinical studies aimed at optimizing the genome editing protocol in HSCs in GMP conditions will allow the translation of this strategy to clinics.

550. Bioinformatic Guided Design of a Lentiviral Vector for X-Linked Chronic Granulomatous Diseases Mimics the Endogenous Expression Pattern and Level of the Native *CYBB* Gene

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Introduction: X-Linked Chronic Granulomatous Disease (X-CGD) is a primary immune deficiency caused by mutations in the *CYBB* gene which encodes for an essential catalytic subunit of the Phagocyte NADPH Oxidase (PHOX) complex. Deficiencies in the PHOX complex results in the inability of phagocytic cells to properly mount an immune response and as a result patient suffer from life-threatening bacterial and fungal infections. **Methods:** We employed a bioinformatic-guided approach to elucidate the endogenous elements that regulate the native *CYBB* locus to design a lentiviral vector possessing strict lineage and temporal specific expression. Our analysis revealed 15 putative endogenous elements contained within a 600kb topologically associated domain (TAD) regulating the native *CYBB* gene. A lentiviral library was constructed to functionally validate each putative enhancer element and an 8.8kb candidate lentiviral vector was designed containing the vital endogenous elements. Current genomic databases were then deployed to elucidate the minimally defined boundaries of each enhancer. When designing the new core elements, many variables were considered including 1) DNase1-accessibility, 2) transcription factor binding, 3) epigenetic histone modifications and 4) sequence conservation. These modifications led us to generate a new 5.9kb candidate vector with higher titer, infectivity and expression.

Results: Using this novel construct to express an mCitrine reporter, we demonstrated strict lineage specific expression *in-vitro* with high levels of expression in mature neutrophils and monocytes differentiated from human cord blood (CB) CD34+ hematopoietic stem and progenitor cells (HSPCs). High levels of expression were also seen in RAMOS (B-lymphocyte cell line) while no expression was detected in Jurkats (T-lymphocyte cell line). We then transduced human CB CD34+ HPSCs and transplanted the cells into NSG mice to demonstrate *in vivo* specific expression. At 16 weeks post-transplantation, mice were harvested and the expression of mCitrine was evaluated across different hematopoietic lineages. Our vector demonstrated high-level of expression in the mature neutrophils (CD66b+ CD15+ CD11b+ CD16+), higher but lower expression in the bulk myeloid cells (CD33+), high expression in the B-cell lineage (CD19+), while having minimal expression in either the T-cell lineage (CD3+) and the HSPCs (CD34+ CD90+ CD38-). To demonstrate functional correction, Lin- cells were harvested from a mouse model of X-CGD and were transduced with our lead therapeutic vector to express a codon optimized version of the therapeutic $Gp91^{phox}$ transgene. The transduced X-CGD Lin- cells were then differentiated *in-vitro* to produce mature neutrophils and restoration of oxidase activity was measured using the dihydrorhodamine and nitroblue tetrazolium assays. Neutrophils transduced with our vector was able to restore oxidase activity to wildtype levels in the murine model of X-CGD. **Conclusion:**Our bioinformatically designed vector demonstrated strict lineage-specific expression at wildtype levels and was able to correct a murine model of X-CGD. These findings validate our bioinformatic-based design approach and have yielded a novel lentiviral vector with promise to advance to the clinic for the treatment of X-CGD.

551. Editing Hematopoietic Stem Cells at the FOXP3 Locus: A Gene Editing Approach to Treat IPEX Patients

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Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare monogenic disorder due to mutations in FOXP3. The master transcriptional regulator, FOXP3, is required for development and survival of natural regulatory T cells (nTreg) , a T cell subset critical for maintaining immune tolerance. IPEX subjects suffer from early onset, progressive, multiorgan autoimmunity only partially responsive to aggressive pharmacological immunosuppression. Allogeneic hematopoietic stem cell transplantation can be curative; however, ongoing immunologic dysfunction and other transplant related complications lead to significant morbidity and mortality. HSC gene therapy using lentiviral vectors is being developed as a potential therapeutic strategy. This approach, however, will require LV that can precisely replicate the complex endogenous control elements within the FOXP3 locus capable of initiating and maintaining endogenous levels of FOXP3 expression while limiting genotoxicity risk due to the random nature of LV integration. An alternative therapeutic approach in IPEX is to harness the host cell DNA repair machinery to achieve targeted integration of a FOXP3 cDNA within the endogenous locus, thereby preserving the upstream and downstream sequence elements required for regulated expression. Based upon the robust in vivo survival advantage for nTreg in Foxp3 mutant mice and IPEX subjects, we predict that engraftment > 5% homology directed repair (HDR)-edited HSC would provide therapeutic benefit. In this study, we demonstrate efficient HDR-editing of FOXP3 in control and IPEX HSC using co-delivery of CRISPR/Cas9 (delivered as RNPs) and a recombinant AAV6 donor template in association with a low-density HSC culture protocol. We first established gRNAs and donor reporter cassettes to target exon 1 of the FOXP3 locus. A gRNA that mediated disruption in > 92% of the FOXP3 alleles in CD34+ cells was used for all subsequent experiments. Using healthy donor, mobilized peripheral blood CD34+ HSC, we observed average HDR-editing rates of ~40% in vitro (range 28-61%, 6 independent donors).Following adoptive transfer into NBSGW recipient mice, an average of 8% (range 1-29%) of human cells within the bone marrow maintained the HDR cassette at 16 wks post-transplant. Equivalent ratios of human cell engraftment and myeloid and lymphoid lineages were recovered in recipients of mock and HDR-edited cells indicating that editing did not perturb differentiation in vivo. Based on these findings, we next generated an HDR donor that introduced a promoter-less, codon optimized, FOXP3 cDNA cassette into the endogenous locus to enable accurate spatiotemporal regulation. Employing a ddPCR assay, we were able to show successful editing and recovery of the edited cells from the bone marrow of 85% of transplanted NBSGW mice. The average engraftment rates were observed to be 8% (range 0.4-17%). To directly assess HDR-editing rates in T lineage cells derived from HSCs, we used the OP9-Delta1 culture system to differentiate HDR-edited cord blood derived, CD34+ cells along the T cell lineage in vitro. After 14 days in culture, both CD4+ and CD8+ T lymphocyte populations were sorted and found to contain the desired cDNA edit. Finally, we isolated CD34+ cells from a small volume cord blood sample isolated at birth from an IPEX subject and successfully performed HDR-editing. Our combined data demonstrate first proof-of-principle HDR editing in control and IPEX patient CD34+ cells showing efficient FOXP3 editing in human HSC and sustained engraftment of HDR-edited HSC cells at levels predicted to provide clinical benefit. Taken together, these findings support pursuit of a gene editing approach as a potential long-term therapy for IPEX patients.

AAV Vectors - Virology and Vectorology

552. Evolving New AAV Strains that Demonstrate AAVR Independence

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A new generation of AAV capsids is emerging that promises improved transduction profiles in large animal models and potentially, a clinical setting. However, the underlying biology of why these engineered or evolved AAV variants are improved remains unexplored. We recently reported a novel synthetic AAV variant, AAVhum.8, derived by iterative evolution of AAV serotype 8, which displays markedly enhanced transduction of human hepatocytes in vitro and in vivo, as well as robust evasion of neutralizing antisera. To further our understanding of this

novel capsid, we investigated the infectious pathway of AAVhum.8. This synthetic variant is not dependent on common AAV glycan attachment factors, including heparan sulfate, sialic acid or galactose, which are utilized by natural AAV strains for cell surface attachment. Glycan-deficient cell lines and a panel of lectins suggest AAVhum.8 binds to the trimannosyl core of an N-linked glycan. While, the recently identified host factors GPR108 and RNF121 remain essential for transduction, we demonstrate that AAVhum.8 is independent of AAVR. Interestingly, while the AAVhum.8 capsid binds AAVR, transduction in different AAVR knockout (AAVR KO) cell lines is unaffected. From a structural perspective, we show that individual capsid surface epitopes can contribute to distinct host glycan usage. Further, grafting the novel sequences evolved on AAVhum.8 onto other serotypes (e.g., AAV6, AAV9, AAVrh.10) partially phenocopies the ability to transduce human hepatocyte cell lines in an AAVR-independent manner. Further, these new AAV strains retain their ability to utilize their original glycan attachment factors in a synergistic fashion. The ability of AAVhum.8 to efficiently transduce cells independently of AAVR suggests exploitation of a distinct cellular trafficking mechanism. Our studies lay the foundation for engineering and evolving new, synthetic AAVR-independent capsids, which expand the AAV vector toolkit for clinical applications.

553. Ex Vivo Characterization of Novel AAV Capsid Variants for Delivery of GJB2 Gene Therapy for Congenital Hearing Loss

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Background: Congenital hearing loss is the most common inherited sensory disorder. Of the over 100 genes associated with hereditary deafness, most are expressed within the cochlear duct of the inner ear, specifically within the mechanosensory organ of Corti, which is responsible for transducing mechanical sound stimuli into neurochemical signals. Mutations in genes expressed in cells of the cochlear lateral wall, which provide ionic and trophic support to the organ of Corti, can also contribute to congenital hearing loss. The GJB2 gene encodes the protein Connexin26 which forms gap junctions in multiple cell types of the cochlea and mutations in this gene represent the most common cause of genetic hearing loss. In order to develop gene therapies for GJB2-deficiency, AAV capsids which exhibit tropism in multiple cell types of the cochlea are needed. We conducted a head-to-head evaluation of novel and previously described AAV capsid variants in an optimized ex vivo whole organ cochlear explant model to assess cochlear tropism. **Methods:** P3-P4 Sprague-Dawley rat pups or P2-P3 mouse pups were used for all experiments. Whole organ cochlear explants that included the lateral wall in addition to the mechanosensory cells were established on Day 0, allowed to acclimate overnight, then were treated with AAV for 120 hours continuously or for 48 hours followed by washout and an additional 72 hours in media without AAV. Several previously characterized or novel AAV variants were tested at various titers; all capsids utilized the CBA promoter to drive the expression of a GFP reporter construct for rapid visualization of cellular transduction. Target cell types included the cochlear lateral wall, support cells of the organ of Corti as well as the spiral limbus. Explants were fixed and stained with phalloidin and anti-GFP. For evaluation of tropism, explants were imaged on a Zeiss LSM880 confocal microscope and GFP coverage was quantified. Potential cellular toxicity was also assessed in explants. **Results:** Initial optimization experiments identified that a 120-hour continuous treatment of cochlear explants with AAV provided the greatest levels of GFP coverage; therefore, all subsequent capsid screening was conducted using this protocol. None of the AAV capsids tested showed signs of toxicity within the organ of Corti cells. Comparative analysis among existing and novel capsids identified several with significant transduction efficiency throughout the cochlea, including several novel capsids with greater GFP coverage than previously published variants that have been widely used. Results also identified capsid-specific differences in tropism consistent with previous in vivo studies. **Conclusions:** Using an optimized ex vivo model for the assessment of AAV tropism within the cochlear duct, we identified novel AAV capsids with favorable tropism profiles for GJB2 gene therapy. Furthermore, tropism profiles for several of these novel capsids surpassed AAV variants that had been previously published for use in the inner ear. These results support the further development of an AAV-based gene therapy for GJB2-related hearing loss.

554. Development and Validation of a CE-SDS Method for Purity Determination of AAV8

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to move charged molecules through a gel matrix by means of an electric current. Although this method has been used for years, SDS-PAGE is a relatively poor technique for quantitative protein purity due to sample preparation artifacts, densitometry variability and resource requirements. Because of its automated, quantitative nature, capillary electrophoresis (CE) is now commonly used for recombinant protein purity analysis. For recombinant monoclonal antibody analysis, CE-SDS is now the preferred method industry wide. Encouraged by the regulatory success of monoclonal antibody purity by CE-SDS, analytical scientists are working toward utilizing CE-SDS to assess purity of AAV capsid proteins to ensure quality and safety of these products. This study provides DOE (design of experiment) details used in robustness studies as part of development, as well as parameters examined during full method validation. Here we demonstrate that CE-SDS can be accurately and reproducibly employed as a platform technique in a QC setting for monitoring purity of AAV capsid proteins in release samples. Most importantly, this technique can be used to obtain comprehensive understanding of fragment related characteristics of in process materials.

555. Evolution of Modified AAV in Rhesus Macaque Brain

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Strategies for the development of AAV variants including rational design and directed evolution have enabled discovery of AAV serotypes with tailored properties. However, these strategies generally use in vitro systems or mice, and results do not always translate to human use. Here, we report an ongoing directed AAV evolution screen in nonhuman primates (NHPs) to identify AAVs with therapeutically optimal brain transduction. We first generated peptide-modified AAV libraries by addition of random peptides into AAV1, AAV2, and AAV9, followed by two rounds of in vivo enrichment via delivery into the cerebrospinal fluid (CSF). The input library, and viral DNA recovered from 14 brain regions collected after each round, were subjected to NGS, along with RNA from the round 2 tissues. Our NGS data show successful enrichment of barcodes from AAV1, AAV2, and AAV9 libraries yielding different candidates enriched for each serotype. For AAV1 and AAV2, we found a range of top candidate sequences for each distinct tissue, with limited to no uniformity among top hits. Thus, for AAV1 and AAV2 libraries, the extent of enrichment for any given sequence was low, and it was variable amongst the different brain regions. In contrast, the same top hits were enriched for AAV9 across all tissues. One of these, "1999," was the top hit in all of the tissues tested in both DNA and RNA. Future single cell technologies will reveal the distinct cellular targeting characteristics of the top hits from each of the enriched AAV1, AAV2 and AAV9 candidates. Overall our approach provides a rapid method to develop tailored AAVs for transduction of primate brain.

556. Development of a Vectorized Antibody Platform for Liver and Skeletal Muscle Gene Transfer

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Vectorized antibody delivery with adeno-associated virus (AAV) gene transfer is a promising approach to treat a number of inherited, infectious, and complex diseases. However, immunological barriers including host anti-drug antibody (ADA) responses present a formidable challenge towards obtaining sustained therapeutic antibody expression and activity. In previous studies, ADA detection coincided with loss of antibody activity following intramuscular administration of AAV vectors regulated by ubiquitous or muscle-specific promoters. Additional data suggests that liver-specific expression of a vectorized antibody can prevent humoral immune responses via tolerance induction. While encouraging, liver-directed expression may diminish over time due to hepatic turnover and subsequent loss of the therapeutic transgene. To bypass such challenges and improve therapeutic outcomes, a recent report by Colella et al. utilized a tandem promoter system composed of liver and muscle-specific regulatory elements to prevent anti-transgene immunity and maintain durable levels of acid alpha-glucosidase (GAA) production in a murine model of Pompe disease. We hypothesized that such promoter systems may provide similar advantages in the context of vectorized antibody gene transfer. Here we developed a tandem promoter cassette, termed LMTP6, that provides tissue-specific expression within liver and muscle. LMTP6 demonstrates enhanced activity in cell lines representative of hepatocytes and skeletal muscle compared to conventional tissue-specific promoters. Following intravenous injection in mice, AAV vectors driven by LMTP6 provide high liver-directed expression of a monoclonal antibody designed to inhibit plasma kallikrein as a possible intervention for hereditary angioedema (HAE). Furthermore, this vector demonstrates high antibody expression following intramuscular delivery. Current experiments are defining the immunologic profile of this promoter system with respect to prevention of humoral and cell-mediated immune responses. Collectively, these data identify a possible platform to bypass immunological barriers and achieve sustained expression of vectorized antibodies.

557. Abstract Withdrawn

558. Characterization of Novel AAV Vectors Engineered for Muscle Gene Delivery

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Recombinant adeno-associated viral vectors (rAAV) demonstrate great promise as the leading platform for clinical gene delivery. A variety of AAV serotypes currently exist and enable transduction of multiple tissues for the treatment of many genetic and other complex diseases. For Duchenne Muscular Dystrophy (DMD), gene therapy is under investigation to replace the absent dystrophin with a smaller, functional micro-dystrophin (uDys). Since muscle constitutes a large proportion of body mass, a high dose of vector administered systemically is necessary. Identification and development of more muscle-tropic vectors should improve safety and efficacy of DMD gene therapy. Previously, we described the novel capsid AAV-SLB101, which yielded superior expression of uDys protein when compared to the natural serotypes AAV8 and AAV9 *in vitro*, in both mouse and human DMD skeletal muscle cells. In addition, biodistribution and uDys expression were both significantly increased in heart and quadriceps in DMD^{mdx} mice. In this study, additional capsids were generated and characterized alongside AAV-SLB101 and naturally occurring serotype controls. *In vitro* assays have been developed to measure and interrogate virus binding and uptake, as well as transduction of rAAVs in multiple disease relevant cell types. More information on the capacity and specificity of engineered rAAVs to bind and enter DMD muscle and other related cells will enhance our understanding of muscle tropism of these novel capsids, and ultimately may lead to the design of more efficacious muscle-tropic vectors.

559. Enhanced Transduction of Human Hematopoietic Stem Cells by AAV6 Vectors: Implications in Gene Therapy and Genome Editing

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We have previously reported that of the 10 most commonly used adeno-associated virus (AAV) serotype vectors, AAV6 is the most efficient in transducing primary human hematopoietic stem cells (HSCs) *in vitro* as well as *in vivo* (*Cytother*., 15, 986-998; 2013; *PLoS One*, 8: e58757, 2013; *Sci. Rep*., 2016. 6: p. 35495, 2013). More recently, polyvinyl alcohol (PVA), was reported to be a superior replacement for human serum albumin (HSA) for *ex vivo* expansion of HSCs (*Nature*, 571: 117-121, 2019). Since HSA has been shown to increase the transduction efficiency of AAV serotype vectors in general (*Gene Ther*., 24: 49-59, 2017), we evaluated whether PVA could also enhance the transduction efficiency of AAV6 vectors in human hematopoietic cells. A dose-dependent increase of up to 9-fold was observed. The improvement in the transduction efficiency is due to PVA-mediated improved entry and intracellular trafficking of AAV6 vectors in human hematopoietic cells. PVA also mediated up to 12-fold enhancement in the transduction efficiency of AAV6 vectors in primary human HSCs *in vitro* (**Figure 1A, B**) as well as up to 7-fold increase in murine hepatocytes *in vivo* (**Figure 1C-E**). Taken together, our studies suggest that the use of PVA is an attractive strategy to further improve the efficacy of AAV6 vectors, which has important implications in the optimal use of these vectors in the potential gene therapy and genome editing for human hemoglobinopathies such as beta-thalassemia and sickle cell disease.

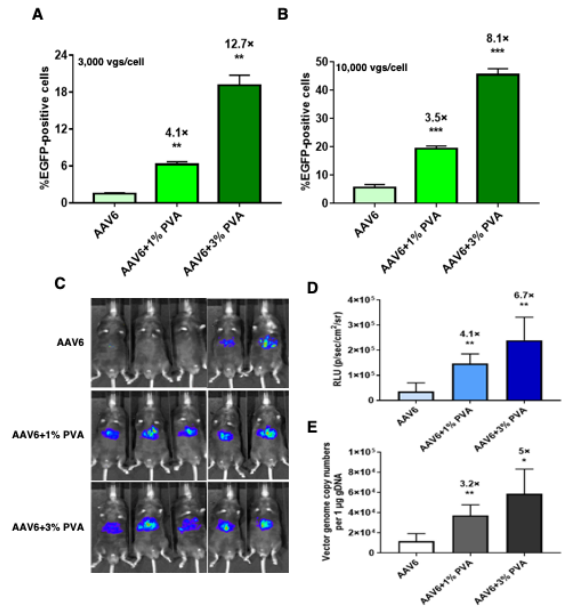

Figure 1. PVA increases the transduction efficiency of AAV6 vectors in primary human HSCs in vitro, and in primary murine hepatocytes in vivo. Primary human bone marrow-derived $CD34^+$ cells were transduced with scAAV6-CBAp-EGFP vectors at 3x103 (**A**) and $1x10^4$ (**B**) vgs/cell, respectively, with or without pre-incubation with 1% or 3% PVA87 at 4°C for 2 hrs. Transgene expression was determined by flow cytometry 48 hrs post-transduction. Statistical significances are indicated as $^{**}p < 0.01$, $^{***}p < 0.001$. ssAAV6-CBAp-FLuc vectors, with or without pre-incubation with 1% or 3% PVA87 at 4°C for 2 hrs, were injected via tail-vein in C57BL/6 mice at $1x10^{10}$ vgs/mouse. Whole-body bioluminescence images were acquired 2-weeks post-vector administration (**C**). Quantitation of bioluminescence signal intensity is shown as photons/second/cm^2/steradian (p/sec/cm^2/sr) (D). AAV6 vector genome copy numbers in mouse liver were quantified by qPCR using FLuc-specific primers (**E**). Statistical differences are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$.

560. Machine-Guided Directed Evolution of rAAV Combinatorial Capsid Libraries in Insect Sf9 Cells

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The widespread successful use of recombinant Adeno-associated virus (rAAV) in gene therapy has driven the demand for scale-up manufacturing methods of vectors with optimized yield and transduction efficiency. The Baculovirus/Sf9 system is a promising platform for high yield production; however, a major drawback to using an invertebrate cell line compared to a mammalian system is a generally altered AAV capsid stoichiometry resulting in lower biological potency. Hereby, we introduce a term of a structural and biological "fitness" of an AAV capsid as a function of two interdependent parameters - 1) packaging efficiency (yield), and 2) transduction efficiency (infectivity), both parameters critically dependent on AAV capsid structural proteins VP1/2/3 stoichiometry. To identify an optimal AAV capsid composition, we have developed a novel Directed Evolution (DE) protocol for assessing structural and biological fitness of Sf9-manufactured rAAV for any given serotype. The approach involves the packaging of a combinatorial capsid library in insect Sf9 cells followed by a library screening for high infectivity in human Cre-recombinase expressing C12 cells. One single DE selection round complemented by Next-Generation Sequencing (NGS) and guided by *in silico* analysis identifies a small subset of VP1 translation initiation sites (TIS, also known as Kozak sequence) encoding "fit" AAV capsids characterized by high production yield and superior transduction efficiencies. The protocol includes the following features:

newly designed AAV TR cassette plasmid vector incorporating 1) a combinatorial capsid gene library expressed in Sf9 cells; 2) GFP reporter gene; and 3) Cre-recombinase-controlled flip Lox-sites activated in C12 cells;

the complexity of AAV capsid library of ~10e5 TIS permutations allows for the assembly of rAAV capsids with dynamic VP1:VP2:VP3 stoichiometry;

combinatorial libraries are subjected to orthogonal selection pressures: Sf9 insect cells favor most productive capsid ratios for greater yield (e.g., with a higher VP3 content); the mammalian cells preferentially select for more infectious AAV (e.g., with a higher VP1 content);

NGS analysis and unique double barcoding combinations allowed sequencing all theoretical capsid gene permutation with ~100x coverage overlap;

optimal "fit" capsid composition providing for both good yield and infectivity is identified by a machine-guided workflow using several consecutive filtering steps.

As a proof of principle, we generated a combinatorial plasmid library of AAV2 capsid gene variants incorporating all 65,536 possible permutations in the VP1 Kozak sequence for standard ATG initiation (NNNNNNNNATGGC) and ~15,000 permutations incorporating non-canonical initiation codons. Using the described workflow, we have identified the optimal VP1 TIS sequence generating VP1:VP2:VP3 with favorable stoichiometry and biological potency. Using similar algorithm, we extended these findings to other AAV serotypes manufactured in insect Sf9 cells.

561. Characterizing SAAV's Structure, Cell Binding, Trafficking, and Antibody Escape Properties

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Adeno-associated viruses (AAVs) are utilized and developed as clinical gene therapy vectors. AAV mediated gene therapy relies on the virus' ability to interact with its cellular receptor, and traffic to the nucleus via the endo-lysosomal pathway. The virus is then transported through the nuclear membrane where genome uncoating and gene expression occurs. Pre-existing antibodies can neutralize AAV infection at any point within this trafficking pathway, reducing the efficacy of treatment. Presently, there is a high seroprevalence of AAVs in the human population. For these reasons, there is a need for AAV vectors that can evade the pre-existing immune response. One possible source for human immune system evading AAVs, which do not circulate within the human population, are non-human infecting AAVs. One such AAV is serpentine AAV (SAAV). SAAV clades with other reptilian AAVs, which diverged from the ancestral lineage of human AAVs. We postulated that the general human population would have limited exposure to SAAV compared to human AAVs, making it an excellent candidate for gene delivery applications. Cell binding assays on different CHO cell lines identified terminal sialic acid as the primary attachment receptor for SAAV. This is consistent with the receptor utilized by some other human AAVs, such as AAV5. This study also used single particle cryo-electron microscopy (cryo-EM) to characterize the SAAV capsid at physiological pH (pH 7.4) and under conditions it is exposed to during cellular trafficking (pH 6.0, 5.5, and 4.0). Our atomic maps and models (at a resolution of 3.15, 2.73, 3.39, and 2.66 Å respectively) of SAAV structures demonstrate that the capsid remains intact with structural variations observed at the atomic level at the different pHs. Finally, native immunodot blot of SAAV probed with several individuals' human sera illustrate that it is not recognized by any of the sera tested, unlike the AAV2 and AAV5 used as controls. Our data illustrates the usefulness of SAAV as a gene delivery vector with the ability to evade the human adaptive immune response and thus increase clinical efficacy.

562. Structural Characterization of AAV3B and Novel Clade C Variants by Cryo-EM Single-Particle Reconstruction

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Cryo-EM and X-ray crystal structures of the major prototypical AAV serotypes and select variants have enhanced our understanding of the natural features that define stability and functionality. They have also provided vital insights into tissue tropism, antigenicity, and regulation of AAV biology that directly fuels therapeutic capsid selection and development. Clade C AAV, or AAV2/3-hybrids, are natural variants that are closely related to AAV2 and AAV3 serotypes. Despite the huge interest in the discovery of AAVs with novel properties for adaptation to gene therapy, clade C variants have been largely unexplored, and no capsid structure is presently available. Phylogenetics of known clade C AAVs, along with novel variants from our discovery pipeline, support the clade's distinction from closely related serotypes. These variants were clustered and ranked by relative abundance from human patient samples and screened for production as recombinant viral vectors. From this analysis, two closely related clade C variants, v513 and v551, were chosen for structural characterization by cryo-EM alongside AAV3b, which was previously solved from X-ray crystal data. *De novo* reconstructions from single-particle micrographs and atomic model refinement have yielded unbiased high-resolution electron density maps for all three capsids, with current resolution estimates between roughly 2.5 and 3.2 Å for each, using the gold-standard approach. In comparison to existing AAV3b crystal structures, cryo-EM of AAV3b demonstrates significant conformational differences, particularly at the three-fold protrusion. This finding highlights the flexibility of key regions that govern AAV functionality and the importance of methodology in the differential analysis of AAV structures. The two AAV2/3 variants differ in sequence length by a single amino acid in VP3, outside known variable regions, but are otherwise nearly identical (99%). Despite differences in length, the conformational similarity observed between the pair of variants appears quite high, although significant changes to capsid stability and transduction potential are noted. As expected, AAV2/3 structural differences from other AAVs appears largely confined to known variable regions. Taken together, our characterization of AAV2/3 and AAV3b by cryo-EM provides for a more complete view of capsid features and characteristics that may be considered during the development of clinical vectors. We

present these findings in conjunction with ongoing efforts to improve the resolution of key functional regions of interest, including the internalized vector genome. While icosahedral symmetry greatly facilitates capsid structure reconstruction, it does so at the expense of genomic resolution. Despite this inherent bias, a relatively high degree of density is observed beneath the three-fold axis in each of the three structures, which we attribute to nucleic acids of the vector genome. Accordingly, we detail our efforts in the asymmetric reconstruction of these three rAAV, and the potential implications of full-particle resolution on novel therapeutic vector discovery.

563. Barcoded AAV Pools for Fast and Semi-High-Throughput Comparing and Selecting AAV Variants

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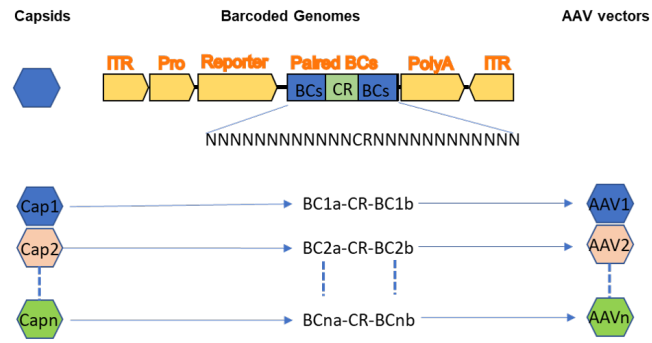
AAV vectors are powerful tools for gene delivery and gene therapy. Hundreds of identified natural AAV serotypes and increasing number of engineered variants allow us to find better capsids for each cell type and tissue, which is vital for effective gene therapies. However, there has been no good method to quickly identify an unique capsid for each target. Currently, the common way to compare AAV variants is to test them side-by-side individually, whereas it is difficult to generate, control and handle hundreds of AAV vectors in every lab or company. Moreover, limited candidates decrease the chance to find better AAV variants. Development of random DNA barcode capsid library has been used to overcome the above disadvantages. This approach, however, involves a selection process that requires multiple rounds of screenings to identify real functional capsids. Due to the involvement of viral replication and packaging processes, the obtained capsids may not represent the best vectors that efficiently infect the targeting cells. To solve this problem, we have developed a novel way for rational capsid comparison and selection. In the method, each capsid variant packages a DNA barcoded genome carrying a reporter gene. The unique pair of barcode is located between coding sequence and Poly-A signal. Presence of reporter gene allows enrichment of transgene-expressing cells and avoids the viral replication and packaging steps. The DNA barcodes and transcribed RNA barcodes screenings allow the evaluating of putative capsids for each target. The efficacy of each AAV candidate is obtained by directly comparing the quantity of barcodes before and after infection rather than comparing with other AAV variants. Results obtained are more actual and not affected by the quality of vectors. Moreover, hundreds of AAV vectors can be generated at once, pooled and tested together. The pre-made barcoded AAV pools allow us to fast and semi- high-throughput compare and select AAV variants both in vivo and in vitro.



564. Development of Novel Improved Bioengineered AAV Variants for Liver-Targeted Gene Therapy

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In recent years, gene therapy based on recombinant Adeno-Associated Virus (rAAV) led to a number of exciting clinical outcomes, with therapeutic benefits reported in patients suffering from genetic conditions affecting the eye, central nervous system, and liver. In fact, rAAV is thus far the only *in vivo* viral gene delivery tool to achieve regulatory approval by the US Food and Drug Administration or the European Medicines Agency. However, accumulating preclinical and clinical data clearly indicate that natural AAV variants, on which most of the current clinical programs are based, are not efficient at targeting primary human cells, nor is data from preclinical models predictive of clinical performance. Novel, bespoke bioengineered AAV variants developed to specifically and efficiently targeting human tissues of choice are quickly becoming the new reality of gene therapy. The process of bioengineering can also be employed to alleviate the reactivity with the pre-existing neutralising antibodies, improve manufacturability, physical stability and other important properties of the AAV particle. The Children's Medical Research Institute (CMRI) in collaboration with LogicBio Therapeutics have established an advanced AAV Development Program, with the goal to develop the next generation of AAV vectors for liver-targeted gene delivery. The Program builds on CMRI's established expertise in capsid bioengineering, using proprietary AAV selection methods based on Directed Evolution, *in vivo* Directed Genetic Drift, capsid

domain swapping, direct mutagenesis, and *in silico* methods. The capsid selection and evaluation process benefits from CMRI's access to normal and patient primary hepatocytes and a wide-range of biologically predictive models. The AAV Development Program was specifically designed to develop AAV variants for expediate clinical development. Building on LogicBio's product development expertise, the AAV development and validation pipeline, in addition to stringent functional testing, includes also evaluation based on vector manufacturability and immunological profiles. In addition to outlining the vector selection and validation pipeline, we will present a set of next generation clinically-ready, bioengineered AAV variants custom-selected for highly efficient functional transduction of primary human hepatocytes with improved manufacturability and enhanced resistance to pre-existing human neutralising antibodies.

565. Modification of the AAV Expression Cassette to Enhance Transgene Translation

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One of the major challenges for gene therapy is generating enough transgene expression to produce sufficient therapeutic protein. This problem is exemplified by the example of alpha 1-antitrypsin (AAT) deficiency. Effective treatment requires a serum therapeutic threshold in humans of 11 μM AAT protein. Optimization of the AAV expression cassette to enhance the stability and translation of the transgene mRNA transcripts is one strategy for increasing the amount of therapeutic protein expression. We hypothesized that introducing known translation enhancer elements (TEEs) that bypass the dependency on rate-limiting initiation factors could increase the efficiency of translation initiation and thus the rate of translation. TEEs selected from viral proteins, highly expressed mammalian proteins, and synthetic sequences were cloned into the 5' or 3' untranslated region (UTR) of the AAV transgene expression cassette consisting of the CAG promoter, AAT transgene, and β-globin polyadenylation signal. TEEs were screened *in vitro* in human liver cell lines Huh7 and HepG2 by measuring AAT expression in cell culture supernatant by ELISA. Six of the tested TEEs (AcNPV, AMV4, TMV, AAT, albumin, Syn21, TEE-1) enhanced AAT expression in both Huh7 and HepG2 cells compared to the wild-type AAT construct. AAT mRNA levels of constructs with TEEs were equivalent to the wild-type construct suggesting that enhancement occurred post-transcription. AAV8 vectors were generated with the best four TEE containing expression constructs from the *in vitro* study (AcNPV, AAT, albumin, TEE-1), and intravenously administered ($5x10^{10}$ gc) to C57Bl/6 mice. Serum was collected every 2 wk for AAT quantification. The most robust TEE (AcNPV, derived from *Autographa californica* nuclear polyhedrosis virus P10 gene) enhanced serum AAT levels by 40% in mice at 4 wk post-administration. However, TEEs from AAT, albumin, and TEE-1 did not increase AAT expression *in vivo*. Screening of the four TEEs *in vitro* in the mouse liver cell line H2.35 showed a similar result to the *in vivo* mouse studies, suggesting potential differences in the ability of TEEs to enhance translation in mouse and human. These results suggest that TEEs can enhance translation of a transgene even in the context of a strong promoter such as CAG and could be a useful method of increasing the therapeutic protein output from AAV vectors.

566. Administration Volume Alters Recombinant Adeno-Associated Virus 8 Transduction Efficiency and Biodistribution *In Vivo*

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The eye is an attractive target for gene delivery due to its ease of accessibility via multiple injection routes. Subconjunctival (SC) administration of AAV vectors exhibit capsid serotype-dependent transduction in the cornea and broad transduction of ocular/peri-ocular tissues, demonstrating its suitability to treat ocular diseases. In addition to viral capsid serotype, physiological factors such as body temperature, starvation, and administration volume affect AAV vector transduction efficiency. Herein, the relationship between injection volume, vector transduction, and vector biodistribution was evaluated in wild type mice using multiple doses of recombinant AAV8. A single SC injection of AAV8 vectors, resulted in efficient transduction of the ocular muscles; in contrast, a ten-fold higher injection volume of the same dose exhibited little to no detectable transduction in the ocular muscles, and trended towards higher transduction efficiency in the peripheral cornea. The transduction pattern was a dose-independent phenomenon that correlated well with vector genome biodistribution. Taken together, these results indicate that injection volume dramatically alters recombinant AAV8 tissue transduction and biodistribution in the ocular and peri-ocular compartments following SC injection. Therefore, optimization of multiple experimental factors is necessary to achieve maximal transduction efficiency and desired tissue targeting, while concurrently decreasing vector production burden and immunological concerns.

567. Abstract Withdrawn

568. Tropism-Modified High-Capacity Adenoviral Vectors Open the Door to Full-Length Dystrophin Genome Editing Approaches for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe X-linked muscle-wasting disease caused by a variety of mutations in the dystrophin-encoding *DMD* gene that, spanning more than 2.4 Mb, is one of the largest human genes known. The length of the *DMD* coding sequence (11 kb) combined with the DNA packaging limitations of most commonly used viral vector systems has restricted viral vector-assisted targeted chromosomal insertion of transgenes encoding full-length dystrophin. We aim at developing an all-inclusive *ex vivo* DMD genetic therapy involving knocking-in a "healthy" copy of a full-length dystrophin-encoding transgene into the *AAVS1* safe-harbour locus in human myogenic cells. To overcome size constrains, we investigated the

feasibility of using tropism-modified high-capacity adenoviral vectors (HC-AdVs) as they can efficiently transduced human myogenic cells and host up to 37 kb of foreign DNA. HC-AdVs encoding EGFP-tagged full-length dystrophin (EGFP::FL-Dys) or Cas9:gRNA^{AAVS1} complexes reached high titers and were shown to package intact recombinant vector DNA. Importantly, transduction efficiencies above 90% could readily be obtained in patient-derived myoblasts and HeLa cells as assessed through flow cytometry and immunofluorescence microscopy. Donor templates consisting of a EGFP::FL-Dys transgene flanked by large stretches of *AAVS1* homologous sequences (5kb) led to stable chromosomal insertion and expression of EGFP::FL-Dys in up to 30% of the targeted cells. These results bode well for converting this HC-AdV platform into an ex-vivo genetic therapy for DMD aiming at complementing defective *DMD* alleles regardless of their mutation(s).

569. *In Vivo* RNA-Driven Selection of CNS-Tropic AAV Using 3rd Generation Capsid Libraries

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AAV-based gene therapy has emerged as the most promising approach to develop effective therapies for neurological diseases. Discovery of AAV9 BBB-crossing properties heralded a new era in CNS gene transfer using less invasive delivery approaches through the vasculature or into CSF. The discovery that AAV9 crosses the BBB after systemic delivery has revolutionized the field of gene therapy for neurological diseases. Despite limited neuronal transduction in the brain, the exceptional transduction of motor neurons in the spinal cord after systemic treatment, has led to the approval of an AAV9 gene therapy for spinal muscular atrophy. Replicating this astounding success for other neurological diseases affecting the CNS more broadly will be a major challenge unless another technological leap yields new AAV capsids capable of delivering genes to a majority of cells throughout the CNS. Until recently, *in vivo* directed evolution of AAV capsid libraries yielded new AAV with modestly improved CNS gene transfer efficiency. In 2016 Deverman and colleagues demonstrated the power of restricting the *in vivo* library selection process to functional capsids using an elegant Cre-based recombination approach conducted in Cre transgenic mice. Unfortunately, AAV-PHP.B and AAV-PHP.eB capsids with more than 100-fold enhanced CNS gene transfer over AAV9 in mice displayed only modestly improved properties in other species including non-human primates. We have developed new capsid libraries that use cell-type specific promoters to drive transgene expression and *in vivo* selection relies on RT-PCR amplification of library sequences from total tissue RNA. These 3rd generation AAV expression libraries can be used for in vivo selection in any species of choice. We used the human synapsin 1 promoter to drive transgene expression in neurons for *in vivo* selection of AAV9 and AAVrh10 capsid libraries to target CNS by systemic administration. Three 7-mer peptide libraries were built for each capsid with complexities higher than $1x10^9$ individual clones in some instances. Illumina NGS showed equal representation of clones in the plasmid and packaged libraries, with virtual absence of nonsense codons in the latter as expected. Libraries were infused into C57BL/6J mice at $1x10^{12}$ vg and one month later there was evidence of skewing in peptide representation. In the second round conducted at lower doses and total brain RNA isolated after 1 week, there was clear evidence of clonal enrichment with some peptides representing upwards of 5% of all reads. The top 50-100 clones from each library will be compared for CNS gene transfer efficiency as a sub-library with degenerate DNA sequences that will also include AAV-PHP.eB and the newly developed AAV-F capsid. Selection in NHP is also ongoing and we anticipate presenting some initial data from those screens.

570. Role of Terminal Galactose in Cellular Uptake, Intracellular Trafficking, and Tissue Tropism Using Adeno-Associated Viruses Isolated from Human Stem Cells (AAVHSCs)

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AAVHSCs are a class of hematopoietic stem cell (HSC)-derived Clade F adeno-associated viruses (AAVs). AAVHSCs have been shown to transduce a variety of disease-relevant tissues. Here we report efforts to characterize their mechanism and kinetics of cellular entry. Many viruses bind glycosylated proteins or lipids on the cell surface for attachment and entry in order to invade the host cell. Thus far, AAV9 (Clade F) is the only AAV reported to bind galactose, making it unique among the AAVs that have been studied. Using mutant CHO cell lines expressing different terminal glycans, we now show that AAVHSCs also utilize terminal galactose as a primary receptor for efficient cellular binding and entry. By treating cells with neuraminidase, which exposes additional surface galactose, we were able to improve transduction efficiency with a self-complementary GFP vector by up to 100-fold. Increased expression correlated to an increase in total vector genomes and nuclear vector genomes. In this study, we sought to determine whether naturally occurring subtle mutations in capsid protein sequence can alter the binding affinity. Here we show that capsids containing the 505R residue in VP3 also possess differences in galactose binding and kinetics of nuclear accumulation following neuraminidase treatment. In addition, we identify that one of the naturally derived Clade F AAVs, AAVHSC16, does not bind to galactose *in vitro* and has reduced transduction efficiency in the liver *in vivo*. This study demonstrates that AAVHSC binding to surface galactose increases transduction efficiency, and that differences among the family of AAVHSCs demonstrate varying levels of tropism for the liver.

571. Targeting the Rodent Peripheral Nervous System Efficiently and with Greater Specificity through Intravenous Delivery of AAV Capsids Evolved by Multiplexed-CREATE

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BBE, Caltech, Pasadena, CA

The peripheral nervous system (PNS) is critical in regulating end-organ function and feedback signal transmission to the central nervous system (CNS). The sensory and enteric nervous systems (SNS and

ENS), key components of the PNS, are of increasing interest to both scientific and clinical communities. With the proper gene delivery vectors, SNS gene therapy could be applied to neurological disorders such as neuropathic pain whereas ENS gene therapy could address the gastrointestinal symptoms, including dysregulated digestion and transit, common to many disorders including Parkinson's. To achieve precise genetic manipulation within the PNS however, more efficient and targeted gene delivery vehicles are needed. Adeno-associated viruses (AAVs) have become the vector of choice for gene delivery in vivo but non-invasive systemic administration of natural serotypes targets the PNS with mixed efficiency and specificity. Attempts to circumvent these limitations by direct administration of AAV are often either surgically challenging (e.g. for dorsal root ganglia (DRG)) or have incomplete coverage (e.g. for ENS). In prior work we have shown that modification of the AAV9 capsid by directed evolution generates AAV variants that target the CNS or selected PNS regions by systemic delivery (Chan et al, 2017). To further explore the potential of targeting PNS with engineered-capsid, we utilized the advanced selection platform Multiplexed-CREATE (Kumar et al, Nature Methods, 2020), that builds upon our prior method CREATE (Deverman et al, Nature Biotechnology, 2016). M-CREATE is a high-confidence AAV selection platform that uses next generation sequencing (NGS) and Cre-transgenic mouse lines (e.g. neuron-specific) to perform positive selection in vivo on PNS areas of interest (including DRG, small and large intestine), and a post-hoc negative selection against off-targets (including liver). After 2 rounds of in vivo M-CREATE selection in C57BL/6J adult mice, we recovered several variants that were positively enriched in the PNS areas of interest compared to their parent capsid AAV9. Preliminary NGS analysis revealed two novel AAV variants: AAV-PNS1, with the greatest relative enrichment between the PNS and off-target tissues, and AAV-PNS2 with the greatest overall enrichment in the PNS compared to AAV9. *In vivo* validation in C57BL/6J adult mice by intravenous delivery of the variants packaging nuclear-localized EGFP under a strong ubiquitous CAG promoter (ssAAV-CAG-NLS-EGFP) showed enhanced EGFP expression in neurons of the nodose ganglia and DRG. Consistent with NGS enrichment data, AAV-PNS1 showed greater specificity to the nodose ganglia and DRG by exhibiting lower overall transduction in peripheral organs including liver (an otherwise strong AAV9 target), while AAV-PNS2 showed higher overall efficiency in targeting both the ENS and SNS. The preliminary outcomes described here demonstrate the potential of M-CREATE to identify designer AAVs with greater efficiency and specificity towards the PNS in adult mice and thereby enabling basic and pre-clinical research.

572. A Novel AAV Capsid with a Potential of Crossing NHP Blood-Brain Barrier

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The adeno-associated virus (AAV) vectors with the ability of crossing blood-brain barrier (BBB) represent an attractive systemic drug delivery tool for neurological diseases. AAV-PHP.B capsid was engineered and developed in adult GFAP-Cre transgenic mice and showed great enhancement on CNS transduction after IV injection. However, AAV-PHP.B does not recapitulate the improvements in nonhuman primates (NHP) after IV infusion due to lack of LY6A which mediates the crossing of brain endothelium of C57BL/6 mice by the capsid. We here aimed to develop novel AAV capsids in NHP for translational applications using the **C**re recombination-based AAV targeted evolution (CREATE) method. As no Cre-transgenic NHP model is available, we delivered GFAP-Cre via AAV vector as well as AAV capsid library into adult NHP. The capsid sequences were recovered by Cre-dependent PCR from tissues in different brain regions and analyzed by next-generation sequencing (NGS). The enriched sequences were selected for next round of screening. After two rounds of selection, one variant was identified and showed improved transduction in mouse and monkey primary brain microvascular endothelial cells (BMVEC) compared to AAV9. More interestingly, the capsid exhibited dramatically enhanced transduction (>10x) in primary human BMVEC and SH-SY5Y human neuroblastoma cells. The ongoing *in vivo* studies would validate the NHP CNS tropism and biodistribution of this capsid variant.

573. Hardwiring Tissue-Specific AAV Transduction in Mice Through Engineered AAVR Expression

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Adeno-associated virus (AAV) vectors are leading candidates for many gene therapies currently in development. In addition, AAV vectors are used in small animal studies for biomedical research to express a transgene or for genome engineering. Strategies to enhance AAV transduction rates and achieve tissue selective expression are expected to significantly improve the utility of AAV vectors in clinical and preclinical studies. This will be aided by a better understanding of the cellular factors critical for AAV binding, entry, and transgene expression. Previously we identified AAVR (also named KIAA0319L) as a key multi-serotype receptor critical for AAV transduction. Here, we harness regulated AAVR expression to develop a versatile mouse platform, which allows for superior tissue-specific AAV expression *in vivo*. We generated AAVR knockout mice with a transgenic cassette that can drive AAVR expression in a highly tissue- and cell-type specific manner through crossing with Cre-lines. We demonstrate that we can genetically hardwire AAV9 transduction to cell-types of choice thereby drastically altering the *in vivo* tropism of the normally broadly tropic AAV9 vector. This re-targeting to desired cell-types coincides with a near-complete de-targeting to other tissues. We further demonstrate that AAVR overexpression leads to strong increases in *ex vivo* and *in vivo* transduction rates. These data suggest that AAVR expression can be used to increase AAV transduction efficiency and to develop better animal models of human disease.

574. Abstract Withdrawn

575. Recombinant Adeno-Associated Virus Vector Tropism in Human Retina

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The human retina is an attractive target of AAV-mediated gene therapy, however a major ongoing challenge is that AAV vector tropism within the tissue remains poorly defined. As a result, hit-or-miss vector selection methods have led to very advanced clinical trials, with resultant data continuing to contradict that obtained from animal models, manifesting most often as lack of therapeutic effect. In this study we hypothesised that human post-mortem retina is a useful model for testing recombinant AAV tropism and can be used to compare AAV vector transduction efficiency. Retinal explants were prepared 24-72 h post-mortem from donor tissue provided by the Queensland Eye Bank and transduced using AAV2/2, AAV2/5, AAV2/6 or AAV2/8 with 0.5-1×10^{10} vector copy genomes expressing tdTomato or secreted luciferase reporter genes. Trans-retinal imaging allowed real time longitudinal monitoring of retinal explants, revealing tdTomato expressing cells within one week of transduction and continuing strong expression beyond 2 months of culture. Transduction of human retinal cells using AAV2/6 was robust, showing tropism to morphologically diverse cells including trans-retinal cells with the characteristics of Müller glial cells. Trans-retinal imaging following transduction with recombinant AAV2/8 vector showed tropism to cells within or directly adjacent to retinal blood vessels walls, corresponding to retinal arterioles, venules and the superficial capillary plexus on retinal cryosections. Secreted luciferase showed rapid increase within the first week in culture, reaching maximum levels at 2-3 weeks, with lowest expression induced by AAV2/2 vectors. This study showed that post-mortem human retina contained viable tissue beyond 24 h post-mortem which can be further maintained in tissue culture for several months. We provide evidence that post-mortem human retinal explants are likely to be a highly externally valid model for gene therapy pre-clinical investigations. While AAV2/8 is known for poor transduction of human cells, our human retinal explant model has revealed a narrow tropism for a subpopulation of retinal blood vessel-associated cells, underlining the importance of optimising viral vectors to target tissues and cells using the best available models.

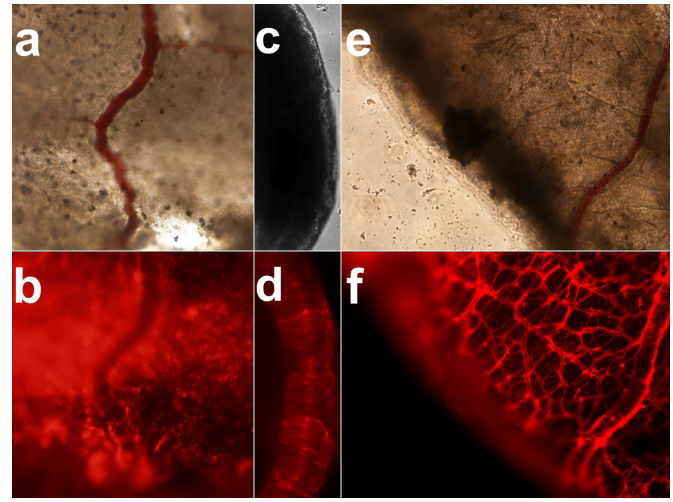

Fig. 1: Representative images of post-mortem human retinal explants in culture. Explants were transduced using AAV2/6 (**a**-**d**) and AAV2/8 (**e**-**f**) vectors containing CMV-tdTomato reporter gene. **a**, retinal explant two weeks post AAV2/6 transduction captured in bright field. **b**, the same explant in the Cy3 spectrum. tdTomato expression is visible as scattered red dots across the entire retinal surface. **c**, retinal explant in tissue culture, folded inwards giving an *in vivo* cross section two weeks post transduction with AAV2/6, in bright field. **d**, the same explant captured in the Cy3 spectrum showing tdTomato-expressing Müller type cells traversing the full thickness of the retina. **e**, retinal explant in culture in bright field two weeks post transduction with AAV2/8. **f**, the same explant captured in the Cy3 spectrum. tdTomato expression is visible within, or directly adjacent to, retinal blood vessel walls.

576. Inhibiting GSK3αβ Signaling Impacts AAV Transduction Events

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Our lab previously reported a 3-5 fold increase in transgene expression while co-administering insulin with rAAV. Preliminary studies focused on determining which portion of transduction is involved: uptake, trafficking, or gene expression. The Pi3k/Akt pathway impacts a significant amount of cellular activities including dynamin clathrin mediated endocytosis. While it is well established that insulin activates this pathway, we had to confirm that this pathway is functional in our cell lines and that it can be activated by insulin. Hep3B human hepatoma cells downregulate phosphatase and tensin homolog (PTEN), a modulator of the dephosphorylation of phosphatidylinositol triphosphate (PIP3) to phosphatidylinositol diphosphate (PIP2). Due to this downregulation baseline Akt phosphorylation is increased. Despite an innate increase in Akt activation, treating Hep3B cells with insulin resulted in a 2.5 fold increase in phospho-Akt. Insulin was co-administered with the Pi3k inhibitor Zstk-474 along with AAV2-CMV-EGFP to cultured Hep3B for 6 hours and GFP levels were measured 72 hours later by FACS using side scatter analysis. These cell culture studies were done without FBS to reduce signaling events from effectors in FBS. In-line with previous studies, there was a statistically significant increase in transduction when administering

insulin. This insulin enhanced AAV2-CMV-eGFP transduction was reduced to insulin-free levels by treating with a Pi3K inhibitor. As a control, the Pi3K inhibitor was co-administered with AAV2-CMV-eGFP without insulin and transduction was not impacted. Following successful inhibition of Pi3K, GSK3 (glycogen synthase kinase) became the priority downstream target. GSK3 is a constitutively active serine/threonine kinase stimulated by the insulin and Wnt pathways. GSK3 has a plethora of known substrates, including dynamin I. Dynamin I phosphorylation by GSK3β is responsible for both clathrin mediated endocytosis and activity dependent bulk endocytosis. When treating cultured Hep3B cells with the potent GSK3 inhibitor CHIR99021 in the absence of serum there is a twofold effect: the quantity of insulin entering the cells is markedly reduced in serum deprived cells, and the fluorescent intensity of cells transduced with AAV2-CMV-eGFP is significantly lower than cells without CHIR99021. FITC labeled insulin was administered to serum deprived cells for 6 hours and the quantity internalized was measured using FACS with side scatter analysis. There was no significant difference in the quantity of internalized FITC-insulin when Pi3k was inhibited, but a statistically significant decrease in cells with GSK3α/β inhibited. Conversely, in human embryonic kidney cells (HEK293T), inhibition of GSK3 lead to a larger amount of FITC-insulin internalization. Again, insulin was co-administered with the GSK3 inhibitor CHIR99021 along with AAV2-CMv-EGFP to cultured Hep3B for 6 hours and GFP levels were measured 72 hours later by FACS using side scatter analysis. Cells with GSK3 inhibited during AAV administration showed a marked decrease in transduction from both insulin treated and untreated populations when compared to the control. This process is being explored in other cell types to account for the variations in cancer cell signaling.

577. Characterization of the Ly6a / PHP.B Interaction and Implications for Vector Engineering

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Introduction: AAV9-PHP.B, a gene-therapy vector based on adeno-associated virus 9 (AAV9), crosses the blood brain barrier (BBB) in C57BL/6 mice with unprecedented efficiency. AAV9-PHP.B achieves this brain-transduction phenotype through its interactions with Ly6a, a cellular receptor expressed on the surface of microvascular endothelial cells in the murine brain. Unfortunately, the endogenous function of the murine Ly6a receptor is currently unknown and it does not have a human orthologue. Here we dissect the interaction between PHP.B and Ly6a to determine the elements that are essential for transduction and BBB crossing. **Methods and Results:** First, we investigated how modulating the binding affinity between the AAV9-PHP.B capsid and the Ly6a receptor impacts transduction efficiency of the vector both *in vitro* and *in vivo*. Vectors with low to moderate affinity for the Ly6a receptor have the best transduction of tissue in the central nervous system (CNS) and can transduce peripheral organs such as the liver. Vectors with the highest affinity for the Ly6a receptor have low transduction in both CNS and liver tissue. Biodistribution analysis of these affinity variants shows a clear negative relationship between Ly6a affinity and liver localization, but no relationship between Ly6a affinity and CNS localization. Next, we investigated how modulating the valency of the interaction between the AAV9-PHP.B capsid and the Ly6a receptor impacts binding and transduction efficiency of the vector *in vitro*. We demonstrate that monomeric Ly6a does not have sufficient affinity for the AAV9-PHP.B capsid to produce binding. However, presentation of the Ly6a receptor in a multivalent context enables robust and specific binding to AAV9-PHP.B. We also generated chimeric capsids containing varied ratios of the AAV9-PHP.B and AAV9 capsid proteins. Capsids produced with a 1:2 ratio of AAV9-PHP.B to AAV9 transduce Ly6a-expressing cells with the same efficiency as capsids containing 100% AAV9-PHP.B. **Conclusions:** Here we discuss the role of affinity and valency in the targeted design of vectors to overcome delivery obstacles in humans. This work improves our understanding of how AAV vectors can engage with proteinaceous attachment receptors, and how the strength of this receptor engagement influences tissue targeting.

578. Enabling Temporal Control of Gene Expression in the Inner Ear after AAVAnc80 Mediated Delivery

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Hearing loss is the most common sensory deficit, affecting over 5 million people in the USA. Over 150 genes have been associated with non-syndromic hereditary hearing loss, but hearing loss and deafness can also occur as a result of noise damage, exposure to drugs, and age. Viral vector- mediated gene transfer can provide a fully functional gene to the inner ear and potentially enable hearing improvement or preservation. AAVAnc80 has been shown to transduce multiple cell types in the inner ear and is therefore an optimal tool for gene delivery. While non-syndromic hereditary hearing loss is caused by single gene mutation(s) that can be restored by gene augmentation, the complex etiologies underlying environmental, drug, or age induced deafness require alteration of multiple genetic components to restore function. AAV vector mediated delivery permits long-term gene expression however, in some cases, the temporal control of a sequence of interest may be desirable (e.g., where constitutive expression is not needed and/or may be associated with toxicity). Therefore, we explored a gene regulation system that permits targeted proteasomal degradation based on a protein destabilizing domain. This method modulates expression of one vector component while maintaining the constitutive expression of other vector components. We examined the efficacy of this system in the inner ear using the reporter system mScarlet. Delivery of AAVAnc80-mScarlet without a destabilizing domain resulted in high expression of mScarlet in mouse cochlear cells. In contrast, mScarlet expression was absent following delivery of AAVAnc80-mScarlet fused to a destabilizing domain due to mScarlet degradation. However, when the animals are treated with a small molecule drug that could attach to the destabilizing domain and prevent degradation, mScarlet expression was detected in various cells of the transduced cochlea. To conclude, in the absence of the small molecule drug, transgene expression was not evident, while after the addition of the drug, expression was

clearly detected. This method can temporally and reversibly control the expression of various proteins in the cochlea following AAV vector mediated gene delivery.

579. Computational Method for Characterization and Analysis of Viral Vectors

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Advancements in gene and immune therapies and approval of products for various diseases reinforce the promises of these therapies to treat challenging disorders. Viral vectors are important tool for introducing the genetic modifications into cellular genome and their progeny. Analysis and evaluation of viral vectors is highly important to unravel the genotoxicity and immunogenicity of vectors and analyzing the therapeutic efficacy. We have developed a computationally efficient workflow for characterization and analysis of viral vectors. Our analysis suite provides different modules for handling various aspects of viral gene therapy data. The first module is designed to identify possible contaminant sequences in vector preparations. It provides the distribution and frequency of each known contaminant as well as viral vector reference sequence. Additionally, fragment size for each identified contaminant sequence is estimated. Multiple contaminant sequences can be identified and evaluated simultaneously in each sample. This mode has wider usability for detecting known sequence in any viral and non-viral next-generation sequencing data. Another module allows the analysis of internal vector fusions or breakpoints distribution. It identifies the fusion position of each breakpoint components and can handle analysis of multiple viral vector references. The detailed information of each breakpoint region is generated including positions of fusion regions, identity, span, sequence information etc. The third analysis module is designed for the characterization of viral vector and host genome fusion distribution profile to analyze the transduced cell population and the clonal contributions. The retrieved genomic positions are annotated for nearby genomic features and the detailed information about each vector genome fusion event including positions, strand, span, identity etc is provided. This module also allows analysis of multiple vector references together in a sample. Our computational workflow is programmed in Bash and Perl. It covers wide range of analysis requirements for viral gene therapy short-read sequencing data. The toolkit has additional usability in investigation of viral cancers and insertional mutagenesis screens.

580. Interactions of AAV with Cellular Receptor AAVR by *Cryo*-Electron Microscopy

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Genomic screening identified a cellular protein, AAVR, as an essential entry factor for most serotypes of Adeno-Associated Virus (AAV) [1], motivating renewed structural characterization of virus receptor interactions. AAV-binding was localized primarily to the first two extracellular PKD domains, which, with heterologous over-expression of several ectodomain constructs [1, 2], has opened the door to *cryo*-Electron Microscopy (EM) at increasing detail, now at ~2.5 Å resolution, in complexes with now three different types of AAV. *Cryo*-Electron Tomography at lower (~3 nm) resolution provided context. Binding of a fusion protein containing all 5 of the extracellular domains of AAVR revealed sites on the exterior of AAV2 where proximal domains were bound firmly, but distal domains, attached by flexible linkers, were oriented in various configurations [3]. More saturated binding was achieved with a His-tagged 2-domain construct that bound in the same location. In part through elimination of flexible regions, the resolution has been extended to 2.5 Å, beginning to reveal hydrogen-bonding interactions. Only PKD domain 2 was seen at high resolution, consistent with its stronger binding to AAV2 [2]. By contrast, the structure of a complex with AAV5, that has measurable binding only to PKD1 [2], shows only PKD1. When the structures are superimposed, the binding sites for the different domains of AAVR bound to AAV2 and AAV5 overlap at only a single amino acid. The N-terminal end of PKD1 is located near the 5-fold axis of AAV5. It is not tied down strongly by interactions until it bridges over the canyon to a binding site on the side of a 3-fold spike. The flexible linker between domains is not seen in either structure, but PKD2 then casts an elliptical footprint as it rises up the shoulder of a different spike that is related by 5-fold symmetry. This is an area where mutation of surface residues can have significant impact upon cellular transduction. For both AAV2 and AAV5, structures with some of the neutralizing monoclonal antibodies indicate overlap with bound AAVR domains, casting new light on possible neutralization mechanisms.
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581. Rational Designed Haploid Viruses for Liver Targeting and Neutralization Escape

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Adeno-associated virus (AAV) vector-mediated gene delivery has achieved impressive therapeutic effect in many clinical trials. Notably, the US Food and Drug Administration has approved two AAV gene therapy reagents using AAV2 delivering RPE65 for inherited vision loss and AAV9 delivering SMN gene for spinal muscular atrophy. AAV vectors have also successfully been applied in hemophilia via liver targeting. However, there are two challenges faced in AAV liver targeting in human clinical trials after systemic administration: low efficacy of liver transduction, and high prevalence of neutralizing

antibodies (Nabs). Therefore, it is imperative to explore effective approaches for the enhancement of AAV transduction and Nab evasion. In our previous study, we demonstrated that haploid AAV2/8 produced from co-transfection of two capsid helper plasmids showed a 4-fold higher transduction in the liver than parental AAV8. Furthermore, the haploid virus AAV2/8 showed greater potential for escaping AAV2 Nabs compared to parental AAV2. These results indicate that haploid viruses might potentially acquire advantage from parental serotypes for enhancement of transduction and Nab evasion. Recently, some studies showed that AAV3B was able to efficiently transduce human hepatocytes in a humanized mouse model, and our prior study found that AAV7 transduced human hepatocytes more efficiently than other serotypes. To explore whether haploid AAV vectors have higher human hepatocyte transduction in humanized mice, we made haploid viruses AAV3/7 in ratios 1:1, 1:3 and 3:1, respectively. The yield of haploid viruses was similar to that of parental viruses. We transduced Huh7 cells with the haploid viruses and their parental AAV3 and AAV7. AAV3 had the highest transduction efficiency, and AAV7 the lowest. Next, haploid AAV vectors encoding self-complementary GFP transgene were systemically administered into humanized mice. At week 3 post AAV injection, the mice were perfused and the livers were collected for analysis of human hepatocyte transduction by flow cytometry. The results showed that haploid AAV3/7 1:3 achieved the highest transduction in human hepatocytes among the haploid vectors and parental AAV3 and 7. Consistent with our previous result, AAV7 had higher transduction than AAV3 in human hepatocytes. Furthermore, we studied whether the haploid virus AAV3/7 could escape neutralizing antibodies against AAV3 or 7. When anti-AAV3 or anti-AAV7 mouse sera were used, results showed that the haploid AAV3/7 1:3 escaped anti-AAV3 Nabs, but not anti-AAV7. However, in human sera and IVIG, we did not find the haploid vectors escaped neutralization, perhaps because all individual human serum samples and IVIG had high titers of anti-AAV3 and AAV7 neutralizing antibodies. We believe that the haploid AAV3/7 1:3 could escape anti-AAV3 neutralization in humans similar to the results obtained from mouse sera if we test more human samples. In summary, the haploid vectors, which have the ability for high transduction efficacy and Nab evasion, might be potential candidates for future clinical trials.

582. A Truncated AAV2 Capsid Protein with a Potential Impact on Vector Potency is a Product of Non-Canonical Translation Initiation

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A key objective of vector manufacturing is the generation of high quality product that complies with strict requirements for purity, potency and safety. The AAV producer cell line (PCL) platform utilizes HeLaS3 cells engineered to contain the AAV vector sequence as well as the AAV *rep a*nd *cap* genes. AAV vector is produced following infection with a wild-type adenovirus (Ad5). Unlike AAV2 vectors produced via triple transfection (TTX), PCL-derived AAV2 vectors show a truncated VP1 (tVP1) species migrating just below VP1 on SDS-PAGE. Given the critical role of VP1 in viral infectivity, this truncated protein may adversely affect vector potency. Notably, tVP1 is also detected in wild-type AAV2 (wtAAV2) produced in Ad-infected HEK293 cells. However, it is not prevalent in PCL-generated vectors of other serotypes (AAV1, 5, 8, 9). LC/MS analysis of an AAV2 vector and wtAAV2 showed that the tVP1 N-terminal residue was an acetylated alanine (Ala35). AAV2 is unique in that the VP1 amino acid sequence in this location is Pro34-Ala35 whereas for most other serotypes it is Ala34-Asn35. When VP1 synthesis is prohibited via introduction of a stop codon at the N-terminus, tVP1 persists, indicating that it is not a VP1-derived proteolytic product. Interestingly, the Cricket Paralysis Virus is known to initiate viral protein translation at an Ala via a 5' cap-independent mechanism involving an IRES element where a Pro residue preceding the initiator Ala is also critical[1]. Likewise, substitution of either Ala35 or Pro34 in AAV2 VP1 with a stop codon inhibits generation of tVP1; however, tVP1 is still detected when a stop codon is substituted for Lys33. Furthermore, an Ala35Asn substitution reduces the prevalence of tVP1. These results suggest that tVP1 may be generated via an IRES mechanism. To assess whether tVP1 affects vector potency, AAV2-SEAP vectors produced via PCL (wt VP1 and Ala35Asn) and TTX were evaluated in an *in vitro* potency assay. All were comparable, suggesting that the amount of tVP1 should be monitored, but is not critical to product quality.

[1]Wilson *et al* MolCellBiol 2000 20:4990

583. Efficient Retinal Transduction by AAV Variants Identified via Directed Evolution and Screening in Non-Human Primate

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PURPOSE: Intravitreal injection (IVI) of AAV is a promising approach for treating a number of common retinal diseases, e.g. diabetic retinopathy and age-related macular degeneration (AMD). IVI can be performed in clinic rather than a surgical suite, thereby increasing accessibility of gene therapies to larger patient populations. Using directed evolution of an AAV2-based capsid library in non-human primate (NHP), we previously identified a number of capsid variants with enhanced retinal transduction. The goal of this study was to characterize transduction, following IVI in macaque, of three of these, DGEDF, P2-V2 and P2-V3 relative to benchmark AAV2 across a dose range similar to that being utilized in ongoing clinical studies of other AAVs. We also evaluated whether these novel capsids were amenable to purification via a commonly used affinity capture methodology. **METHODS:** AAV2, AAV-DGEDF, AAV-P2-V2 and AAV-P2-V3 vectors containing CMV/chicken-beta promoter driving green fluorescent protein (GFP) were packaged by triple transfection (ttfx) in HEK293T cells and purified by iodixanol gradient followed by anion exchange chromatography and buffer exchange into balanced salt solution. Macaques were pre-screened for anti-AAV2 NAb with a NAb50 of ≤5 utilized as inclusion threshold. Vectors were IVI in

macaques at 1e10vg or 1e11vg in a volume of 100ul. GFP expression was documented in life by fluorescent fundus photography. Animals were sacrificed 6 weeks post injection and retinal sections were stained with cone arrestin antibody and DAPI, and evaluated via confocal microscopy. Area of transduction and intensity measurements were performed on fundus images using Image J. AAV-DGEDF, AAV-P2-V2 and AAV-P2-V3 vectors were also packaged by ttfx in HEK293 cells and purified by iodixanol gradient followed by affinity capture using POROS CaptureSelect AAVX affinity resin. Total vgs were determined for load and elution fractions. **RESULTS:** Transduction of macaque retina at 1e11vg mediated by DGEDF, P2-V2 and P2-V3 was substantially improved over AAV2 in both area transduced and intensity of fluorescence within the central retina. Area of expression in the central 'foveal ring' was 3.5X, 3.2X and 4.0X larger for DGEDF, P2-V2 and P2-V3, respectively relative to that for AAV2. Intensity of fluorescence similarly was 4.25X, 3.25X and 4.2X higher, respectively. At 1e10vg, GFP expression was only observed in eyes receiving capsid variants (not AAV2), therefore a comparison was not possible at this dose. In all cases, variants at 1e10vg outperformed AAV2 at the 10 fold higher dose. Both in life images and microscopy of retinal sections indicated that the majority of retinal cells expressing GFP were retinal ganglion cells and müller glia. All variants resulted in transduction of cone photoreceptors within the foveal pit at the 1e11vg dose. All variants were efficiently purified using AAVX resin. **CONCLUSIONS:** We have shown three novel AAV capsid variants to exhibit increased transduction relative to AAV2 in IVI macaque. Both total area and magnitude of transduction were increased and a clear dose response was observed. All variants were efficiently purified using a common, scalable method suitable for large scale GMP manufacturing. We have recently shown that another variant identified in the same library screen, but not included here, has the ability to avoid neutralization by anti-AAV NAbs in human vitreous samples. Taken together, these novel capsids have high potential for addressing common retinal diseases like AMD and diabetic retinopathy.

584. Context-Specific Function of the Engineered Peptide Domain of PHP.B

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Making adeno-associated virus- (AAV-) based gene therapy efficient and safe requires improving the AAV capsid to 1) overcome poor transport through tissue barriers; and 2) redirect the broadly tropic AAV to disease-relevant cell types. Peptide or protein-domain insertions into AAV surface loops can achieve both engineering goals. However, it remains unclear the extent to which domain insertions disrupt or depend on specific AAV capsid structural elements to maintain normal function. Here we examine AAV9-PHP.B, an engineered variant containing a 7-amino-acid insert that facilitates interaction with the receptor Ly6a, resulting in transport across the blood-brain barrier (BBB) in C57BL/6 mice. We found that any disruption of the PHP.B 7-mer peptide from the AAV9 HVRVIII context eliminated Ly6a binding and BBB-transport, including a simple graft of the peptide to the analogous loop in AAV1. Comparative structural analysis of AAV9-PHP.B and AAV1-PHP.B revealed that the engineered loop conformation is not fixed in either context. We propose a model in which PHP.B interacts with Ly6a using an extended interface that includes extant AAV9 residues. We discuss the implications for AAV-capsid engineering and the transfer of engineered activities between serotypes.

AAV Vectors - Preclinical and Proof-of-Concept Studies

585. A Mechanistic Target of Rapamycin (mTOR)-Inhibiting shRNA Delivered Intravitreally via rAAV as a Novel Therapeutic Strategy for Diabetic Retinopathy

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Among the leading causes of vision loss in developed nations, diabetic retinopathy (DR) is characterized by retinal neovascularization, a process that is driven by vascular endothelial growth factor (VEGF). This has led to the development of anti-VEGF-based treatments for this condition, which are generally delivered via intravitreal injection. However, these therapies require frequent administration, negatively affecting patient compliance and treatment outcomes. Combined with safety concerns regarding long-term VEGF suppression, a gene therapeutic focusing on a non-VEGF target may prove to be a better alternative for the treatment of DR. One such target is mTOR, which is well-suited for addressing various aspects of DR, as the involvement of additional cellular factors and symptoms beyond angiogenesis have shown it to be a multifactorial condition. We have previously demonstrated the ability of a mTOR-inhibiting shRNA packaged alongside a stuffer DNA to maintain proper genome size and delivered via rAAV2 (rAAV2-shmTOR-SD) to markedly reduce retinal neovascularization in a rat model of oxygen-induced retinopathy. Here, the long-term therapeutic efficacy of rAAV2-shmTOR-SD was explored in a streptozotocin-induced diabetic mouse model. Dextran-FITC staining performed on the retinas of C57-B6 mice intravitreally injected with the therapeutic virus vector exhibited significantly less vessel leakage, a major aspect of pathological angiogenesis, when compared to untreated control mice or those administered a virus vector containing a scrambled control shRNA. These results were confirmed by retinal vascular histology showing that mice treated with rAAV2-shmTOR-SD had markedly lower levels of pericyte loss, which is associated with weakened vessel walls. Furthermore, H&E staining showed a reduction in the thinning of the nuclear cell layers, demonstrating the ability of rAAV2-shmTOR-SD in helping maintain retinal integrity by exerting a cytoprotective effect, whereas TUNEL assays revealed that it possesses anti-apoptotic properties as well. Finally, co-immunostaining used to

determine retinal tissue tropism showed that the therapeutic virus vector was well-supported in glial cells, which play a major role in the progression of DR. These data combine to build upon our previous work to further demonstrate the promise of rAAV2-shmTOR-SD as a potential gene therapeutic for the treatment of DR.

586. Gene Therapy for Wilson Disease Using RAAV to Restore ATP7B Gene Function

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Wilson Disease is a rare genetic disorder that disrupts proper copper clearance from the body, caused by mutations in the human ATP7B gene. This gene encodes for a copper-transporting ATPase 2 protein, which is involved in loading copper onto Ceruloplasmin to secrete it into the bloodstream, and excreting excess copper into the bile. The presence of high levels of copper causes toxicity primarily to hepatic cells, and over time to the central nervous system and other peripheral tissues, leading to cellular degeneration. Current treatments for Wilson Disease target heavy metal toxicity by removing excess copper from circulation with chelating agents, zinc, and in some cases liver transplant. We sought to pursue gene therapy as a therapeutic modality to restore normal gene function by using recombinant adeno associated virus (rAAV) vectors to deliver functional ATP7B. To this end we generated multiple constructs with optimized variations of the human 5.9kb ATP7B gene, including the use of different liver-specific promoters to drive the expression of the transgene, and removal of 3 to 4 metal binding domains (MBD) to circumvent viral packaging restrictions. These vectors were produced using two different platforms: triple transient transfection of HEK293 cells and adenovirus induction of a HeLa producer cell line. The functionality and efficacy of multiple vectors were tested in vivo in the Atp7btx-j and Atp7b-/- mouse models. In these models, the presence of the transgene and vector genome were detected and quantified both by PCR and fluorescent in situ hybridization. In addition, we measured a significant decrease of copper accumulation in the liver, both by Timm's sulfide staining and inductively coupled plasma mass spectrometry (ICP-MS). The restoration of copper homeostasis was evident by improvement in liver pathology. The totality of these studies enabled us to choose the final therapeutic product candidate for our upcoming Wilson Disease clinical trial.

587. Enhancement of Adeno-Associated Virus Mediated Insulin Gene Therapy Using Teniposide in Diabetic Mice

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Gene therapy using adeno-associated viral (AAV) vectors holds considerable promise for the treatment of inherited dysfunctional single gene and many complex acquired disorders. The success of gene therapy using AAV vectors depends on two main factors: 1) the efficiency of cell transduction, 2) and persistence of introduced gene, which requires eliciting minimal or no host immune response upon vector administration. Previous studies have suggested that some small molecules can enhance the transduction efficiency of AAV vectors and reduce the vector dose that is below the immunological response threshold. This study explores the use of Teniposide, a chemotherapeutic agent, in improving the efficacy of gene therapy using AAV. Streptozotocin-induced diabetic mice were used as a model of type 1 diabetes. We co-administered Teniposide with a self-complimentary AAV vector encoding an insulin gene designed for liver-specific expression. Teniposide co-treatment resulted in up to 3-fold decrease in AAV dose compared with the vector alone while reducing the diabetic hyperglycemia to comparable degrees. A single treatment with Teniposide, co-administered with AAV, did not cause any systemic toxicity. While the mechanism of action remains to be fully elucidated, our data suggest that Teniposide co-administration could significantly enhance AAV transduction in vivo, and provide a novel and useful strategy to improve the clinical efficacy of gene therapy.

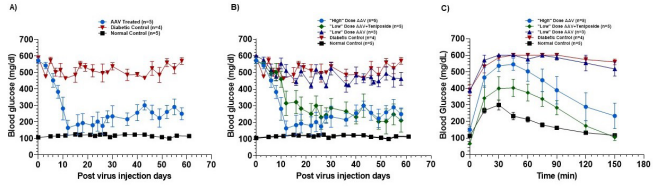

Figure 1. A) ***Blood glucose measurements in STZ-induced diabetic mice after injection of AAV vector containing the insulin gene therapy treatment.*** AAV treated mice exhibited lowered blood glucose levels. Treatment dose: $5x10^{12}$ vg/kg AAV. **B)** ***Blood glucose measurements in STZ-induced diabetic mice following injections of AAV vector and Teniposide.*** "High" dose: $5x10^{12}$ vg/kg AAV. "Low" dose with Teniposide: $1.5x10^{12}$ vg/kg AAV + 20 mg/kg Teniposide. "Low" dose: $1.5x10^{12}$ vg/kg AAV. **C)** ***Intraperitoneal glucose tolerance test.*** Blood glucose measurements over the course of two and a half hours after intraperitoneal injection of 3gm/kg glucose. "High" dose: $5x10^{12}$ vg/kg AAV. "Low" dose with Teniposide: $1.5x10^{12}$ vg/kg AAV + 20 mg/kg Teniposide. "Low" dose: $1.5x10^{12}$ vg/kg AAV.

588. "Para-Retinal" Vector Administration Into the Deep Vitreous Increases Transgene Expression in NHP Retina

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The eye is advantageous for exploring gene therapy, as the small closed compartment requires minimal vector quantity and is somewhat protected against immune system activation. Vector delivery to the retina currently is performed by sub-retinal administration or intravitreal injection. Sub-retinal application has the advantage of placing the vector within a few hundred microns of the target cells; but it requires surgical manipulation of the retina tissue, and distribution limited to the injection site. Subretinal vector dosing is used for Luxterna®, the FDA-approved LCA therapy. Intravitreal application is minimally traumatic to the eye and retina and is preferable in reaching a larger expanse of the retina. Intravitreal application is used for the Gensight LHON vector. Exploratory ocular retinal therapies are often developed in the tiny mouse eye and then face the challenge of scaling to the larger human eye with 800 times more vitreous volume. Further, the geometry of mouse versus human eyes is substantially different: the mouse vitreous is only 1 mm thick between the retina and back of lens, whereas it is 16 mm in human. Hence intra-vitreous injection in mouse places the vector immediately adjacent to the retina, whereas in human the vector must diffuse to reach the retina after a mid-vitreous injection. The large AAV virus size (20-25 nm; 3700 kDa) impedes rapid diffusion, and capsid surface charges may further slow movement to the retina surface from a mid-vitreous injection site.

We explored adjusting the vector delivery larger eyes to overcome the physical limitations of vector diffusion, by applying the vector near the retina surface, in a method we call "Para-Retinal" deep-vitreous administration. When tested in NHP eyes (smaller than human but substantially larger than mouse), we found that vector expression in the NHP fovea was substantially greater than after mid-vitreous application (using a myc-tagged vector equivalent to our human ocular trial for X-linked retinoschisis: scAAV8-hRSP/IRBP-hRS/myc, at 3e11 vg/ eye). Para-retinal administration is minimally invasive and is done with direct viewing of the retina, such as with the new ocular imaging systems (e.g., Zeiss Lumera microscope with Alcon NGENUITY visualization system). We further confirmed in New Zealand White rabbit that para-retinal dosing provided substantially greater vector concentration near the retina at one hour after deep vitreous injection. Note that as the rabbit vitreous depth is 6 mm vs. 16 mm in human, one expects even greater limitation of vector distribution in the larger human vitreous. Our results demonstrate improved retinal foveal transduction with para-retinal application and show the potential to achieve greater retinal transduction for clinical application in human through a para-retinal injection method when coupled with a novel AAV vector that exhibits better retinal transduction in NHP following intravitreal administration.

589. Pilot Study: AAV-*CRB2* to Protect Against Blindness Due to Loss of CRB Proteins in Müller Glial Cells

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Loss of CRB1 and/or CRB2 proteins in the mouse retina results in a CRB-dose dependent retinal degeneration depending on the levels of CRB proteins in Müller glial cells (MGC) and/or photoreceptor cells. *Crb* mutant mice lacking CRB proteins specifically in MGC might allow testing of adeno-associated viral (AAV) *CRB* gene therapy vectors. Here, we present a *Crb* double mutant MGC-specific retinitis pigmentosa Cre-recombinase mouse model with complete loss of CRB1 and a >40% reduction of CRB2 in MGCs. The double mutant retina's showed slow progression of gliosis and disruptions at the subapical region and adherens junctions at the outer limiting membrane. Photoreceptors were lost at the peripheral and central superior retina, while gross retinal lamination was preserved. MGC-specific Cre expression in *Crb* double mutant mice did, whereas single mutant mice lacking CRB1 did not, result in electroretinography (ERG), optokinetic head tracking (OKT) or morphological deficits. Further, we challenged the retina by intravitreal injection of DL-α-Aminoadipic Acid (AAA; dose escalation: PBS, 100 µg, 150 µg, 200 µg) to induce gliosis and retinal disorganization. Wildtype mice and $Crb1^{KO}$ mice showed little changes in OKT/ERG responses and morphological changes at 100 µg AAA. The $Crb1^{KO}Crb2^{LowMGC}$ mice were more sensitive to AAA-induced-gliosis showing a strong decrease in inner/outer photoreceptor length and OKT deficits. Finally, we tested the rescue of vision (as measured by OKT) and inner/outer segment photoreceptor length by intravitreal application of AAV2/ShH10Y-CMV-*CRB2* or AAV2/ShH10Y-CMVmin-*CRB1* in *Crb* double mutant mice prior to 100 µg AAA application. AAV2/ShH10Y-CMVmin-*CRB1* did not rescue OKT responses over 100 µg AAA injected eyes (n=8). AAV2/ShH10Y-CMV-*CRB2* rescued OKT responses and inner/outer segment photoreceptor length (n=4). The results suggest that CRB expression in MGCs is important for retinal cell adhesion, and that *CRB1* patients might benefit from application of AAV-*CRB2* gene therapy vector to MGCs

590. Gene Therapy for Metachromatic Leukodystrophy (MLD) That Crosses the Blood-Nerve and Blood-Brain Barriers in Mice and Non-Human Primates

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Metachromatic leukodystrophy, commonly known as MLD, is an inherited autosomal recessive lysosomal storage disorder with a great unmet medical need. This fatal neurodegenerative disease occurs in three forms: late infantile (prevalence of 1 in 40,000), juvenile and adult. The late infantile and juvenile forms represent the majority of the MLD patients and mortality at 5 years is estimated at 75% and 30%, respectively. MLD is most commonly caused by mutations in the *ARSA* gene and patients suffering from the disease are deficient in arylsulfatase-A (ARSA) enzyme. The disease is characterized by accumulation of supraphysiologic levels of lipids (sulfatides) to toxic levels in the nervous system and peripheral organs. These excess sulfatides lead to the destruction of myelin, a key protective layer of the nerve fibers also involved in conduction velocity of action potential propagation. Of note, ARSA pseudodeficient individuals are reported to have approximately 10-15% of normal levels of ARSA enzyme activity and display no clinical symptoms, suggesting that partial levels of normal ARSA activity are sufficient for the proper metabolism of sulfatides in humans. Based on this genetic validation in humans, we set our minimal therapeutic threshold at 10-15% of normal human brain ARSA activity. Herein, we report preclinical gene therapy data where a single intravenous dose of HMI-202 (AAVHSC15-human-ARSA (hARSA)) crosses the blood-nerve- and blood-brain-barriers (BNB and BBB) in juvenile non-human primates and in the ARSA KO murine model of MLD. In the ARSA KO mice, hARSA expression patterns were nearly identical to that of murine ARSA (mARSA) distribution in the nervous system of wild type age-matched littermates, in both neuronal and glial cellular profiles. In adult ARSA KO mice, we show a dose-response relationship in hARSA activity, transcript and vector genome copies in the central nervous system (CNS) where normal levels of hARSA activity are detected as early as 1 week following administration (earliest time-point of collection) and exceeding normal levels sustained out to 26 weeks post-dose in the CNS (end of study). A similar CNS hARSA expression profile was detected 1 week post-dose in ARSA KO neonates and sustained out to 12 weeks (end of study). In addition, we demonstrate that HMI-202 can modulate key disease-associated biochemical markers including myelin and lymphocyte (MAL) transcript levels, lysosomal-associate membrane protein-1 (LAMP-1) accumulation, and sulfatide levels in the CNS of treated MLD mice. In summary, HMI-202 demonstrates the ability to achieve enzymatic activity levels at or above the targeted therapeutic threshold, rapid onset of expression, sustainability, broad biodistribution and biological effect on sulfatide levels in a murine disease model and in

NHPs. Based on these preclinical data, IND-enabling studies of HMI-202 are ongoing to support the development of HMI-202 as a gene therapy for the treatment of MLD.

591. Development of a Clinical Candidate AAV3 Vector for the Potential Gene Therapy of Hemophilia B

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Although recombinant adeno-associated virus serotype 8 (AAV8) and serotype 5 (AAV5) vectors have shown clinical efficacy in a number of Phase I/II clinical trials for gene therapy of hemophilia B (Nathwani *et al*, *N Engl J Med*, 365: 2357-2365, 2011; Nathwani, *et al*, *N Engl J Med*, 371: 1994-2004, 2014; George *et al*, *N Engl J Med*, 377:2215-2227, 2017; Miesbach *et al*, *Blood*, 131:1022-1031, 2018), it has become increasingly clear that these serotypes are not optimal for transducing primary human hepatocytes (Li *et al*, *Mol Ther*, 23: 1867-1876, 2015; Wang *et al*, *Mol Ther*, 23: 1877-1887, 2015). We have previously reported that among the 10 most commonly used AAV serotypes, AAV serotype 3 (AAV3) vectors are the most efficient in transducing primary human hepatocytes *in vitro* (Glushakova *et al*, *Mol Genet Metab*, 98: 289-299, 2009; Ling *et al*, *Hum Gene Ther*, 21: 1741-1747, 2010) as well as *in vivo* in mice "humanized" with human hepatocytes (Vercauteren *et al*, *Mol Ther*, 24: 1042-1049, 2016). These studies suggested that AAV3 vectors expressing the human clotting factor IX (hFIX) may be a more efficient alternative for the potential gene therapy of hemophilia B in humans (Berns and Srivastava, *Gastroenterol Clin*, 48 :319-330, 2019). In our present studies, we have systematically optimized the promoter as well as the hFIX transgene cassette inserted between two AAV3 inverted terminal repeats (ITRs), and encapsidated into an AAV3 vector. Initial candidate vector designs were screened by transient transfection into HepG2 cells *in vitro* as well as by *in viv*o murine hydrodynamic plasmid injection. The lead candidate vector selected includes a 294 nucleotide (nt) enhancer/promoter, a 92 nt intron, a liver-codon optimized human fIX Padua (R338L) transgene, and a synthetic 49 nt polyadenylation sequence between a 146 nt 5' AAV3 ITR and a 125 nt 3'deltaTRS AAV3 ITRs designed to generate self-complimentary (sc) vector genomes. Functionality of AAV3 packaged vector particles was verified by transduction of HepG2 cells at MOIs ranging from 10^3 to 10^6 vgs/cell and measurement of fIX production rate into conditioned medium as well as intracellular genome copy number. Subsequently, the lead candidate vector design was tested in mouse models in vivo as follows. Since AAV3 vectors do not transduce mouse hepatocytes, the expression cassette was packaged into AAV8 capsids, and $1x10^{10}$ vgs were injected i.v. in hemophilia B mice. AAV3-hFIX vectors were also injected in "humanized" mice at $5x10^{11}$ vgs/mouse and fIX levels were evaluated 3-weeks post-vector administration. In both models, therapeutic levels of fIX were observed in plasma. Thus, our studies have led to the development of a clinical candidate scAAV3 vector, which is likely to be more efficacious at reduced doses, without the need for prophylactic immune suppression, for clinical gene therapy of hemophilia B.

592. Hijacking HBV Pol to Induce Apoptosis Specifically in Infected Hepatocytes *In Vivo*: A Novel Approach for Potential Treatment or Cure

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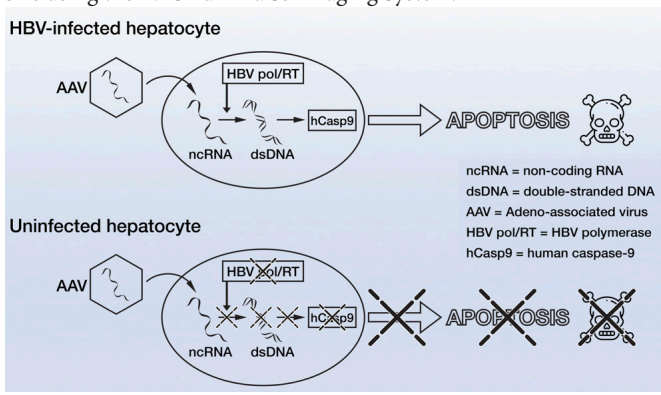
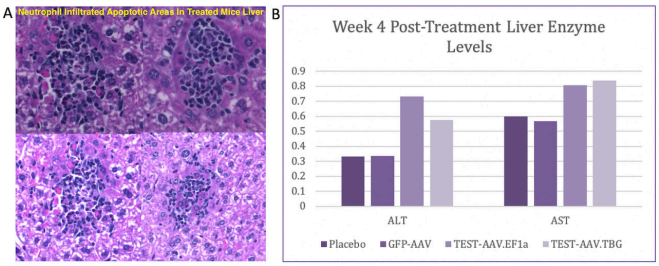
Background: We have previously demonstrated a novel *in vitro* proof-of-concept to co-opt the Hepatitis B Virus (HBV) polymerase (pol) to induce apoptosis of infected hepatocytes. HBV transgenic mice were used to evaluate *in vivo* the efficacy and specificity of the mechanism of our approach. We further conducted xenotransplant studies to evaluate the utility of our vector as a potential for HBV treatment or cure. **Methods:** AAV particles were packaged with a proprietary vector construct that expresses a non-functional non-coding (nc)RNA flanked between sequences specific to the reverse transcriptase (RT) domain of HBV pol (HBV pol/RT). The ncRNA would be recognized by HBV pol/RT and get reservely transcribed into double-stranded (ds)DNA which is encoded to overexpress caspase-9 (casp-9)(Fig 1). HBV transgenic mice, five per group, were injected with AAV particles expressing vectors under EF1a or liver-specific thyroxine binding globulin (TBG) promoter, a GFP vector or placebo. Peripheral blood was monitored weekly for liver enzymes and HBeAg. Heart, lungs, kidneys and liver were harvested on days 14 or 28, and evaluated for tissue distribution of AAV VP by IHC and Western Blot. Casp-9, caspase cleavage products and double staining of HBV core proteins with casp-9 were also evaluated by IHC. In addition, HBV pol-expressing or non-expressing HepG2-Red-Fluc cells were implanted into the liver of nude mice with matrigel to facilitate local tumor growth. Mice were then treated with AAVs expressing the test vector. At various timepoints, mice were injected with D-Luciferin substrate to quantify the changes in tumor size using the IVIS Lumina S5 Imaging System.



Results: Organs harvested from transgenic mice on day 14 post-treatment showed significant casp-9 expression in liver and kidney cells expressing HBV but not in those not expressing HBV. Kidney tissues of mice treated with the vector carrying TBG promoter had no increased casp-9 expression compared to untreated and GFP controls.

Moreover, cells in other organs that were stained positive for treatment AAV particles exhibited no casp-9 overexpression. Livers harvested on day 28 post-treatment showed diffuse apoptotic neutrophil-infiltrated areas in treatment groups but not in controls (Fig 2A). At week 4 in treatment groups, ALT level increased 1.8-2.2-fold and AST levels increased 1.4-fold compared to GFP-AAV and placebo controls. (Fig 2B). There were no significant changes in peripheral HBeAg. The results of the nude mice xenotransplantation cure study will be presented.



Conclusion: A novel AAV vector to hijack HIV pol specifically induced overexpression of Casp-9 and apoptosis in HBV-expressing cells in vitro and in vivo. The lack of decrease in peripheral HBeAg was expected because regenerating liver tissue in transgenic mice constitutively express the antigen. Although additional models and studies are needed, the data suggest a potentially new pathway to treat or cure HBV infection.

593. In-Vivo Expression of Human Opsin in Mouse Bipolar Cells Using Novel AAV Capsids

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Inherited retinal degeneration are characterized by a progressive photoreceptor degeneration and ultimately lead to cell death and severe visual impairment. Gene replacement approach is effective for treating the degeneration in early stages, however, may not effective at late-stage disease once irreversible cell death has taken place. Optogenetics is an alternative strategy that aims to restore vision by expressing microbial and human opsin to reactivate residual retinal neurons, such as bipolar cells, in late-stage photoreceptor diseases. However, to date effective targeting of bipolar cells with adeno-associated vectors (AAV) has not been achieved. The aim of this study was to investigate expression of human opsin in bipolar cells of wild-type and retinal degeneration mice using novel AAV capsids. We generated different vector libraries using a combination of three different AAV capsid variants (AAV2-7M8, AAV8-BP2 and AAV2-4YF) carrying opsin tagged with a fluorescent marker (YFP) driven by bipolar cell specific promoter (grm6). The vectors were injected subretinally and intravitreally into wild-type (C57BL/6) and retinal degeneration (*rd*^{1}) mice. Six weeks after injections, retinas were harvested and immunostained with anti-YFP and anti-PKC alpha antibodies and examined under confocal microscopy for protein expression. Our results demonstrate that human opsin can be ectopically expressed in ON-bipolar cells in wild-type and rd1 retinas via an intravitreal and subretinal injection using all three novel AAV capsids. As expected for the grm6 promoter expression was restricted to the bipolar cells. Early results show that there are no major differences in the level of expression between the variants delivered by intravitreal or subretinal injection. Ongoing quantitative analysis of the level of expression may reveal an optimal capsid and the vector delivery method with respect to wild-type or degenerate retina. In conclusion, the bipolar cell layer of the wild-type and degenerate retina can be targeted effectively with human opsin. Achieving high levels of optogene expression in bipolar cells has potential to restore useful visual function, setting the stage for future trials in human patients.

594. *In Vitro* Fabry Disease Correction in Patient iPSC-Derived Cardiomyocytes and Endothelial Cells Using an Evolved and Optimized AAV Gene Therapeutic (4D-310)

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Introduction Fabry disease is an X-linked disorder in which mutations in the *GLA* gene (encoding α-galactosidase A, AGA) result in reduced or absent AGA enzyme activity and consequent accumulation of globotriaosylceramide (Gb3). Gb3 is considered cytotoxic to cardiomyocytes and endothelial cells (kidney/heart/neuron) resulting in significant morbidity and shortened life expectancy. Administration of recombinant AGA (ERT) appears to slow disease progression in some tissues. Likely due to poor uptake into cells, for example in the heart, there remains significant unmet medical need. Thus, there is a compelling need for a durable treatment such as a single administration intravenous gene therapeutic targeted to key tissues that express *GLA* cell-autonomously, reducing Gb3 and thereby improving clinical outcomes. Using an industrialized directed evolution approach ("Therapeutic Vector Evolution") performed exclusively in non-human primates, we have identified and characterized an AAV capsid variant that can efficiently target key organs in Fabry disease, particularly heart. We engineered this capsid variant to carry and express the *GLA* gene (4D-310). A key component of translating 4D-310 into clinical development is to evaluate its tropism, expression and function in human cell models of Fabry-diseased cardiomyocytes and endothelial cells. **Methods** To generate human cell models, both normal fibroblasts and Fabry patient-derived fibroblasts (harboring the W162X pathogenic mutation in the *GLA* gene that typically results in absent AGA protein activity) were reprogramed into induced pluripotent stem cells (iPSCs). Fibroblasts were reprogrammed through a single non-integrative RNA transfection. After robust characterization of Fabry iPSCs, cells were differentiated into cardiomyocytes and endothelial cells. Cardiomyocytes were differentiated using Wnt modulation and glucose deprivation. Endothelial cells were differentiated through sequential Wnt inhibition and VEGF stimulation followed by purification through CD31^{+} sorting. Cell models were transduced at various multiplicities of infection (MOI) with our AAV capsid carrying either an EGFP reporter gene or a codon optimized *GLA* gene (4D-310). AGA expression was detected by flow cytometry to analyze transduction efficiency.

Activity of AGA was assayed using an optimized AGA fluorometric activity assay, that measures enzymatic glycolipid substrate cleavage. Gb3 accumulation, a clinical hallmark pathology of Fabry disease, was assessed by immunocytochemistry. **Results** The evolved novel AAV capsid carrying an EGFP reporter gene showed highly superior transduction efficiency versus wildtype AAV serotypes (1, 8, 9) in normal iPSC-derived human cardiomyocytes, achieving 72% at MOI 100 and almost 100% at higher MOIs. Transduction with 4D-310 in Fabry diseased iPSC-derived cardiomyocytes and endothelial cells led to rapid, dose-dependent AGA protein expression and activity, well above basal levels. In addition, transduction resulted in efficient clearance of accumulated Gb3 in cardiomyocyte and endothelial cell models. **Conclusions** These data demonstrate that this next-generation evolved capsid is highly tropic towards human cardiomyocytes *in vitro*. We successfully differentiated Fabry patient iPSC-derived cardiomyocytes and endothelial cells and developed assays to evaluate the functional activity of the transgene product. 4D-310 results in rapid cell-autonomous dose-related AGA activity intracellularly, resulting in clearance of Gb3, the accumulation of which is considered central to the pathogenesis of Fabry disease in humans.

595. Transgene Immunogenicity Has Significant Effects on Gene Therapy in a Mouse Model of Adult Polyglucosan Body Disease

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Background: Glycogen branching enzyme (GBE) catalyzes the creation of branches during glycogen synthesis. In patients with adult polyglucosan body disease (APBD, also known as adult form glycogen storage disease type IV), insufficient GBE activity causes formation of less-branched, poorly soluble glycogen aggregates (polyglucosan bodies) in liver, muscle, and the CNS. We previously reported the use of an AAV vector expressing human GBE in infant APBD mice with plausible results (Yi, H, *et al*., Hum Gene Ther, 2017). However, this approach showed no effect in adult mice 6 months post AAV administration, likely caused by T-cell-mediated immune response to the human protein. As the APBD mice produce residual endogenous GBE like human APBD patients, we speculated that mouse GBE would not be immunogenic, and the use of mouse GBE in mice would mimic the use of human GBE in human patients, providing relevant information for future gene therapy in human APBD. **Methods**: Human and mouse GBE cDNAs were individually cloned into an AAV vector (AAV-hGBE and AAV-mGBE) that contains a CMV enhanced chicken beta-actin hybrid promoter and both were packaged as serotype 9. Six-week-old APBD mice were injected intravenously with either vector at a dose of $2.5x10^{13}$ vg/kg (n=8 each group) and euthanized 6 weeks later. Age-matched untreated (UT) mice were used as controls (n=6). GBE expression and correction of glycogen accumulation in tissues were assessed. Fixed livers were used for immunohistochemical detection of CD4+ and CD8+ cells. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured to evaluate liver damage. Muscle functional tests (four-limb wire-hang, Rota-rod, and treadmill) were performed before euthanization. **Results**: Both vectors significantly elevated GBE activity in liver, heart, and skeletal muscle (quadriceps), and AAV-mGBE led to significantly higher GBE activities. Neither vector significantly elevated GBE activity in the brain (Fig. 1A). During the 6-week treatment period, UT mice accumulated significant amount of glycogen in liver, quadriceps and brain over baseline (before injection); AAV-hGBE significantly slowed glycogen accumulation in liver and quadriceps but not in the brain; AAV-mGBE completely prevented glycogen accumulation in liver and quadriceps and significantly reduced the accumulation in the brain (Fig. 1B). Muscle functional tests demonstrated generally better treatment outcome from AAV-mGBE than from AAV-hGBE (not shown). Despite the lowered liver glycogen level in the AAV-hGBE-treated mice than UT, their plasma AST and ALT activities were significantly higher, likely a result of strong cytotoxic T cell response as indicated by the pronounced positive CD4 and CD8 stains in liver of these mice. AAV-mGBE treatment did not cause obvious T cell response and significantly lowered AST and ALT activities (Fig. 1C & D). **Summary:** We conclude that transgene-induced immune responses play a significant role in the efficacy of AAV-mediated gene therapy in APBD mice. The use of mouse GBE in the mouse model is analogous to using human GBE in human patients, thus this study predicts encouraging outcome of gene therapy for APBD patients if expressing human protein.

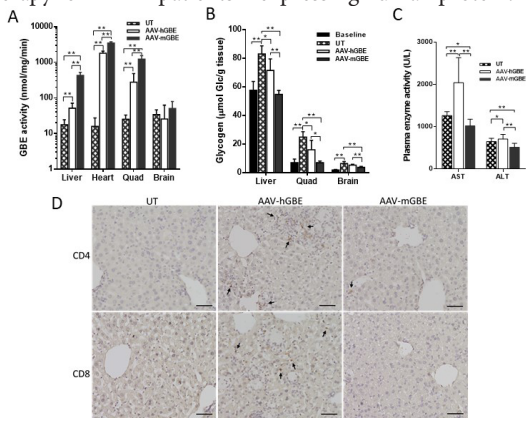

Figure 1. Comparison of AAV-hGBE and AAV-mGBE treatment in APBD mice. Mice were injected with AAV at 6 weeks of age and examined 6 weeks later. A, tissue GBE activities. B, tissue glycogen contents. Baseline, untreated mice at 6 weeks of age (n=6). C, plasma AST and ALT activities. D, immunohistochemical staining of liver sections with anti-CD4 and CD8 antibodies. Arrows indicate CD4+ or CD8+ lymphocytes. Scale bars = 50 μm. In *A-C*, data are presented as mean ± SD; n=6 for UT, n=8 for AAV-hGBE and AAV-mGBE. *p<0.05, **p<0.01.

596. Transient Correction of the Phenotype in a Cardiac Mouse Model of Friedreich Ataxia Using AAV9-CAG-FXN: Toxic Effect of Supraphysiological Expression of the Frataxin Transgene

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Friedreich ataxia (FRDA) is a rare autosomal recessive ataxia characterized by a progressive spinocerebellar ataxia and cardiomyopathy. FRDA results from a GAA repeat expansion within the first intron of the FXN gene leading to reduced transcription and residual levels of the encoded protein called frataxin. Frataxin is a ubiquitously expressed mitochondrial protein involved in the biogenesis of iron-sulfur clusters, which are essential inorganic prosthetic groups. In individuals with FRDA, cardiomyopathy represents the main cause of premature death. Herein, we performed a preclinical evaluation of an AAV9-based gene therapy approach using a mouse model of the disease that reproduces the cardiac phenotype (MCK conditional Fxn knockout mouse). AAV9 containing a cassette for the expression of human frataxin was administered intravenously after symptom outset at varying doses. Longitudinal echocardiography and weight recording were performed to assess phenotype progression. After initial correction of the cardiac phenotype observed by echocardiography, we observed a decrease in weight and worsening of cardiac function in a dose-dependent manner. Molecular analyses of vector genome copy number determination and transgene mRNA and protein expression were performed on isolated tissue samples. Together, our data indicates that too high expression of frataxin can have detrimental effects on mice and suggests that the therapeutic index for frataxin expression is an important consideration for clinical gene therapy efforts for FRDA.

597. Systemic Administration of AAV5-HPS1 to Prevent Hermansky-Pudlak Syndrome-Assocoated Pulmonary Fibrosis in Mice

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Introduction: Hermansky-Pudlak Syndrome (HPS) is a rare autosomal recessive disorder, due to mutations in ten different genes, manifesting with oculocutaneous albinism (OCA) and bleeding diathesis because of defects in lysosomal-related organelles (LROs). HPS-1 patients have mutations in *HPS1*, and middle-aged adults with HPS-1 develop pulmonary fibrosis (HPSPF), a devastating and lethal. HPSPF is characterized by irreversible and progressive fibrosis of the lung parenchyma and interalveolar septa, ultimately leading to respiratory failure. Histologic features of HPSPF include foamy alveolar macrophages and enlarged alveolar epithelial type II cells (AECII). There is no FDA-approved drug for HPSPF treatment. The identification of effective therapy has been hindered by the lack of a good model that represents the human HPS genotype and phenotype. Because *HPS1* variants are loss-of-function mutations, gene therapy is considered a possible strategy for treatment. **Objectives and Hypothesis:** Our goal is to establish gene therapy for HPS-1 using a novel HPS-1 murine model. For this study, we aim to systemically administer adeno-associated virus (AAV) to an *Hps1* knock out mouse model to prevent pulmonary fibrosis. **Methods:** *Hps1* was deleted in C57BL/6J using CRISPR-Cas9. Knockout mice were characterized by examining Hps1 expression, analyzing lung histology, assessing fibrosis in lungs, and measuring lung function tests. For AAV-GFP and AAV-Hps1 therapy in mice, we generated AAV that harbored the open reading frame of murine Hps1 driven by a CMV promoter. For control, AAV with GFP was used. One-day old mice were injected with 10^{12} genome-containing (GC) particles of AAV-GFP or AAV-Hps1 per animal through the facial vein. Organs were collected for analysis at 1, 3, and 6 months after injection.**Results:** We established a complete *Hps1* knock out mouse model ($Hps1^{1\Delta/\Delta}$) that had low *Hps1* mRNA expression. The $Hps1^{1\Delta/\Delta}$ murine model demonstrated defects in LROs, including: melanocytes (reduced ear, coat, and tail pigmentation), AECII (enlarged and foamy cells), and platelets (prolonged bleeding time). Analysis of tissues one month after AAV injections revealed that GFP was expressed in the lungs. We expect that continued analysis at planned timepoints will show correction of the LRO defects and lung function abnormalities observed in $Hps1^{1\Delta/\Delta}$ mice. **Conclusion:** Generation of an improved HPS-1 mouse model allowed us to explore AAV-mediated gene therapy in HPS-1.

598. Intravitreal Injection of AAV Expressing Soluble VEGF Receptor-1 Variant Tested in a Streptozotocin-induced Mouse Model as a Potential Gene Therapy for Diabetic Retinopathy

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Marked by the formation of leaky vessels within the retina, pathological angiogenesis is the defining feature of diabetic retinopathy (DR) and is a process driven by vascular endothelial growth factor (VEGF). A major cause of irreversible blindness in developed nations and currently affecting approximately 100 million patients worldwide, DR constitutes a growing global health concern for which gene therapy may prove to be greatly beneficial in addressing. This is because currently available treatments for DR, generally employing anti-VEGF strategies, require frequent intravitreal injections and is often burdensome to both patients and healthcare providers. Here, we investigate the therapeutic potential of a recombinant AAV2 expressing a soluble variant of the VEGF receptor-1 (rAAV2-sVEGFRv-1) in a long-term study performed using a streptozotocin-induced diabetic mouse model. C57/B6 mice were intravitreally injected with rAAV2-sVEGFRv-1 or a therapeutic dose of bevacizumab, which multiple studies have shown to be as effective in treating DR as approved therapeutics and is often used off-label due to the economic advantages it offers over the latter. Overall, the therapeutic efficacy of rAAV2-sVEGFRv-1 compared favorably to bevacizumab in addressing various aspects of pathological angiogenesis and DR as a whole. rAAV2-sVEGFRv-1, which was previously shown to effectively transduce the retinas of C57/B6 mice and therein exert anti-VEGF activity in a laser-induced model of choroidal neovascularization, here exhibited its ability to lessen the extent to which vessel leakage occurred, as well as its incipient causes. The former was determined via Dextran-FITC staining, whereas retinal histology and H&E staining revealed that the therapeutic virus vector reduced pericyte loss, an aspect of DR pathophysiology linked with weakened vessel walls, and blocked the thinning of the nuclear cell layers, which is associated with decreasing retinal integrity, respectively. As a demonstration of the main advantage of gene therapy over currently used DR treatments, whose dosing schedules range from monthly to trimonthly, the previous assays all showed that a single administration of rAAV2-sVEGFRv-1 remained therapeutically efficacious at the time of sacrifice three months later, well after the effects of bevacizumab began to fade. Co-immunostaining showed that rAAV2-sVEGFRv-1 was well supported by the ganglion cell layer, and the therapeutic virus vector was additionally determined to possess anti-apoptotic qualities via TUNEL assay. Taken together, these results suggest that rAAV2-sVEGFRv-1 may be able to serve as an effective and convenient gene therapy-based solution for the treatment of DR.

599. Developing an Optimized Cardiac Reprogramming Cocktail for Gene Therapy in Humans

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Heart failure affects an estimated 38 million people worldwide and is typically caused by cardiomyocyte loss or dysfunction. Due to the limited ability of cardiomyocytes to regenerate, *in vivo* reprogramming of non-myocytes into functioning myocardial cells using a cocktail of genes has great potential to treat heart failure. However, current human reprogramming cocktails are complex and include several different combinations of transcription factors, epigenetic regulators, kinases, microRNAs and/or small molecules, making it challenging to develop a reprogramming gene therapy for clinical application. We have developed a novel human cardiac reprogramming cocktail consisting of only two transcription factors. This new cocktail robustly reprograms cardiac fibroblasts *in vitro,* generating cells that exhibit calcium transients and express numerous cardiomyocyte-specific genes. Importantly, these reprogramming factors can be engineered into a single AAV cassette of ~4.7kb in size. We have also identified several microRNAs that further improve the reprogramming efficiency of this cocktail and can fit into one AAV cassette together with the two transcription factors. We are currently advancing cardiac reprogramming for clinical use by developing a novel AAV variant to deliver the reprogramming factors to target cardiac cells and evaluating safety and efficacy in animal MI models.

600. *In Vivo* Transduction of Murine Hematopoietic Stem Cells after Intravenous Injection of AAVHSC15 and AAVHSC17

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AAVHSCs are a group of Clade F AAVs originally isolated from human hematopoietic stem cells (HSCs). Understanding the transduction properties of recombinant versions of AAVHSCs in HSCs, particularly *in vivo*, will support their development for HSC-directed gene therapies. It has been established that AAV transduction of HSCs can be transient or result in very low levels of gene expression, which is difficult to detect with conventional methods. To more sensitively investigate AAVHSC transduction efficiency of HSCs *in vivo*, we utilized a loxP-STOP-loxP-tdTomato (Ai14) transgenic reporter murine model. In Ai14 murine cells, Cre expression induces tdTomato reporter expression. Here, we intravenously infused AAVHSC15 and AAVHSC17 expressing Cre:eGFP for detection of transient (tdTomato$^+$) and stable (eGFP$^+$) expression in HSCs from Ai14 mice. AAVHSC15.Cre:eGFP and AAVHSC17.Cre:eGFP vectors were injected retro-orbitally at a dose of 6E11 or 1.2E12 vector genomes (vgs) per mouse. At two weeks

post-injection, mice were euthanized and bone marrow was isolated by standard procedures. Isolated bone marrow was depleted of red blood cells, filtered and the resulting cells stained for Lin, Sca-1 and c-Kit (LSK, markers of progenitor cells). AAVHSC trafficking to the hematopoietic niche was evident by the distinct tdTomato-positive population in the isolated femoral bone marrow, specifically, 6 to 8% of bone marrow LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) cells. Higher levels of vector genomes were observed in the tdTomato-positive LSK cells compared to tdTomato-negative LSK cells. Subsequently, peripheral blood (red blood cell depleted) from treated Ai14 mice and non-injected control) was evaluated at 2, 6, 10, 14, 18 and 21 weeks (terminal) post-injection. tdTomato-positive cells were detected in the peripheral blood of treated animals at all time points tested, with up to 21% at 21 weeks, demonstrating a dose-response. The identification of tdTomato-positive cells in the peripheral blood long-term is indicative of LSK cell transduction, followed by differentiation and daughter cell proliferation. tdTomato-positive cells were identified in B-cell, T-cell and erythroid lineages evident by expression of cell-specific markers CD19, CD3e, and TER119, respectively. Thus, AAVHSC15 and AAVHSC17-transduced and -modified cells were capable of hematopoiesis. Furthermore, the percentage of tdTomato-positive LSKs at 21 weeks post-injection in the bone marrow was 7-13%, indicating a population of active transduced progenitor cells that are capable of self-renewal and long-term stability. In summary, AAVHSC15 and -17 productively transduced mouse hematopoietic stem cells after retro-orbital IV injection, providing an opportunity for HSC-directed gene therapies for long-term expression and disease treatments.

601. Proof-of-Concept Studies in Mongolian Gerbils Support Intravitreal Gene Replacement Therapy of Human L-Opsin for Blue Cone Monochromacy

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Blue cone monochromacy (BCM) is a rare X-linked retinal disease characterized by the absence of L- and M- opsin expression in cone photoreceptors. The disease is manifested by decreased visual acuity, significant deficiencies in color discrimination, photophobia and nystagmus. Recent studies have demonstrated the feasibility of recombinant AAV vectors delivered subretinally to drive cone-specific expression of visual opsins in opsin-deficient animals. However, subfoveal injection that would be required to restore opsin expression in foveal cones would be unacceptable in the diseased central retina in BCM patients. We are developing ADVM-062 (AAV2.7m8-MNTC-OPN1LW) for cone-specific expression of human L-opsin (hLopsin), optimized for the intravitreal (IVT) delivery. Functional activity of this IVT-injected vector was evaluated in cone-rich Mongolian gerbils (*Meriones unguiculatus*) that naturally lack L-opsin. We previously reported long-term ADVM-062 activity by electroretinography (ERG) at a single time point of 90 weeks post-dose. In the current study, we further evaluated ADVM-062 by assessing the time course of its functional activity, as well as localization of L-opsin protein in cones. Animals were dosed IVT with ADVM-062 at 3.9E11 vg/eye or with vehicle. ERGs were recorded at baseline, 3, 6 and 12 weeks post-dose. All ERGs were recorded following adaptation to a rod-desensitizing background. Activity of endogenous gerbil cone opsins was assessed using 440 and 513 nm flashes. Activity of the transgene opsin was assessed using 630 and 660 nm flashes. ERGs were recorded to a series of increasing flash intensities and to cone-isolating flicker frequencies of 10 and 25 Hz. Naïve gerbil retinas had low amplitude ERG responses to long wavelength stimuli, presumably driven by the endogenous M-opsin sensitivity that extends weakly into long wavelengths. Administration of vehicle-control by IVT injection did not alter baseline ERG responses. In contrast, a single IVT dose of ADVM-062 resulted in a statistically significant augmentation of ERG responses to long-wavelength stimuli ($P<0.0001$ for both 630 and 660 nm wavelengths, 2-way RM ANOVA), while responses to 440 nm and 513 nm stimuli were unaffected. The increase in sensitivity to longwave light was detected as early as 3 weeks post-dose. Increase in 25Hz flicker ERG responses to 660 nm light were consistent with the cone-specific nature of this sensitization. Thus, ERG recordings demonstrated that ADVM-062 was pharmacologically active. The cone-restricted expression of hLopsin was assessed by immunofluorescence in animals IVT-injected with 3.0E11 vg/eye ADVM-062-myc (AAV2.7m8-MNTC-OPN1LW-myc), designed to express human L-opsin with a C-terminal myc-tag. In conclusion, a single IVT dose of ADVM-062 can effectively transduce gerbil cones to sensitize them to long wavelength stimuli as early as 3 weeks following a single IVT injection. These findings support the development of ADVM-062 as a potential IVT-delivered treatment for BCM.

602. Expression-Functional Correlation and Validation of a Surrogate Marker for DAPC Restoration in LGMD2E Mouse Model

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Background: Limb-girdle muscular dystrophy type 2E (LGMD2E) is caused by mutations in β-sarcoglycan (SGCB) leading to protein deficiency, loss of formation of the sarcoglycan complex, and loss of stabilization of the dystrophin-associated protein complex (DAPC). The sarcoglycans and sarcospan are integral proteins critical for stabilizing the DAPC and providing mechanical support to the sarcolemma. *SGCB-/-* mice have been shown to concurrently display loss of additional sarcoglycans (α, γ, and δ). Evidence suggests sarcospan may also be lost in the absence of SGCB. Further evaluation of *SGCB+/-* mice will provide insight into a potential expression-function correlation. Additionally, we hypothesize that other sarcoglycan and sarcospan expression may be able to serve as a surrogate marker for functional restoration of DAPC following SGCB gene transfer. We characterized *SGCB+/-* mice and assessed the ability for SGCB gene transfer to restore sarcoglycan and sarcospan expression in *SGCB-/-* mice and tested their utility as a surrogate marker for DAPC restoration. **Methods:** Transcriptional and translational regulation of SGCB along with functional outputs were assessed in *SGCB+/-* mice. Sarcoglycan and sarcospan protein expression in skeletal and cardiac muscle from untreated and vector-dosed *SGCB-/-* mice was evaluated

by immunofluorescence staining and western blot and compared to functional outputs following gene transfer. **Results:** *SGCB*+/- mice were not found to have any significant dystrophic phenotype and a correlation of expression to function is still being evaluated. Sarcoglycan and sarcospan expression in *SGCB*-/- was significantly reduced, both of which were restored following SGCB gene transfer. **Conclusion:** Preliminary co-localization studies confirmed restoration of the DAPC following SGCB gene therapy. This data suggests the potential for additional sarcoglycans and sarcospan to serve as a surrogate marker for functional restoration of the DAPC.

603. Development of an AAV5-Based Gene Therapy for Dyslipidemia

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Angiopoietin-like 3 (ANGPTL3), a key regulator in lipoprotein metabolism, has recently been identified as an attractive target for reducing TG and LDL-C and cardiovascular disease risk. In addition, ANGPTL3 has been identified as a potential target for the treatment of NASH. Individuals who are homozygous or compound heterozygous for null variants in ANGPTL3 have levels of plasma LDL-C and TGs that are approximately 70% lower than those in persons without such variants. They also have reduced risk of insulin resistance and diabetes and cardiovascular disease. Most importantly, ANGPTL3 deficiency is not associated with an adverse phenotype. We are developing an AAV based gene therapy encoding an artificial miRNA targeting ANGPTL3 for treatment of dyslipidemia. To this end, we have designed and constructed a series of miRNAs, based on our miQURE™ platform, against conserved regions of ANGPTL3. The miRNAs were screened *in vitro* for their ability to knockdown luciferase reporter constructs and endogenous ANGPTL3 mRNA in Huh-7 cells. The best candidate was selected for AAV production and the AAV vector was tested in WT mice at three different doses. A dose-dependent knockdown of ANGPTL3 plasma levels of up to 90% could be achieved. The AAV vector was also tested in the APOE*3-LEIDEN.CETP mouse model that possesses human characteristics with respect to lipid metabolism and responds similarly as humans to all registered hypolipidemic drugs. The AAV-treated dyslipidemic mice showed a significant reduction in TG and total cholesterol without any signs of adverse effects. In conclusion, we here report effective silencing of ANGPTL3 in WT- and APOE*3-LEIDEN.CETP mice leading to lowering of cholesterol and TG. These data warrant further investigation of this approach for effective long-term treatment of dyslipidemia following a one-time therapy.

604. Development of a Novel Cone-Rod Dystrophy 6 (CORD6) Mouse Model to Assess AAV-CRISPR/Cas9-Based Therapies

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Purpose: Cone-rod dystrophy 6 (CORD6) is caused by autosomal dominant mutations in *GUCY2D,* the gene encoding retinal guanylate cyclase-1 (retGC1). We previously showed that AAV- delivered CRISPR/Cas9 can selectively and efficiently target *GUCY2D* and *Gucy2e* in non-human primates and wild type mice, respectively. These experiments established the ability to perform somatic gene editing, but did not determine whether an AAV-CRISPR/Cas9 based approach would confer therapy in a disease setting. Our overarching goal is to establish proof of concept for this approach in mouse models that replicate the CORD6 patient phenotype. Previously described transgenic mice (CORD6-Tg) containing the CORD6-causing R838S *GUCY2D* mutation under the control of a photoreceptor-specific promoter exhibit loss of retinal structure/function similar to patients. Our preliminary data shows that AAV-CRISPR/Cas9 targeted to R838S *GUCY2D* prevents loss of retinal structure/function in these mice. While the CORD6-Tg model mimics aspects of the human disease, it does not accurately reflect its genetics. We recently developed an R838S *GUCY2D* knock-in (KI) mouse via targeted replacement of endogenous *Gucy2e* exon 13 with exon 13 from *GUCY2D* (contains the CORD6-causing R838S mutation). These mice allow us to assess editing at the natural genomic location in a model where the number of wt and mutant alleles are balanced. The purpose of this study is to 1) optimize CRISPR/Cas9 editing in the CORD6-Tg mouse, and 2) characterize our novel R838S-KI mouse model of CORD6 to confirm whether it mimics the CORD6 phenotype. **Methods:** *CORD6-Tg mice.* Two AAV vector plasmids: 1) hGRK1-SaCas9 and 2) U6-*GUCY2D*-gRNA were packaged in AAV44.9(E531D) using triple transfection. Right eyes of CORD6-Tg mice were subretinally co-injected at P14 with AAV44.9(E531D)-hGRK1-SaCas9 and AAV44.9(E531D)-U6-*GUCY2D*-gRNA. Left eyes were injected with AAV-U6-*GUCY2D*-gRNA alone. Electroretinogram (ERG) and optical coherence tomography (OCT) data was collected 4, 10, and 20 weeks post-injection (p.i). Additional in-life data collection is on-going. *R838S-KI mice:* Retinal structure and function of heterozygous R838S-KI mice (carry one mutant human exon 13 and one wt mouse exon 13), and WT littermates were characterized by OCT and ERG analysis at 4, 8, 12, and 20 weeks of age. **Results:** *CORD6-Tg* mice: Photoreceptor structure and function were significantly improved in right eyes for at least 10 weeks post-injection. Data collection at later time-points is on-going. *R838S KI mice*: Significantly reduced retinal structure and function were observed in heterozygous R838S KI mice by 8 weeks of age compared to WT littermates. Data collection for this experiment continues. A cone survivability assay will be performed at the conclusion of the study to further quantify the extent of cone cell loss. **Conclusions**: Preservation of mouse photoreceptor structure and function was achieved via targeted knockout of the *GUCY2D(R838S)* sequence in CORD6-Tg mice using AAV-CRISPR/Cas9. We generated a novel R838S-KI mouse that allows us to assess editing at the natural genomic location. Preliminary results show that this model also mimics the human condition. The R838S-KI mouse is therefore an ideal model in which to test potential therapies.

605. AAVF-ABCD1 as a Potential New Vector for X-linked Adrenoleukodystrophy Gene Therapy

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Adrenoleukodystrophy (ALD) is a neurodegenerative disease caused by mutations in the gene encoding the peroxisomal ATP binding cassette transporter (ABCD1) which help transport acylCoA-activated very long-chain fatty acids (VLCFA) into the peroxisome for degradation. Adrenomyeloneuropathy (AMN) is the most common phenotype of ALD leading to spinal cord degeneration and peripheral neuropathy. Our previous work demonstrated that AAV9-mediated ABCD1 gene transfer via intrathecal (IT) delivery achieves protein expression of ABCD1 in spinal cord but limited delivery to brain tissue. To this aim, a new vector AAVF was recently developed, which mediates greatly enhanced transduction of the CNS in mice after IV and IT delivery routes compared to AAV9. Astrocyte and neurons were the main targeting cell type in both brain and spinal cord. Here, we performed IT delivery of AAVF-ABCD1 and compared to AAV9-ABCD1. AAVF-ABCD1 at 3e10gc/mice achieved similar expression level of ABCD1 in spinal cord and DRG in comparison to AAV9-ABCD1 at 1e11gc/mice. As we observed before, AAV9-ABCD1 didn't transduce mouse brain tissue very well by the IT delivery, in contrast to AAVF-ABCD1, which led to significant amount of ABCD1 expression in mouse brain (around 63% of the spinal cord expression level), consistent with the result from the earlier AAV-GFP report. Similar to AAV9-ABCD1, AAVF-ABCD1 was also detected in peripheral liver tissue but not in heart organ. CNS tissues following AAVF-ABCD1 IT delivery were harvested for very long chain fatty acid (VLCFA) analysis, the result is pending. In conclusion, after IT delivery AAVF-ABCD1 has at least 3 times high transduction efficacy in spinal cord compared to AAV9-ABCD1 and remarkable improvement in brain transduction. Further study on the dosing and distribution of AAVF-ABCD1 as well as functional assays will help to corroborate the feasibility of AAVF-ABCD1 in ALD gene therapy.

606. Exploring Retinal Gene Therapy Using Adeno-Associated Viral (AAV) Vectors in *Crb1* Mutant Rats

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Variations in the *CRB1* gene are a major cause for recessive retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) in children. The brown Norway rats from breeder Janvier (BN-J) have naturally occurring inherited insertion-deletions in exon 6 of the *Crb1* gene causing retinal degeneration. The *Crb1* rats exhibit an early onset phenotype with disruptions at the outer limiting membrane (OLM), rosettes, a disorganized retinal lamination starting at postnatal day 10 (P10), and rapid loss of retinal activity at 1 month of age as measured by electroretinography (ERG). Here, we explored to rescue the early onset degenerative phenotype by adeno-associated viral (AAV) *CRB* application in the *Crb1* rat retina. Tropism of different AAV capsids show that subretinal injection of AAV9.CMV.*GFP* transduces photoreceptor cells and retinal pigment epithelium (RPE), whereas intravitreal injection of ShH10Y.CMV.*GFP* is efficiently transducing Müller glial cells. In addition, using immuno-electron microscopy, we detected the subcellular localization of CRB1 protein at the OLM at the subapical region of Müller glial cells whereas CRB2 is present at the subapical region of Müller glial cells and photoreceptor cells. At post-natal day 5 (P5), one eye of the *Crb1* mutant rats was intravitreally injected with either 1µl of ShH10Y.CMVm.*CRB1* (1.9exp10 vg) or ShH10Y.CMV.h*CRB2* (3.4exp10 vg) whereas the other retina was injected with PBS as a control. Other *Crb1* retinas were subretinally injected at P5 or P17 with AAV9.CMV.*CRB2* (1.8exp10 vg) with no injection in the other retina. AAV treated retinas were analyzed by electroretinography (ERG), optokinetic head tracking response (OKT) and morphology. We observed no significant differences between AAV-injected versus non-injected or PBS-injected eyes. In conclusion, no rescue nor toxicity was observed in the rats treated with the AAV-*CRB1* or AAV-*CRB2* gene therapy vectors. Future research could focus on earlier treatment of *Crb1* rat retinas. Preliminary morphological analysis of one-month old *Crb1* mutant rat retinas injected intravitreally at P3 with ShH10Y.CMV.*GFP* (7.9exp10 vg) showed significant Müller glial cell transduction. Current research is focusing on the treatment of *Crb1* mutant rats using intravitreal injections at P3 of ShH10Y.CMVm. *CRB1* as well as ShH10Y.CMV.h*CRB2.*

607. Multiple Species Comparison of AAV8 Biodistribution Following Intravenous Administration

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Recombinant adeno-associated viruses have demonstrated their effectiveness in clinical gene therapy applications. Understanding the tissue biodistribution and cellular transduction of AAV capsids in various animal species and strains is essential when selecting efficacy and toxicology models to support development of a clinical candidate gene therapy vector. It is also important to consider how AAV production methods (e.g. scale of manufacturing) may influence cellular transduction. Here we provide a comprehensive evaluation of AAV8 biodistribution and transduction efficiency in mice, rats and non-human primates. First, we describe the production and quality control assessment of approximately 6E+15 AAV8 viral particles expressing a secreted reporter gene (SRG) under control of the ubiquitous EF1α promoter and intron regulatory elements. Next, this AAV8 preparation was administered intravenously in both genders of C57Bl6 mice, Sprague Dawley rats, Wister Kyoto rats and cynomolgus macaque at doses ranging from 1e12 to 1e14 vg/kg. At necropsy tissue dissection and sampling of multiple organs was conducted using careful methods and engineering controls to prevent potential cross-contamination of DNA between tissues. Vector genome copy biodistribution and *SRG* mRNA were analyzed with high accuracy and

efficiency in several tissues (including Liver, heart, skeletal muscle and spleen). In addition, in-life blood samples were collected to analyze SRG protein levels. This comprehensive data set provides important insight into the inter-species differences of AAV8 biodistribution and transduction efficacy and may serves as a guide to evaluate additional AAV capsids in preclinical models.

608. Design and Evaluation of Novel AAV Viral Constructs with High Transduction Efficiency in Animal RGCs and Neuronal SH-SY5Y Cells

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Ocular gene therapy has recently gained recognition as a new approach to many complex retinal diseases. For example, adeno-associated virus (AAV) was utilized in a clinical trial as a vector for gene therapy in RPE65 mutations, causing inherited retinal dystrophy and retinitis pigmentosa. Gene manipulation offer numerous advantages over other therapeutic drugs. The aim of this study was to design an AAV viral construct to deliver and express green fluorescence protein (GFP) (720 bp) in the animal retina targeting RGCs which can only be achieved by specific promoter. We have developed a novel AAV2 viral vector with modified small sequence (1054 bp) hybrid promoter (cytomegalovirus + chicken β actin (CAG2)) and an expression enhancer (Woodchuck Hepatitis virus post-transcriptional regulatory element (WPRE)) (592bp) with high RGCs transduction efficiency when injected intravitreally in the animal eye. The expression of GFP was imaged live in the animal 8 weeks after a single intravitreal injection ($3.1x10^{10}$ gc) of AAV2 viral construct with or without WPRE. Optical coherence tomography (OCT) and fundus imaging (Phoenix Micron IV) was performed and analyzed at 8 weeks. The efficiency of AAV2 transduction was also evaluated in retinoic acid differentiated SH-SY5Y neuronal cells *in vitro*. The enhanced GFP expression was confirmed by fundus imaging in live wild type mice with fluorescently labelled RGCs expressing GFP in AAV2.CAG2.GFP.WPRE injected mice retina, whereas no GFP expression was seen in AAV2.CAG2. GFP treated mice. No noticeable variation in individual retinal layer thickness was observed in these animals on OCT scans after intravitreal injection of AAV2. Subsequently, the eye and optic nerve were harvested, and confirmed for eGFP expression using western blot and immunohistochemistry. Western blot showed significantly elevated eGFP expression in AAV2.CAG2.GFP.WPRE treated cells as compared to AAV2.CAG2.GFP treated cells: mean±SEM; control *vs* AAV2.CAG2.GFP *vs* AAV2.CAG2.GFP.WPRE; 89.29 ± 6.52 vs 358.4 ± 18.12 vs 452.2 ± 53.72, p=0.0006 n=3 in each group). The expression of eGFP in cells treated with AAV2.CAG2.GFP and AAV2.CAG2.GFP. WPRE respectively were also verified by immunofluorescence staining. Both novel viral vectors supported efficient transduction of RGCs after intravitreal injection *in vivo* and neuronal cells *in vitro*. Also, this construct can hold an additional gene (~1800 bp) along with the GFP gene. Modulation of genes in the RGCs can therefore be achieved using these novel AAV constructs as targeted retinal gene therapy and may be adapted to neurodegenerative diseases, such as glaucoma.

609. Transduction Profiles of Engineered Adeno-Associated Viral Capsids in Mouse and Marmoset

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Adeno-associated viruses (AAVs) remain promising vectors for gene therapy due to stable expression *in vivo* and a strong clinical safety record. Unfortunately, naturally occurring AAV serotypes are inefficient transducers of many gene therapy relevant tissues, requiring high viral doses to achieve therapeutic efficacy for many disease indications. Fortunately, AAVs are amenable to engineering efforts to improve tissue tropism and specificity. Past efforts have produced AAV variants with improved transduction capabilities for clinically relevant cell populations, including crossing the blood-brain-barrier (BBB) in mice (AAV-PHP.eB) [1], but the efficacy of those variants has not translated across all strains and species. With the aim of enhancing viral tropism for refractory targets, libraries of AAV9 were selected for novel characteristics using Multiplexed Cre-recombination-based AAV targeted evolution (M-CREATE) [2]. Viral genomes from capsids that transduced tissues of interest across rodent Cre-lines are selectively amplified and recovered through the M-CREATE method, allowing simultaneous positive and negative selection of AAV variants. Systemic administration of AAV libraries through intravenous injection permits non-invasive transduction of tissues where direct administration is difficult, enabling subsequent variant selection within gene therapy relevant cell populations. We are presenting data on three novel engineered capsids: variant AAV-CAP.A4, which was identified for improved transduction in mouse lung tissue, and variants AAV-CAP.B10 and AAV-CAP.B22 that, when administered systemically, can cross the blood—brain barrier and efficiently transduce neurons in adult mice and marmosets. AAV-CAP.A4 was compared against serotypes AAV5 and AAV9 for lung tissue transduction after systemic injections of 1e11 viral capsids/animal. AAV-CAP.A4 displays a 17-fold higher total lung transduction over AAV9 and a 45-fold improvement over AAV5. In Alveolar Type II pneumocytes, the improvement in transduction over AAV9 and AAV5 reaches 29-fold and 100-fold, respectively. This enhancement in AAV-CAP.A4 transduction is specific to the lung, while the liver and other tissues targeted by AAV9 have similar transduction profiles. A panel of novel AAV9 variants was identified for similar transduction across the BBB as the strongest current neurotropic AAV, PHP.eB [1]. Promising variants were chosen for pooled screening via systemic delivery in adult marmosets, from which AAV-CAP.B10 and AAV-CAP.B22 were chosen for independent screening. Both variants display improved transduction across the brain relative to AAV9. In the marmoset cortex, AAV-CAP.B10 and AAV-CAP.B22 transduce neurons ~15 fold and ~17 fold higher than AAV9 respectively. These novel variants enable robust, non-invasive gene delivery to the adult marmoset brain following IV administration. This work demonstrates that, through the M-CREATE method, novel AAV variants can be developed with sought-after transduction profiles in clinically relevant cell populations. We have also shown that relative improvements to the transduction profile obtained through

in vivo selection in mice can be translated to non-human primates. [1] Chan, K.Y. et al. *Nat. Neurosci.* 20, 1172-1179 (2017). [2] Kumar, S. R. et al., Nature Methods, *In press.*

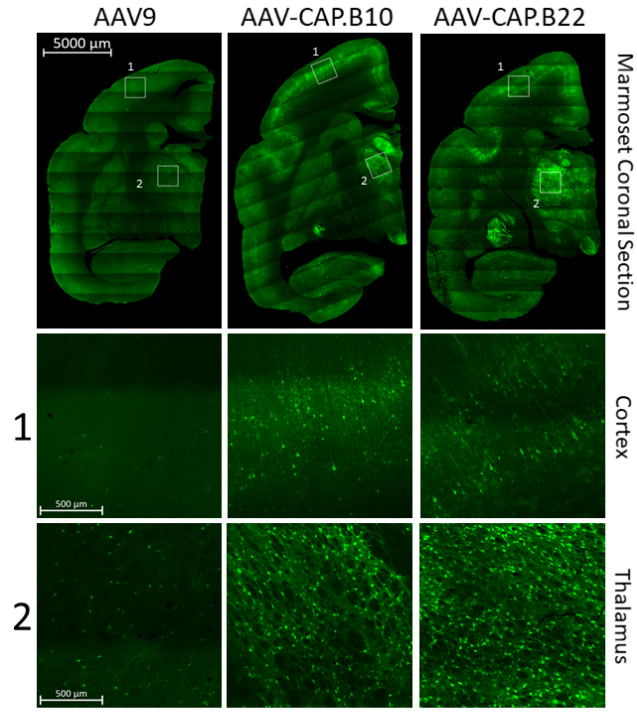

Figure: Comparison of adult marmoset CNS transduction between AAV9, AAV-CAP.B10, and AAV-CAP.B22. Transduced cells expressing CAG-HA-Frataxin are labelled green. Viral variants and serotypes are arranged in columns, with corresponding inserts from cortex and thalamus.

610. Abstract Withdrawn

AAV Vectors - Clinical Studies

611. Improved Motor Function in Children with AADC Deficiency Treated with Eladocagene Exuparvovec (PTC-AADC): Compassionate Use Study

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Background: Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare inherited disorder caused by mutations in the AADC gene. Resulting deficiencies of neurotransmitters required for normal motor function impede motor development within the first months of life. As current treatments yield little improvement, gene therapy has been proposed to restore neurotransmitter production and improve motor function. This study evaluated the efficacy and safety of PTC-AADC, a recombinant adeno-associated virus containing the human cDNA encoding the AADC enzyme, in children with AADC deficiency for up to 5 years post-treatment. **Methods:** An observational study was performed on data from a single-arm, investigator-initiated, compassionate use trial enrolling children >2 (range, 2.00-8.25) years of age with confirmed AADC deficiency (n=8) who received bilateral intraputaminal PTC-AADC (total dose, $1.8x10^{11}$ vg). Primary efficacy endpoint was the proportion of patients achieving key milestones at 5 years as measured by the Peabody Developmental Motor Scale, Second Edition (PDMS-2), compared with a historical control group (n=82). Secondary efficacy endpoints included change from baseline in PDMS-2, Alberta Infant Motor Scale (AIMS), and Comprehensive Developmental Inventory for Infants and Toddlers (CDIIT) scores; change in body weight; and neurologic examination findings related to AADC deficiency symptoms. Putaminal L-6-[^{18}F] fluoro-3,4-dihydroxyphenylalnine (^{18}F-DOPA) uptake on positron emission tomography (PET) was evaluated as a pharmacodynamic endpoint. Safety endpoints included treatment-emergent adverse events (TEAEs) through 5 years and viral shedding. Mean study follow-up duration was 62.5 months. **Results:** At baseline no enrolled patients had full head control, could sit unassisted, or stand or walk with support. Five years post-PTC-AADC, 4 of 8 patients exhibited full head control and could sit unassisted (P=0.0002 vs control), while 2 of 8 could stand with support (P=0.045 vs control) (table).

Table: Number of Patients Achieving Key Motor Milestones

Motor Milestone	Timepoint	PTC-AADC Group(n=8)	Historical Control Group(n=82)	*P* Value
Full head control	1 year	4	-	-
	2 years	4	-	-
	5 years	4	0	0.002
Sitting unassisted	1 year	2	-	-
	2 years	4	-	-
	5 years	4	0	0.002
Standing with support	1 year	0	-	-
	2 years	0	-	-
	5 years	2	0	0.0454
Walking with assistance	1 year, 2 years	0	-	-
	5 years	0	0	N/A

Least-squares mean PDMS-2, AIMS, and CDIIT total scores increased from baseline to 5 years (all P<0.0001), with onset of improvement as early as 3 months for PDMS-2 and AIMS and 6 months for CDIIT. From baseline to year 5, mean body weight increased (P=0.027) with steady increases in the majority of patients. The number of patients with AADC-related hypotonia, oculogyric crises, limb dystonia, and stimulus provoked dystonia decreased during the first year after PTC-AADC; improvements in these movement disorders were apparent as early as 1 month post-treatment. Mean putaminal ^{18}F-DOPA PET uptake increased as early as month 6 and continued through 5 years (P=0.0134). All patients experienced ≥1 TEAE, none considered related to PTC-AADC and most of mild or moderate intensity. Eight patients experienced 9 possibly/probably treatment-related dyskinesia episodes; these generally occurred in the first few months post-treatment and resolved within 4 months. Seven patients experienced ≥1 serious AE, none related to PTC-AADC. There were no deaths during the study (1 patient died after the 60 month study period). Viral vector was not detected in blood after PTC-AADC. **Conclusions:** Children

with AADC deficiency achieved sustained improvements in motor function after PTC-AADC, along with rapid and maintained increased putaminal dopamine production. No new safety signals were identified.

612. Use of Prophylactic Steroids to Mitigate Potential T-Cell Response in AAV8-Mediated *hLDLR* Gene Transfer in Subjects with Homozygous Familial Hypercholesterolemia

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Homozygous familial hypercholesterolemia (HoFH), a rare genetic disorder, is characterized by markedly elevated levels of low-density lipoprotein cholesterol (LDL-C) and early atherosclerotic cardiovascular disease. Bi-allelic mutations in the LDL receptor (*LDLR*) gene are the most common cause of HoFH. Reconstituting functional *LDLR* in the liver may be a valuable treatment strategy. We are conducting a first-in-human clinical trial of AAV8.TBG.hLDLR (also called RGX-501) *hLDLR* gene transfer in adult subjects with genetically confirmed HoFH due to *LDLR* mutations. We enrolled 9 subjects, ages ranging from 24-41 years, 6 males and 3 females. There were 3 subjects in each of Cohort 1, Cohort 2, and Expansion Cohort 2 who received a single IV administration at the doses of $2.5x10^{12}$ GC/kg for Cohort 1, and $7.5x10^{12}$ GC/kg for Cohort 2 and Expansion Cohort 2. One subject in Cohort 1 experienced a mild transitory activation of the innate immune system accompanied by hypotension and elevation in transaminases approximately 22 hours post dosing that resolved within a day. All 3 subjects in Cohort 2 experienced initial elevations in transaminases 4-6 weeks post-dosing, attributed to a T-cell response based on corroboration by enzyme-linked immune absorbent spot (*ELISpot*) assay sensitive for T-cell response to the AAV8 capsid or human LDLR transgene. The peak ALTs were 165 (normal range: 6-41 IU/L), 388 (normal range: 10-40 IU/L), and 1469 (normal range: 0-55 IU/L) IU/L in the 3 subjects. All 3 subjects were asymptomatic and responded to treatment with prednisone at 60-100 mg/day, followed by a slow taper, resulting in transaminases decreasing to normal levels. In the expansion of Cohort 2, the protocol was modified to include a 13-week prophylactic steroid regimen of prednisone 40 mg/day for 8 weeks beginning on the day before RGX-501 administration followed by a 5-week taper to mitigate potential T-cell response. The peak ALTs were <1.5x ULN in all 3 subjects. Administration of prophylactic steroids to subjects in Expansion Cohort 2 appears to have mitigated T-cell response and subsequent elevations in transaminases resulting from AAV-mediated gene therapy that were previously observed in Cohort 2. Mitigation of T-cell response may have the potential to preserve transgene expression in transduced hepatocytes.

613. Improved Motor Function in Children with AADC Deficiency Treated with Eladocagene Exuparvovec (PTC-AADC): Interim Findings from a Phase 1/2 Study

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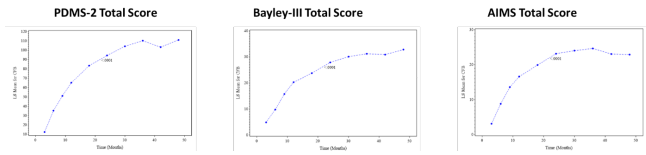
Background: Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare disorder caused by mutations in the gene encoding the enzyme AADC. Resulting deficiencies in monoamine neurotransmitters, namely dopamine, thwart normal motor development, leading to missed motor milestones and subsequent movement disorders, such as oculogyric crises. Current treatments fail to restore dopamine production and improve motor function. Gene therapy replacing the mutated *DDC* gene is being investigated to fulfill this unmet need. We report interim findings from a phase 1/2 study evaluating the efficacy and safety of PTC-AADC, a recombinant adeno-associated virus vector containing human cDNA encoding the AADC enzyme, in children with AADC deficiency. **Methods:** This is an interim analysis at 2 years from a 5-year, phase 1/2, prospective, open-label trial of children with AADC deficiency receiving bilateral intraputaminal injection of PTC-AADC (total dose, $1.8x10^{11}$ vg). The primary efficacy endpoint was the proportion of patients achieving key milestones at 2 years as measured by the Peabody Developmental Motor Scale, Second Edition (PDMS-2), compared with a historical control group (n=82). Secondary efficacy endpoints included changes in PDMS-2, Alberta Infant Motor Scale (AIMS), and Bayley Scales of Infant Development, Third Edition (Bayley-III) scores; change in body weight; and neurologic examination findings related to AADC deficiency symptoms. Putaminal L-6-[^{18}F] fluoro-3,4-dihydroxyphenylalnine (^{18}F-DOPA) uptake on positron emission tomography (PET) was evaluated as an objective measurement of *de novo* dopamine production. Safety endpoints included treatment-emergent adverse events (TEAEs) and viral shedding. The mean duration of study follow-up was 39.9 months. **Results:** A total of 10 patients (median age 34.0 months) were enrolled and received treatment. All completed follow-up through year 1 except 1 patient (withdrawn at 11 months due to influenza B encephalopathy leading to death), and all the others completed follow-up through year 2. The figure shows significant improvements over a 4-year period in 3 motor function scales.

Figure. Mean Change From Baseline in Motor Development Scores for Patients Treated with PTC-AADC



Mean body weight also increased from baseline to year 1 (P=0.0011), and the number of patients with hypotonia, oculogyric crises, limb dystonia, and stimulus provoked dystonia decreased. Mean putaminal ^{18}F-DOPA PET uptake increased by year 1, increasing further through year 2. No viral shedding was detected in either blood or urine. All patients experienced ≥1 TEAE, most of mild or moderate intensity; none were considered definitely related to treatment. Of 131 TEAEs, 27 were possibly related to PTC-AADC, including 18 dyskinesia episodes; most resolved within 6 months with routine management.

Seven patients experienced a total of 18 serious AEs; all resolved except for influenza B encephalopathy in 1 patient that resulted in death. All were considered unrelated to study treatment. **Conclusions:** Children with AADC deficiency achieved improvements in motor function after intraputaminal PTC-AADC therapy, coinciding with evidence of increased *de novo* dopamine expression in the brain. No new safety signals were identified. The findings of this interim analysis add to the growing body of data supporting the efficacy and safety of PTC-AADC in AADC deficiency.

614. Interim Data from the First in Human RGX-121 Gene Therapy Trial for the Treatment of Severe MPS II (Hunter Syndrome)

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Mucopolysaccharidosis type II (MPS II) or Hunter syndrome is caused by a deficiency of iduronate-2-sulfatase (I2S) leading to an accumulation of glycosaminoglycans (GAGs) including heparan sulfate in tissues. Severe MPS II results in irreversible neurocognitive decline and behavioral symptoms that are not addressed by intravenously administered enzyme replacement therapy with recombinant I2S enzyme. RGX-121 is a recombinant adeno-associated virus serotype 9 capsid (AAV9) containing a human iduronate-2-sulfatase expression cassette (AAV9.CB7.hIDS). In an MPS II murine model, RGX-121 administered into the cerebrospinal fluid (CSF) demonstrated dose-dependent I2S activity, reduced levels of GAGs, amelioration of storage pathology in the central nervous system and improved neurobehavioral function. Vector distribution and reduced levels of GAGs were observed in peripheral organs, as well as normalization of liver size and weight. In this phase 1/2, first-in-human, multicenter, open-label, dose escalation trial (NCT03566043), RGX-121 is administered as a one-time injection into the cisterna magna of participants with severe MPS II ages 4 months to 5 years. Assessments include safety and tolerability up to 104 weeks; CSF, plasma and urine biomarkers; immunogenicity; neurodevelopmental scales (Bayley Scales of Infant and Toddler Development or Kaufman Assessment Battery for Children, and Vineland Adaptive Behavior Scales); audiometry; imaging of the brain, liver and spleen; and clinician- and patient-reported outcome measures. Cohort 1 has completed enrollment with at least 1 patient completing 16 months of follow-up. Cohort 2 is actively enrolling and dosing. RGX-121 administration has been well tolerated. In Cohort 1, CSF levels of heparan sulfate, a biomarker of neuronopathic disease in MPS II, showed consistent and durable reductions measured up to 48 weeks. Early neurodevelopmental testing demonstrated stabilization in a patient with pre-existing cognitive decline and ongoing skill acquisition in asymptomatic patients less than 2 years of age.

615. Review of Safety and Interim Analysis of Efficacy in the First in Human Intrathecal Gene Transfer Trial for Giant Axonal Neuropathy

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Giant Axonal Neuropathy (GAN) is a rare childhood onset neurodegenerative disorder of the peripheral and central nervous system. Recessive *GAN* mutations cause loss of function of gigaxonin, a cytoskeletal regulatory protein, leading to progressive sensorimotor and optic neuropathy, CNS involvement and respiratory failure. We present an updated report on a single site, phase I, non-randomized, open label dose escalation gene transfer study for GAN (NCT02362438), a first-in-human intrathecal (IT) AAV9 mediated gene transfer with the longest patient follow-up for an intrathecal trial. 12 GAN patients have been dosed thus far at four dose levels; $3.5x10^{13}$ (1X), $1.2x\ 10^{14}$ (3.3X), $1.8x10^{14}$ (5X) and $3.5x10^{14}$vg (10X), with scAAV9-JeT-GAN with now up to 3 years of follow up. We review safety of intrathecal gene transfer and immune modulation and present an interim analysis of efficacy . GAN natural history study data is used for comparison of outcome measures which include: Motor Function Measure 32 (MFM32), Neuropathy Impairment Score (NIS), Friedreich's Ataxia Rating Scale (FARS), electrical impedance myometry (EIM), ophthalmologic assessments and neurofilament assays. We highlight the overall safety of an intrathecal route of AAV9 based gene transfer and include preliminary safety for the highest human intrathecal dose to date ($3.5x10^{14}$vg). The efficacy interim analysis brings forward several key aspects: 1) feasibility for adequately targeting the nervous system, 2) characterization of an optimal IT dosing regimen that is safe and tolerated, 3) relevance of a carefully controlled natural history cohort for comparison in the absence of a placebo arm, 3) the value of exploratory outcome measures in a complex neurodegenerative disorder. We show that the disease progression compared to natural history is slowed in a dose dependent manner. Efficacy data for patients dosed up to the $1.8x10^{14\ vg}$dose level will be presented. This study is a proof of concept for IT gene transfer as a strategy for gene replacement targeting the central nervous system.

616. Successful Technology Transfer and Clinical Manufacturing for AAV Gene Therapy Vectors: Lessons from Multiple Campaigns

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The use of Adeno Associated Virus (AAV) for gene therapy applications is entering an age of manufacturing industrialization. Nevertheless, the tech transfer processes and clinical manufacturing of AAV gene therapy vectors requires significant coordination across multiple departments within the sponsor company and the Contract Manufacturing Organization (CMO). Given the relative maturity of sponsors and CMOs across the Cell and Gene Therapy space, it is critical that strong relationships are built between relevant SME's (sponsor and CMO) to support communication of technical information early in the project. Effective risk assessment and gap analysis are central to the success of the technology transfer. Gap analysis should include: equipment similarity and compatibility, facility fit, standard procedures, documentation, personnel experience and personnel training related to the AAV manufacturing field. Risks associated with timelines have to be considered and effectively discussed to determine appropriate mitigation early in the project. Voyager Therapeutics has recently completed multiple runs for several separate programs at different CMOs. Here, we present and analyze the main challenges associated with the transfer of knowledge to the CMOs and the impact of these challenges on the overall manufacturing timeline. Our analysis shows that developing and mastering a production process in-house (R&D or pilot), while important, is not the only key to a successful transition to cGMP manufacturing when it comes to AAV production. Early and particular attention has to be given to technology transfer and project planning with the CMO in order meet program timelines and achieve reasonable COGS. Lessons learned from our experience and the framework principles needed to enable a successful technology transfer will be presented.

617. Safety and Tolerability of PF-06939926 in Ambulatory Boys with Duchenne Muscular Dystrophy: A Phase 1b Multicenter, Open-Label, Dose Ascending Study

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Background: Duchenne muscular dystrophy (DMD) is an X-linked disorder caused by mutations in the *DMD* gene resulting in the absence of the protein dystrophin. Children with DMD have progressive muscle weakness and many experience loss of ambulation by age 12, require ventilatory assistance in their teens and suffer premature mortality in their third decade. Mutations that maintain the dystrophin reading frame produce a partially functioning dystrophin protein and results in a milder phenotype known as Becker muscular dystrophy (BMD). PF06939926 is a gene therapy viral construct consisting of a recombinant adeno-associated virus serotype 9 (AAV9) vector and mini-dystrophin gene based on the sequence from a family affected by mild BMD, who maintained ambulation beyond 50 years of age. In a DMD rat model, PF06939926 showed dose-dependent increases in the proportion of muscle fibres expressing mini-dystrophin, the concentration of muscle dystrophin and in improved muscle strength and endurance. This is an ongoing multi-center (United States), open-label, non-randomized, ascending dose study to assess the safety and tolerability of a single intravenous infusion of PF-06939926. (ClinicalTrials.gov Identifier: NCT03362502) **Methods**: Eligible children are male with a genetic diagnosis of DMD and are aged 4-12 years, receiving a stable, daily regimen of glucocorticoids. Children with neutralizing antibodies to AAV9 or T-cell responses to the transgene by Enzyme-Linked ImmunoSpot above a pre-set threshold are not eligible. Approximately 15 children will receive between 1E+14 vg/kg and 3E+14 vg/kg of PF-06939926 as per an inverted terminal repeat-based quantitative polymerase chain reaction (qPCR) drug product titer assay. Enrollment will be staggered within and between different dose cohorts. Formal review by an external data monitoring committee is required prior to dose progression and in the event of pre-defined safety signals. Primary endpoints will be evaluated through 12 months post-treatment and include adverse events, abnormal laboratory and clinically relevant changes in physical and neurological examinations. Other endpoints include muscle biopsy, biomarkers and functional assessments. Available results will be reported at the meeting. **Acknowledgments:** The authors would like to acknowledge the investigators**:** Russell J. Butterfield, MD, PhD; Perry B. Shieh, MD, PhD, and Edward C. Smith, MD, as well as the DMD patient community, for their contribution to the study.

618. Long Term Follow-Up of Integration Profiles in Subjects Enrolled in the First SIN-LV Gene Therapy Trial for Treatment of X-Adrenoleukodystrophy

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X-linked adrenoleukodystrophy (X-ALD) is a severe monogenetic disease that affects between 1:20,000 and 1:50,000 individuals. A successful therapeutic approach represents autologous ex vivo hematopoietic stem cell gene therapy. Our clinical study starting in

2006 was the first to apply such strategy to treat 4 subjects suffering X-ALD and to employ HIV-1 based lentiviral vectors (LVs) with self-inactivating (SIN) configuration. LVs have been meanwhile applied in > 300 different clinical trials. Therefore, long-term follow-up integration site (IS) analyses that may show a consistent diverse gene-corrected cell pool represents a valuable source for underlining the safety of LV gene therapy. IS analysis of samples collected from four subjects (P1: 3 - 6 years (y); P2: 3 - 10 y; P3: 3 - 6 y; P4: 3 - 8 y) was performed applying GCP compliant genomic DNA shearing based S-EPTS/LM-PCR (shearing extension primer tag selection ligation-mediated PCR). IS analysis retrieved in average > 1,000 unique IS per whole blood sample and revealed a consistent, polyclonal hematopoietic reconstitution. No signs of clonal dominance were found, while not a single IS contributed over 5% of total IS detected in two consecutive visits. Though several IS within genes related with severe adverse events in gene therapy have been observed (CCND2, MECOM), none of them indicated a selective advantage. The detection of identical IS in myeloid and lymphoid lineages (overlap:1.1% - 6.0%) confirmed a robust engraftment and long-term survival of transduced hematopoietic progenitor cells. Downstream analysis revealed the characteristic LV insertion profile, with gene-rich regions as main targets (exceeding 77% of IS in all four subjects) and no preference for transcriptional start sites (TSS; 6.5% of all IS are within 10kb up- or downstream of a gene). Interestingly, besides already known genomic regions described for LV clustering, such as KDM2A and PACS1 genes, additional common integration regions have been observed in Mir612, TNRC6C and SMG6 genes. Furthermore, not more than 2.5% of detected IS were found in the vicinity of oncogenes. Overall, the landscape of ALD vector integration over a span of 3 to 10 years was very similar in all four subjects analyzed. Such findings are in line with the subjects' clinical situation, showing long-term positive effects on brain demyelination (presented in a separate abstract by Aubourg et al.). S-EPTS/LM-PCR is a sensitive and unbiased tool for IS analysis. A diverse clonal repertoire in the first four treated X-ALD subjects up to 10 years post therapy indicates no signs of LV genotoxicity.

619. Proposed Patient Population and Outcome Measures for a First in Human Study of PR006, an AAV9-Based Gene Therapy, for Fronto-Temporal Dementia Patients with Pathogenic GRN Mutations

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Objective: To describe the patient population and outcome measures of the Phase 1/2, first-in-human trial of PR006, a recombinant adeno-associated virus serotype 9 (rAAV9) vector-based gene therapy candidate transducing a copy of the human *GRN* gene, for patients with FTD-GRN (PROCLAIM trial). Background: Fronto-temporal dementia (FTD) is a devastating dementia syndrome encompassing a heterogenous group of clinical syndromes characterized by progressive deficits in behavior, executive function, and language. Up to 40% of FTD cases are familial, and about 5% to 10% of those cases are caused by mutations in the progranulin gene (*GRN*). FTD with *GRN* mutations (FTD-GRN) is a rapidly progressing dementia with death occurring within 3 to 10 years after diagnosis and no approved treatment options. Patients suffering from FTD-GRN carry a single mutation in the *GRN* gene, resulting in haploinsufficiency and an approximately 50% reduction in PGRN levels. In FTD-GRN patients, delivery of a functional copy of the *GRN* gene by rAAV9 directly to the CNS may normalize PGRN levels, potentially restoring lysosomal function and slowing neurodegeneration, thus resulting in modification of disease progression. As multiple brain regions are affected in FTD-GRN, broad biodistribution of such therapy is needed. The AAV9 vector is particularly well suited to deliver the *GRN* gene to the CNS, given its ability to transduce multiple CNS cell types, the persistence of expression of the transgene, and its safety record. Selecting the optimal patient population and relevant outcome measures is another critical point for early efficacy biomarker signal detection in FTD-GRN clinical trials. Methods: PROCLAIM is a Phase 1/2 randomized, open-label, ascending dose study to evaluate the safety and efficacy of PR006 in patients with FTD-GRN. The study will evaluate three dose levels of PR006 in ascending dose cohorts. A total of 15 patients will be enrolled in the study and will receive PR006 at a low, mid or high dose, administered sub-occipitally intra cisterna magna. Patients with different FTD-GRN phenotypes will be allowed to participate in the study provided that they are at a symptomatic stage of disease as ascertained by a CDR plus NACC FTLD sum of boxes score of between 1 and 15 inclusive. The primary objective is to evaluate safety and tolerability of PR006, as well as to quantify the change in PGRN levels in blood and CSF. Secondary objectives are to assess pharmacodynamic effects of PR006 on neurofilament light chain protein, clinical decline as assessed by the CDR plus NACC FTLD instrument, as well as immunogenicity. Exploratory objectives include the assessment of clinical symptom burden, MRI measures of cortical atrophy and white matter hyperintensities, and biomarkers of neuroinflammation and astroglial pathology. Results: The PROCLAIM trial is planned to open patient enrollment in 2020. Conclusions: PROCLAIM will assess safety, tolerability, biomarker and efficacy effects of PR006 and will allow selection of an optimal PR006 dose for further clinical development.

620. Dual Routes of Administration in Clinical Trial Design for the Treatment of Friedreich's Ataxia

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Friedreich's ataxia (FA) is the most common form of hereditary ataxia, affecting about 5,000 individuals in the US and 15,000 people worldwide. It is caused by a trinucleotide GAA repeat expansion in the frataxin (FXN) gene that results in decreased activity of iron-sulfur cluster enzymes and energy production. As a consequence, the spinal cord, sensory nerves, and cerebellum degenerate, causing neurological symptoms including uncoordinated movements and sensory impairment, and affecting fine motor function and gait. FA patients often have severe hypertonic cardiomyopathy with cardiac fibrosis and arrhythmia.

Currently, there is no effective treatment for FA. This project is a collaboration to develop an AAV-based gene therapy to target the cardiac and CNS disease manifestations of FA. Preclinical studies show the efficacy of an adeno-associated virus (AAV) engineered to carry the human frataxin FXN gene driven by the ubiquitous chicken β-actin (CBA) promoter. The construct has been shown to produce fully-processed human frataxin (hFXN) protein in animal studies. Toxicology and biodistribution studies in rats and non-human primates demonstrate safety of the investigational product and support an upcoming clinical trial. The proposed clinical trial is an open-label, single ascending dose for the first two cohorts (Phase I/II), followed by a randomized, controlled, double-blind in cohorts three and four (Phase IIb). The study will investigate the safety, tolerability, and exploratory efficacy of concurrent intravenous (IV) and intrathecal (IT) injection of rAAV9-CBA-hFXN in patients with FA. Outcome measures validation, natural history data, and protocol design has been informed by a 5-year longitudinal study in 50 individuals affected by FA. Planned outcome measures of this trial will include safety (Phase I) and mFARs scores (Phase IIb), as well as frataxin protein quantification and other exploratory outcomes in children and adults with FA. We will collect a battery of clinical, physiological and imaging outcome measures including cardiac and brain magnetic resonance imaging (MRI), gait analysis, metabolic exercise testing and muscle mitochondrial oxidative phosphorylation by MRI. In addition, a custom-made health-based application will be used for continuous remote data collection. Patients are screened for anti-AAV9 titer by ELISA assay in an IRB approved pre-screening protocol. Manufacturing of the clinical product following Good Manufacturing Practices (GMP) guidelines is ongoing. Completion of this project will be an important step towards understanding FA and advancing our clinical knowledge.

621. Multiyear Follow-Up of AAV2-ND4 Gene Therapy for Leber's Hereditary Optic Neuropathy

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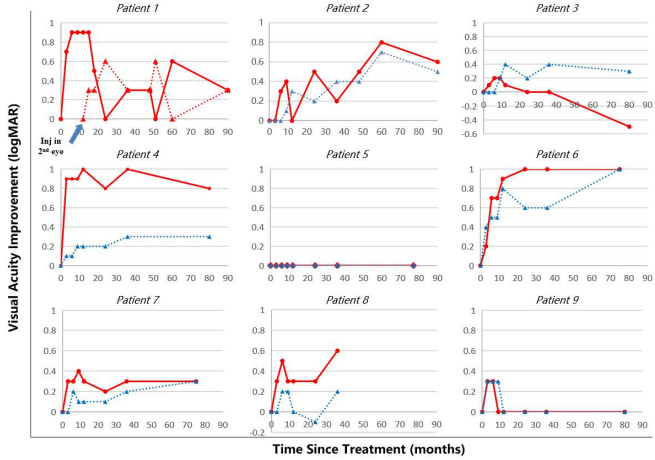
Purpose: Adeno-associated virus (AAV)-mediated gene therapy is under investigation as a therapeutic option for the Leber's hereditary optic neuropathy (LHON). We conducted a prospective, open label clinical trial evaluating safety, tolerability and efficacy of a single unilateral intravitreal (IVT) injection of NR082-1, a recombinant adeno-associated virus (rAAV) expressing the NADH ubiquinone oxidoreductase subunit 4 (*ND4)* gene, in 9 patients with G11778A mutation of the ND4-associated LHON. Thirty-six months follow-up results of these 9 patients have been previously published. Here, we report safety and efficacy data up to 7.5 years of follow-up after a single injection of NR082-1. ***Methods:*** Between 2011 and 2012, nine patients aged between 8 and 60 years old with LHON carrying the ND4-G11778A mutation were enrolled in a prospective, international, open-label gene therapy study (NCT01267422) evaluating the safety, tolerability, and efficacy of a single $1x10^{10}$ vector genome (vg) unilateral IVT injection of NR082-1. The primary endpoints were adverse events or serious adverse events reported during the long-term follow-up visits up to 7.5 years post-treatment. The secondary endpoints included best-corrected visual acuity (BCVA) and visual fields. ***Results:*** Eight out of nine patients were followed up. Of the eight patients (out of nine treated patients), six patients maintained clinically significant (defined as ≥0.3 logMAR, three lines) improvement of their BCVA at 75-90 months follow-up visits. No adverse events such as uveitis, vitreous inflammation, keratitis, crystal damage and immune response were observed. Slit-lamp examination showed no anterior or posterior segment abnormalities. ***Conclusions:*** Gene therapy with rAAV2-ND4 in patients with G11778A mutation of the ND4-associated LHON resulted in sustained, clinically relevant benefit, as measured by BCVA in six out of eight patients who had received a single $1x10^{10}$ vg IVT injection of NR082-1. With a follow-up period of up to 90 months, no late toxic effect from the therapy were reported.



622. Assessment of Residual Full-Length SV40 Large T Antigen in Clinical-Grade Adeno-Associated Virus Vectors Produced in 293T Cells

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One challenge in manufacturing adeno-associated virus (AAV) vectors is to produce large quantities of vectors with high purity and function. In this regard, for manufacturing with a mammalian system, the choice of the cell line is critical. In addition to adenovirus E1A and E1B genes, Human embryonic kidney HEK-293T cells constitutively express SV40 large T antigen with the T antigen providing enhanced helper function for AAV production by stimulating Rep expression (Ogston et al. J Virol 2000; 74:3494). HEK-293T thus represents an excellent cell line for AAV production and has been widely used in GMP facilities to achieve high yield AAV vectors for clinical applications. However, down-

stream purification and quality control for HEK-293T cell-produced AAV vectors require assessment of host cell residual DNA and there is a theoretical concern for any consequences of the presence of DNA encoding SV40 large T antigen DNA (SV40T). In this study, we describe a method for assessment of full-length transcription-competent SV40T DNA in AAV vectors using an AAVrh.10 vector encoding mCherry reporter cDNA as an example. The presence of SV40T in purified vector was assessed by directly analyzing the purified AAV vector (1.4 x 10^{11} genome copies) by qPCR targeting a 129 bp sequence of SV40 T sequence. Less than 100 copies (50-70 copies) of SV40T DNA was detected in the input 1.4 x 10^{11} copies AAV vector genome. Using nested PCR targeting full-length SV40T DNA, no detectable signal was seen in 1.4 x 10^{11} AAV genome copies where the limit of detection was 10 copies SV40T DNA. Finally, as a measure of transcribable full-length SV40T, we assessed mRNA expression in mice following high dose (5 x 10^{12} gc/kg) intravenous administration of AAVrh.10mCherry. SV40T mRNA was assessed in liver, the primary site of transduction following intravenous administration. RT-qPCR analysis demonstrated high levels of transgene mCherry mRNA expression while SV40T mRNA was undetectable. In summary, despite the presence of small segments of SV40T DNA in a purified AAV preparation, this did not translate to any measurable SV40T mRNA in an *in vivo* assay designed to maximize signal using the liver for amplification. This method can be adapted to any other serotype of AAV despite their difference in tissue tropism by simple modification of assessing the SV40T mRNA expression in the primary site of transduction. This highly-sensitive assay, if adapted in the GMP for routine vector quality control and lot release provides assurance that HEK-293T cells can be used safely for high yield and quality AAV vector production for therapeutic use.

623. Design of a Phase 1/2 Study to Evaluate the Safety and Efficacy of an AAV9-Based Gene Therapy in Infants with Type 2 Gaucher Disease

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Objective: To describe the design of the PROVIDE trial, an open-label evaluation of the safety and efficacy of PR001, a recombinant adeno-associated virus serotype 9 (rAAV9) vector-based investigational gene therapy that transduces the human *GBA1* gene for infants with Type 2 Gaucher disease. **Background:** Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder diagnosed by the presence of biallelic pathogenic mutations in *GBA1* or a finding of less than 15% of normal activity of the *GBA1* encoded enzyme glucocerebrosidase (GCase) in peripheral blood leukocytes. GD is a classic loss-of-function disease: *GBA1* mutations causing more profound enzyme deficiencies are associated with earlier onset of GD, faster progression of symptoms, and a higher likelihood of neurological manifestations, termed neuronopathic Gaucher disease (nGD). Infants with Type 2 Gaucher disease, the acute neuronopathic form, have the most severe reductions of GCase. By 6 months of age, in addition to the peripheral manifestations of GD, infants present with progressive and severe neurologic manifestations, including supranuclear gaze palsy and convergent squint, severe stridor and apnea, spasticity, opisthotonos, and failure to achieve motor, behavior, and cognitive milestones. Most children die by age 2. Whereas the majority of peripheral GD manifestations can be attenuated by treatment with enzyme replacement therapies (ERTs), the neurological manifestations of GD do not respond as ERTs do not penetrate the central nervous system (CNS). Thus, there is a high unmet need for an effective treatment for the devastating neurological symptoms of Type 2 GD. PR001 is an rAAV9 vector-based investigational gene therapy that transduces the human *GBA1* gene to the cells of the CNS, and in animal models, restored GCase activity and lysosomal function, reduced GCase glycolipid substrates from toxic accumulation, and reduced other lysosomal defect-driven pathology. The AAV9 vector is particularly well suited to deliver the *GBA1* gene to the CNS, given its ability to transduce multiple CNS cell types, the persistence of expression of the transgene, and existing safety data. **Methods:** PROVIDE is an open-label, Phase 1/2, multicenter study to evaluate the safety and efficacy of single-dose PR001 in infants diagnosed with Type 2 GD. Up to 15 patients with bi-allelic *GBA1* mutations and neurologic signs and symptoms consistent with a diagnosis of Type 2 GD will receive PR001 administered via suboccipital injection into the cisterna magna. The primary objective of this study is to evaluate the safety and tolerability of PR001, including the development of any immune response. Secondary objectives include evaluation of the effect of PR001 on clinical events and measures, including cognition, adaptive behavior, and functioning; and pharmacodynamic changes in GCase enzyme and glycolipids (e.g., glucosylceramide [GluCer] and glucosylsphingosine [GluSph]) in the blood and cerebrospinal fluid (CSF). **Results:** The PROVIDE trial is planned to open patient enrollment in 2020. **Conclusions:** Intracisternal administration of PR001 will provide an opportunity to assess whether PR001 can safely increase GCase activity directly in the CNS of Type 2 GD patients and can fill an unmet medical need by providing meaningful clinical benefit in the management of the neurological manifestations of GD.

624. Continuing Clinical Efficacy and ALDP Gene Expression 5-10 Years after Lentiviral LV-Based CD34+ Cell Gene Therapy in Patients with X-linked Adrenoleukodystrophy (X-ALD)

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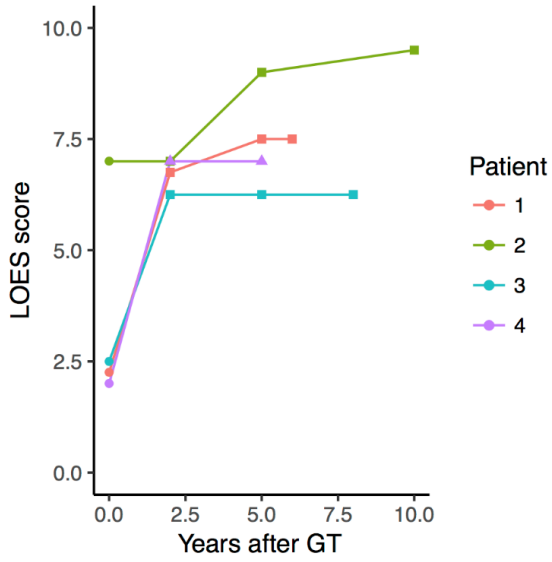
X-ALD is caused by mutations of the ABCD1 gene encoding the ALDP protein. Untreated boys develop a rapidly progressive multifocal brain demyelination and most die before adolescence. Until the end of the 2000s, allogeneic hematopoietic stem cell transplantation (HSCT) at an early stage of brain lesions was the only effective therapy, thought to be mediated by the replacement of brain microglial cells derived from donor bone marrow myelo-monocytic cells. Since allogeneic HSCT is limited by donor-related constraints and carries a major risk of mortality, we attempted lentiviral-based hematopoietic stem cell gene therapy (LV-GT) in 4 patients aged 4.5- 7.6 years who had no bone marrow donor (Cartier & Hacein-Bey-Abina et al 2009). X-ALD became the first monogenic disease of the CNS to be successfully treated with LV-GT and few years later, this was confirmed in a larger group of patients (Eichler 2017). Herein we report a long-term observation of the 4 patients who pioneered LV-GT. All of them survived although they had gadolinium-enhanced lesions at pre-treatment brain MRI that are predictive of death within 5 years in 91% of untreated patients. At the last visit, neurological examination was still normal in 2 patients and showed mild alterations in 2; there was a significant but moderate deterioration of the neurological function score and verbal and non-verbal performance intelligence quotient. At MRI, the mean LOES score, which quantifies demyelination, increased only from 3.4 before LV-GT to 6.7 at 2 years, then remained nearly stable over 5-10 years of evolution (Figure). Mean VCN in PBMC was 0.20 6 months after LV-GT, then between 0.1 and 0.2 from 1 year post GT to date The % of ALDP positive cells showed a consistent decrease in CD15+ granulocytes, CD14+ monocytes, CD3+T lymphocytes, and CD19+B lymphocytes, without relationship with the clinical status. In conclusion, LV-GT has maintained in the long term its positive effects on brain demyelination, despite a marked decrease in VNC and ALDP expression in circulating cells. Our observations await confirmation in a larger cohort and longer follow-up. The Long Term Follow-Up of Integration Profiles in the 4 patients is presented in a separate Abstract by Labik et al. .



Gene Targeting and Gene Correction

625. Targeted Genomic Integration to Restore the NF1 Coding Sequence in Models of Neurofibromatosis Type I (NF1)

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Neurofibromatosis Type I (NF1) is an autosomal dominant disease caused by the loss of function of the protein neurofibromin, a GTPase-activating protein that negatively regulates the Ras signaling pathway. Loss of neurofibromin leads to the formation of malignant and benign neurofibromas originating in non-dividing cells that form the myelin sheath of peripheral nerves, called Schwann cells. This disease effects 1 in 3,000 people worldwide, however there is no effective treatment to reduce the size or number of neurofibromas. Gene editing technology could address the root cause of NF1 by correcting mutations in the *NF1* gene, however current approaches are not broadly applicable due to a series of limitations. First, the 8.6 kb *NF1* coding sequence is too large to be delivered in its entirety via traditional gene therapy vectors. Second, no single patient mutation occurs in the population at a frequency greater than 2% and mutations are distributed along the full coding sequence of the gene, such that no single conventional gene editing approach can address a significant portion of the population. Finally, post-mitotic Schwann cells do not efficiently utilize homologous recombination pathways. Given these challenges, we have developed a CRISPR/Cas9-based strategy to restore the correct *NF1* gene sequence through the non-homologous end joining repair process, which we estimate to be applicable to 90% of NF1 patients.

This strategy includes the delivery of one of two ~5 kb donor cassettes, encoding either the 5' or 3' half of the *NF1* cDNA sequence, depending on the patient mutation, flanked by a *S. aureus* Cas9 (SaCas9) guide RNA (gRNA) target site that corresponds to a sequence in the middle of the *NF1* gene. For mutations occurring in either the 5' or 3' half of the gene, the corresponding donor would be used. Co-delivery of SaCas9 and this gRNA with the appropriate donor leads to a double-strand break in the middle of the *NF1* gene and on both sides of the donor sequence, creating free DNA ends for ligation of the donor sequence into the genomic double-strand break, thereby restoring a wild-type *NF1* coding sequence. We predict that this will result in the production of functional neurofibromin and will lead to a reduction in Ras signaling and a decrease in the size and number of tumors in a NF1 mouse model. First, we designed gRNAs targeted to the middle of the *NF1* gene, between exons 30 and 31, and their editing activity was evaluated in HEK293T cells. Donor cassettes containing the 5' half of the *NF1* cDNA were constructed for each of the top four performing gRNAs with the highest levels of editing activity. The gRNA and donor cassette pairs were then delivered with SaCas9 in HEK293T cells and genomic DNA and RNA were harvested. Sequencing of the genomic DNA with targeted Tn5-based sequencing confirmed that the intended insertion occurred in ~8% of alleles for the top performing gRNA and donor cassette pair. Additionally, successful transcription of the donor neurofibromin-coding sequence and correct splicing to endogenous exon 31 of *NF1* was confirmed through Illumina amplicon sequencing. Two AAV8 vectors were generated encoding SaCas9/gRNA and the associated donor cassette. These were co-delivered by intramuscular injection into wild-type mice. Eight weeks after injection, we collected genomic DNA from these tissues and used PCR across the insertion to confirm the targeted integration of the 5' half of the *NF1* cDNA in the skeletal muscle. Current work includes quantifying the editing efficiency in the skeletal muscle, as well as Schwann cells, where the disease originates. Future studies will evaluate this strategy in both patient cell lines and an NF1 mouse model to determine if restoration of the correct *NF1* sequence can reduce Ras signaling and the number and size of neurofibromas.

626. Using Nanopore Sequencing to Characterize On-Target CRISPR-Mediated Gene Editing

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Gene therapies using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are being developed to treat a myriad of previously intractable genetic disorders. Introduction of the CRISPR components into live organisms is often achieved by adeno-associated virus (AAV) vectors delivering the Cas nuclease and guide RNAs to the tissues and cells affected. The variable consequences of CRISPR editing events at both on- and off-target sequences include small indels, large deletions, inversions, translocations, and integration of the AAV genomic sequence. Each of these results can have lasting effects, requiring the development of better assays to interrogate and more accurately predict these DNA cleavage and repair events. Recently, Hanlon *et al.* and others have reported the integration of AAV vector into endogenous DNA following CRISPR mediated cleavage to be high, using PCR based assays. In order to eliminate the size and sequence specific bias that can be introduced with PCR amplification or polymerase based sequencing technology, we sequenced the tissue of two mouse models treated with AAV-CRISPR in vivo. Both mouse models contain long trinucleotide repeats, one with approximately 130 CGG repeats knocked into the Fmr1 endogenous locus (*Fmr1* KI), and a BAC transgenic model with approximately 72 CAG repeats within the human *ATXN2* gene (BAC-*ATXN2*-Q72). One month following the stereotaxic injection of adult mice in the striatum (*Fmr1* KI) or deep cerebellar nuclei of the cerebellum (BAC-*ATXN2*-Q72) of adult mice, DNA was harvested from the corresponding brain regions and sequenced following Cas9-mediated enrichment of the target loci. Native DNA libraries were sequenced using the Nanopore MinION to capture long reads. We detected deletions of the corresponding trinucleotide repeats in both mouse models, and also sequences that remained unedited at the target loci. We also detected the integration of the AAV-CRISPR delivered cargo sequence at the target locus in the *Fmr1* KI mouse striatum. Target enrichment and sequencing strategies that do not rely on DNA polymerase offer an unbiased look at the genomic sequence, and are a useful tool for characterizing the consequences of CRISPR-mediated DNA editing in vivo. These results are important as we consider how to monitor the safety of gene editing strategies.

627. Correcting Duchenne Muscular Dystrophy Using AAV-Mediated Split Base Editors

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Duchenne Muscular Dystrophy (DMD) is an X-linked degenerative muscle disease with early childhood onset first manifested as muscle weakness that progresses rapidly leading to paralysis and ultimately death of the patients in their 20s. DMD is caused by mutations in dystrophin, an essential gene for maintaining the stability of the muscle membrane by linking the cytoskeleton of the muscle cells with the extracellular matrix. While there is no cure for DMD, exon skipping is emerging as a highly promising approach to correct DMD by inducing the spliceosome to exclude frame-shifted exons from mature mRNA, which creates a truncated dystrophin that ameliorates the symptoms of DMD. Currently, most methods of exon skipping require direct delivery of antisense oligonucleotides to the muscle cells, an approach that is inherently transient, resulting in recurrent invasive and costly procedures over the lifetime of the patient. We have overcome the problems associated with conventional exon skipping by engineering a programable CRISPR base editing exon skipping strategy, which we named CRISPR-SKIP, for disrupting the splice acceptor of target exons. By targeting either base of the conserved AG dinucleotide within splice acceptors, CRISPR-SKIP achieves efficient and permanent exon skipping in the target cells. Our results demonstrate that CRISPR-SKIP can effectively disrupt the splice acceptor of exon 45 in the dystrophin gene, a strategy that can be utilized to restore the dystrophin reading frame in patients with deletions of neighboring exons, which comprise approximately 9% of DMD cases. Overall, the panel of base editors that

we have created can introduce targeted base modifications ranging from 8.6-27.3% of the alleles, which is anticipated to be sufficient to provide a functional correction of DMD. Additional experiments demonstrated that targeted genome editing led to efficient skipping in mRNA and restoration of dystrophin protein expression. Finally, to enable in vivo correction of DMD, we developed a split intein base editing system that is compatible with AAV delivery. We demonstrate that split intein base editors fully recapitulate the activity of their full-length counterparts at the splice acceptor of dystrophin exon 45. In summary, this work describes a novel exon skipping strategy that relies on base editors to enable efficient skipping of exon 45 in the dystrophin gene as well as a platform for *in vivo* delivery of base editing tools.

628. HDR-CRISPR: A Novel System to Promote Cas9-Mediated Homology-Directed DNA Repair

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In the last decade CRISPR-Cas systems have been widely used for genome editing applications. After the introduction of a targeted DNA double strand break (DSB), the predominant non-homologous end-joining (NHEJ) DNA repair pathway ensures high frequencies of targeted gene knockouts. However, using this technology for precise genome editing is still challenging. It requires the harnessing of the homology-directed repair (HDR) pathway and the efficient delivery of a proper donor template, often resulting in HDR frequencies far below the thresholds required for clinical translation. Typically, the frequency of HDR-mediated DSB repair is increased with the use of chemicals that transiently arrest the cells in the S/G2 cell cycle phase when HDR is most active or that inhibit NHEJ. However, the global effects of these drugs pose serious safety concerns if applied in clinically relevant cells. To address this issue, we aimed at increasing the local concentration of factors involved in HDR at the site of the DSB to drive the cell to engage HDR rather than NHEJ for genome editing. We identified 6 candidates which are critical in DSB repair pathway choice and generated 13 different Cas9-fusion proteins (referred to as HDR-CRISPRs). We investigated their impact on DNA repair choice using a traffic light reporter (TLR) based on a non-functional mVenus protein fused to an out-of-frame TagRFP. Upon introduction of a DSB, NHEJ-mediated repair will result in Tag-RFP expression while HDR with the corrective DNA template will restore mVenus expression. With our best-performing HDR-CRISPR, we achieved up to 3-fold increase in HDR-mediated repair. Importantly, simultaneous inhibition of the NHEJ pathway led to an almost even distribution of HDR and NHEJ events. To further corroborate our findings, we used an alternative fluorescence reporter in which the coding sequence of a blue fluorescent protein (BFP) is converted into green fluorescent protein (GFP) by introducing a single nucleotide conversion. We designed single- or double-stranded oligodeoxynucleotides (ODN) harbouring the desired nucleotide change with sequence homology of varying lengths and measured their ability to promote HDR-mediated conversion of BFP to GFP via flow cytometry. We show in this system that HDR-CRISPR increases the frequency of precise editing up to 4-fold. Interestingly, the best-performing HDR-CRISPR were different for the two reporter systems, highlighting that the nature of the donor template may affect the DNA repair pathway choice, thereby requiring the deposition of different DNA repair factors at the DSB site to engage HDR-mediated repair. These results support our hypothesis that the availability of key HDR-promoting factors at the site of the DSB influences the DNA repair pathway choice in a donor dependent manner. We envision that this strategy is readily translatable to clinically relevant systems, opening new opportunities for the future use of CRISPR-Cas systems for precise genome editing applications

629. Combination of CRISPR/Cas9-Triggered Reactivation of Endogenous Fetal Globin and SB100x Transposase-Mediated Gamma-Globin Gene Addition for In Vivo HSC Gene Therapy of Hemoglobinopathies

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The degree of phenotypic correction of beta-thalassemia is closely correlated with the level of gamma-globin expression. We have recently reported that SB100x transposase-mediated gamma-globin gene addition achieved 10-15% gamma-globin of adult mouse globin, resulted in significant but incomplete phenotypic correction in a thalassemia intermedia mouse model. We and others have also shown that reactivation of endogenous fetal globin represents another promising approach for gene therapy of hemoglobinopathies. Here we aimed to combine these two mechanisms to obtain higher levels of gamma-globin expression. Exploiting the large packaging capacity of helper-dependent HDAd5/35++ adenovirus vectors, we generated a HDAd5/35++ vector (HDAd-comb) containing a CRISPR/Cas9 cassette targeting the BCL11A binding site within the HBG1/2 promoters, a gamma-globin gene cassette driven by a 5kb beta-globin mini-LCR, and an EF1α-mgmt^{P140K} expression cassette allowing for in vivo selection of transduced cells. The latter two cassettes were flanked by *FRT* and transposon sites. Upon co-infection with another vector expressing SB100x transposase and Flippase (HDAd-SB), this design allows for the excision/integration of the globin/mgmt cassettes mediated by Flp-SB100x transposase. Concurrently, Flp-*FRT* mediated excision will destroy the HDAd genome, thereby shortening the duration of CRISPR/Cas9 expression and decreasing Cas9-mediated toxicity to long-term repopulating cells. In HUDEP-2 cells, co-transduction with HDAd-SB resulted in over 50% reduction of Cas9 expression by day 10 after transduction, compared to transduction with HDAd-comb alone. We next tested our vectors in "healthy" human CD46/beta-YAC transgenic mice which expresses huCD46 for efficient HDAd5/35++ in vivo HSC transduction and 248kb of the human beta-globin locus that accurately reflects globin switching. After in vivo transduction with HDAd-comb and selection with low doses of O^{6}BG/BCNU, 95% of RBCs expressed human gamma globin. We detected by HPLC that the gamma-globin over adult mouse alpha globin levels reached ~20%, more than 2.5-fold and 1.5-fold higher than in mice transduced with the individual CRISPR/Cas9 or gene addition vector, respectively. Gamma-globin mRNA expression increased to a similar level. In addition, the human gamma over human beta globin expression reached 50% on average, indicating a dramatic switching. Moreover,

compared to the CRISPR vector alone, the cleavage level at the CRISPR target site was significantly higher with HDAd-comb, most likely due to a better survival of genome-edited primitive HSCs. Transplantation of lineage-depleted bone marrow cells from HDAd-comb in vivo transduced primary mice into lethally irradiated C57BL/6J recipient mice exhibited stable, high-level gamma-globin expression. Next, we attempted testing the combined system in a mouse model for sickle cell disease, the Townes model crossed with CD46 transgenic mice. These mice showed characteristic RBCs with sickling phenotype and abnormal percentage (35%) of reticulocytes in peripheral blood. After transduction with HDAd-comb + HDAd-SB and selection, similar levels of gamma globin expression were measured compared to the results from CD45/beta-YAC mice. Although we found that the Townes model only supported partial reactivation of endogenous fetal globin, a complete phenotypic correction was observed in most of the treated mice. Our observations demonstrate that the combined vector system leads to additive gamma-globin expression at a level that could be sufficient for a cure of hemoglobinopathies.

630. Extraction of Guide RNA from a Transcript for Association with Deactivated Cas9

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Advancements in gene targeting using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats and associated protein) are creating new options for gene therapy approaches. For an autosomal dominant disease, a deactivated version of Cas9 (dCas9) could be used in combination with a guide RNA to specifically suppress the disease allele. Such a strategy would require constant provision of the dCas9 and the gRNA and would therefore benefit from AAV delivery to the target cell type. The AAV transgene would need to enable expression of both the dCas9 and the gRNA, which are typically generated from a RNA polymerase II promoter and a RNA polymerase III promoter, respectively. This offers potential safety issues for whilst expression of the dCas9 could be cell-specific, expression of the gRNA would be ubiquitous and the consequences of gRNA presence in non-targeted cells is currently unknown. To investigate future options for transgene design, we attempted to generate a functional gRNA using a RNA polymerase II promoter. A gRNA was inserted after the CAG promoter in a reporter construct in two forms, with additional nucleotides at the 3' end of the scaffold sequence (G1) and with no additional nucleotides (G2). The resulting constructs of pCAG.G1.GFP.WPRE.pA and pCAG.G2.GFP.WPRE.pA were used to transfect HEK293T cells with control samples transfected with a standard pCMV.dCas9.U6.gRNA construct. After 48 hours, GFP expression confirmed functional transcripts were being produced and cells were harvested with cytoplasmic and nuclear enriched fractions isolated for protein and RNA assessments. Western blot analysis confirmed successful enrichment of cytoplasmic and nuclear fractions and RT-PCRs across the 5'UTR revealed successful removal of G1 but not G2 from the transcripts. QPCR assessments were performed using three primer sets targeting GFP, gRNA-GFP and gRNA sequences. Total RNA ratios of the three targets were 1 : 0.9 : 1.3 for cells transfected with the G2 construct and for the G1 construct, ratios of expression were 1 : 0.2 : 0.3. The significant difference in levels of detected gRNA compared to GFP in G1 transfected cells (p=0.0077) suggested degradation of the gRNA. Co-transfection of the pCAG.G1.GFP.WPRE.pA with pCMV.dCas9 and a dual luciferase plasmid containing the target sequence for the gRNA in the 5'UTR of the *Renilla* expression cassette revealed no knockdown of *Renilla* activity. We present encouraging data that indicate a gRNA can be generated from a RNA polymerase II promoter, offering potential in the future for achieving gRNA and dCas9 expression off a single cell-specific promoter. However, further investigations are required to build on these preliminary investigations.

631. Improving the Chemical Synthesis and Performance of Extended-Length Guide RNAs for Genome Editing and Control of Gene Expression

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The landscape of CRISPR-based technologies for genome editing and control of gene expression is expanding rapidly and many of these technologies utilize extended-length guide RNAs that are challenging to produce by traditional RNA synthesis chemistries. Two examples include (i) the SAM CRISPR-activation system (Konerman, et. al., Nature, 2014) which employs guide RNAs around 160-nt long containing two MS2 RNA aptamers appended to stem-loops in the guide RNA to recruit transcriptional activation domains, and (ii) Prime Editing (Anzalone et. al., Nature, 2019) which employs extensions of the guide RNA to direct the replacement or insertion of new DNA sequences into the genome. Using a novel RNA synthesis chemistry (Dellinger et. al. JACS, 2011) we find it straightforward to chemically synthesize long RNA oligos in the size range currently utilized for these applications. We are continuing to further develop synthesis and purification methodologies to enable effective synthesis of even longer RNA oligos. Chemical synthesis of RNAs affords multiple advantages including (i) increased efficacy, (ii) robust and scalable production of highly pure sgRNAs for biotechnological and therapeutic applications, and (iii) greater flexibility in the sgRNA design including the ability to incorporate chemical modifications site-specifically to enhance performance. We are studying the impact of various types of chemical modifications in 163mer guide RNAs designed for the SAM CRISPR-activation system aimed at enhancing their stability in cells while maintaining their guide RNA functionality. Experimental results using qRT-PCR to directly measure the half-lives of these modified guide RNAs transfected into mammalian cells without Cas9 protein, as well as other cell-based assays to monitor the activity of the extended-length modified guide RNAs complexed with Cas9 protein, will be presented. These studies elucidate the types and positions of chemical modifications which do not interfere with the activity of extended-length guide RNAs but can improve their performance and utility, for example, by increasing the duration of their activity when delivered into cells or *in vivo*.

632. Off-Target Analysis Platform Demonstrates High Specificity of Lead Megatal Enzymes

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Gene editing has shown potential for the treatment of genetic disorders and engineering cell-based therapeutics, such as CAR-T cells. Its success in developing human therapeutics is dependent on the ability to develop efficient and specific nucleases that generate a DNA break to drive the editing process. megaTALs, comprising a sequence-specific engineered meganuclease domain appended to a programmable transcription activator-like (TAL) array, offer the benefits of extended target site length, DNA binding and cleavage site specificity, and a monomeric architecture making them attractive for potential therapeutic application. To analyze the specificity of megaTALs, we developed a platform that utilizes genome-wide off-target discovery via *in silico* prediction and oligo-capture assay followed by verification of the discovered off-target sites by targeted sequencing. While oligo-capture identified the majority of the validated off-target sites for a candidate megaTAL, *in silico* prediction could identify only a small number of bona fide off-targets for megaTALs showing the robustness of oligo-capture for off-target identification. We also show that the TAL array within a given megaTAL was not sufficient to drive off-target activity. For validation of activity at discovered putative off-target sites, we employed a multiplexed amplicon sequencing method that can allow verification of indel activity at hundreds of potential off-targets sites simultaneously while maintaining a high sensitivity of ~0.1 % indel frequency. Overall, we show megaTALs can be designed to yield the required high levels of on-target activity and genome-wide specificity for use in human therapeutics.

633. Enhancing Site-Specific Gene Integration Efficiency for Permanent CFTR Gene Correction

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Objective: Using helper-dependent adenoviral (HD-Ad) vectors, we demonstrated that *a human CFTR* minigene can be precisely integrated into a specific chromosomal location through CRISPR/Cas9-mediated homology repair in both pig and human cells. One unique advantage of our system is that following gene integration, there is no residual Cas9 expression, which eliminates the potential risk of the host immune attack on cells with gene integration. In this study, we aim at enhancing the efficiency of gene integration to provide better long-term therapeutic benefits. **Methods:** HD-Ad vectors containing CRISPR/Cas9 and donor template (6 kb *LacZ* or 8.7 kb *hCFTR* cassette flanked by homology arms) were delivered into pig or human cells. To increase integration efficiency, we co-transduced a vector expressing A protein factor. Pig *GGTA1* or human *AAVS1* locus was chosen as a safe harbor to receive transgene integration. CRISPR/Cas9 cleavage and transgene integration frequencies were quantified by digital PCR. The *LacZ* integration was also verified by X-gal staining and β-galactosidase assays. The CFTR function was assessed by membrane potential sensitive dye-based (FLIPR) and Ussing chamber assays. **Results:** We confirmed the site of gene integration by junction PCR and Sanger sequencing. We detected transgene mRNA and protein expression. The *LacZ/hCFTR* integration efficiency was increased by 2-5 fold with protein factors. In $CFTR^{-/-}$ cells, the function of integrated *hCFTR* was measurable. We also detected hCFTR channel response in pig $CFTR^{-/-}$ primary airway cells by the Ussing chamber assay. Immunostaining also confirmed hCFTR expression. **Conclusions:** These results validated the potential of our *CFTR* gene targeting strategy. We showed that Cas9 expression was eliminated following transgene integration, which is important for *in vivo* applications since Cas9 is a foreign protein and elimination of its expression would avoid immune responses to gene-corrected cells.

634. Mapping the Cell-Cell Communication of Arc in the Brain Using *In Vivo* Gene Editing and Expansion Microscopy

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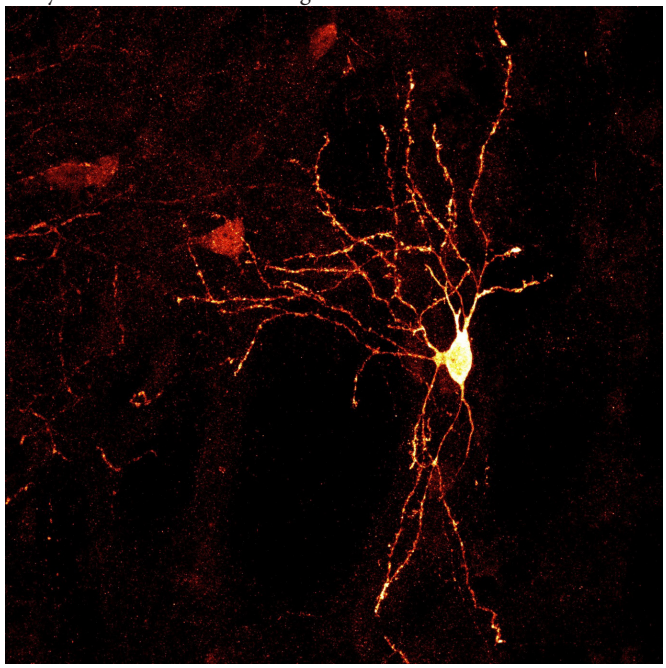
SUMMARY: In this project, we want to develop tools to map brain connections and their circuitries. These tools are based on the signaling pathway of the activity-regulated cytoskeleton-associated protein (Arc). Recently, synaptic protein Arc has gained much attention due to its property to self-assemble and form a virus-like capsid that packages Arc mRNA. The genetic material is then transferred to another neuron where exhibits its function. Using a CRISPR/Cas9 based tool named HiTi (homologous independent targeted integration, we aim to knock in fluorescent probes in Arc. Together with expansion microscopy (ExM), we will be able to visualize the nanoscale Arc vesicles transfer between neurons, allowing for mapping individual connections and study their function. This project also provides a detailed study of the HiTi system on how DNA donor molecules integrate into the genome. **INTRODUCTION:** Arc is an immediate-early gene which expression is essential for synaptic homeostasis. It is involved in memory formation and is a master regulator of synaptic plasticity. Dysregulation of Arc has been associated with neurodegenerative disease. We lack a definite proof of how this cell-cell communication is regulated in the mammalian brain. We are missing what implications this genetic material transfer cause in a healthy brain, and whether this transport changes with a disease. **METHODS:** In our study, we adopted the HiTi system to label the Arc protein on its N-terminal with different fluorescent probes. GFP, mCherry, and the c-myc were inserted in different loci of the 5' untranslated region to study which tag would work best without

hindering the Arc function. We used Illumina deep sequencing to better characterized how the insertion of the DNA donor template occurs in the GOI. Moreover, tagmentation-assisted PCR was carried out to investigate the off-targeting of the HiTi system. Due to the ability of the ExM to spatially retain the localization of small molecules in the gelled matrix, we implemented it in our immunohistochemistry (IHC) protocol. This approach will enable us to visualize the transfer of Arc particles between synaptic boutons. **RESULTS:** Fluorescent cells in striatal and hippocampal sections were found only when the HiTi system targeting Arc was combined with Cas9 protein. IHC analysis showed accumulation of Arc protein in dendritic spines (fig). Following PCR on genomic DNA and Sanger sequencing proved the knock-in of the fluorescent probes in the Arc gene. The sequencing results showed correct insertion of the DNA donor template at the 5' end while at the 3' end, variable indel events occur with both templates. Further analysis, such as Illumina deep sequencing, will elucidate more on the HiTi knock-in mechanism. **CONCLUSION:** The gene engineering HiTi system was efficiently delivered through our AAVs in the mouse brain. Successful gene engineering of Arc, proved the technology to be reliable for gene modification in the CNS. The spatial localization of Arc protein in the dendritic spines was consistent with previous research done in primary culture cells. Moreover, the accumulation of the protein in the post-synaptic compartments explains its importance in synaptic regulation. However, whether the transfer of Arc particles occurs at detectable levels or not between cells remains to be determined. This study will further our knowledge into how cells connect in the brain.



635. Optimization of Electroporation Conditions for Cas9 RNP Mediated Gene Editing

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In the CRISPR-Cas9 gene editing process, Cas9 protein and the sgRNA combine to form a ribonucleoprotein (RNP) complex to elicit gene editing. Cas9 can be delivered to the cells by transfection of a Cas9 plasmid or purified Cas9 protein. Electroporation is an important delivery method for Cas9 protein, plasmid and the sgRNA. The electroporation based gene editing process usually requires optimization and it is not clear why sometimes it fails. Here we found that several widely held notions are incorrect and they affect gene editing. First, it was believed that Cas9 protein and sgRNA takes 20 to 30 minutes to reconstitute. We found that even if electroporation is performed right after mixing the Cas9 protein and the sgRNA with the cells, gene editing level is similar to using longer-time reconstituted RNP. This result suggested that either Cas9 RNP formation is very fast, or they can go into cells separatedly to form RNP later. Second, we found that Cas9 RNP requires about 3% to 10% higher voltage than plasmid electroporation. If the difference is not adjusted, gene editing efficiency will be very low, potential leading to the wrong conclusion that a particular sgRNP is unsuitable. Thirdly, we found that the molar ratio of Cas9 protein and sgRNA is not critically important and optimization on the ratio is not worthwhile. Our work simplified the current electroporation protocol for RNP based gene editing and it may help researchers achieving better experiment outcomes.

636. Abstract Withdrawn

637. A Global Survey of Products of Homology-Directed Repair Catalyzed by CRISPR/Cas Gene Editing in Mammalian Cells

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As CRISPR-Cas systems advance toward clinical application, it is essential to identify all the outcomes of gene-editing activity in human cells. Reports highlighting the remarkable success of homology-directed repair (HDR) in the treatment of inherited diseases may inadvertently underreport the collateral activity of this remarkable technology. We are utilizing an in vitro gene-editing system in which a CRISPR-Cas complex provides the double-stranded cleavage and a mammalian cell-free extract provides the enzymatic activity to promote non-homologous end joining, Micro-Homology Mediated End Joining, and Homology Directed Repair. Here, We Detail the Broad Spectrum of Gene Editing Reaction Outcomes Utilizing Cas 9 and Cas12a in combination with single-stranded donor templates of the sense and nonsense polarity. This system offers the opportunity to see the range of outcomes of gene-editing reactions in an unbiased fashion, detailing the distribution of DNA repair outcomes as a function of a set of genetic tools.

638. Quantitative Modelling Somatic Cell Genome Editing Strategies for Autosomal Recessive and Polygenic Disease

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Patient outcomes from genome editing are not solely dependent on the efficiency of the genome editor. Tissue morphogenesis and selection for edited genomes plays a role in determining systemic outcome. To investigate these factors in a putative gene therapy approach for involving precise correction of a gene *in vivo*, we developed a mechanistic mathematical model for enzyme replacement therapy and enzymatic tissue correction by GAA and a mechanistic model of genome editing in the mouse liver focused on Hereditary Tyrosinemia Type 1 and healthy mice. This model was combined with a previously established model for the central dogma to generate a model for understanding cellular outcomes from the dosing of AAVs, DNA plasmids, mRNA nanoparticles encoding different types of genome editors - spCas9, saCas9 and base editors.

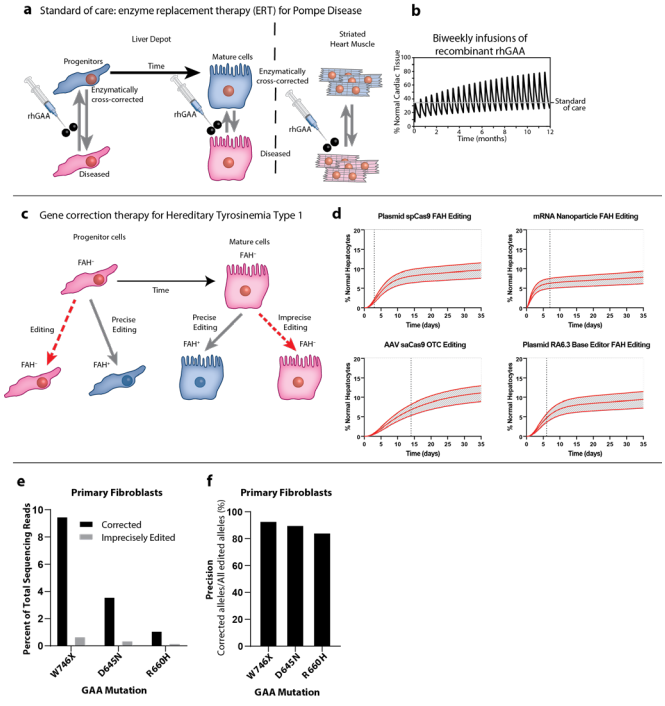

Figure 1: a. Schematic of the model for enzymatic cross-correction, b. percentage of corrected cardiac tissue based on biweekly enzyme replacement therapy c. Schematic of the model for mouse gene editing d. Results from simulation of model in c for various modes of genome editor delivery e. In vitro editing of patient fibroblasts showing in vitro editing f. In vitro editing has high precision This model was augmented with data from RNP mediated editing of mouse muscle and eye tissue to evaluate the probable efficiency of editing the mouse liver. This mathematical model was then combined with a model for the cross-correction of tissues with extracellular acid-alpha-glucosidase (GAA) produced by the liver depot to model a gene therapy for biallelic infantile Pompe Disease - a lysosomal storage disease. Finally, cell therapy for Pompe disease was modeled by considering the lifespan of native and dosed hepatocytes.

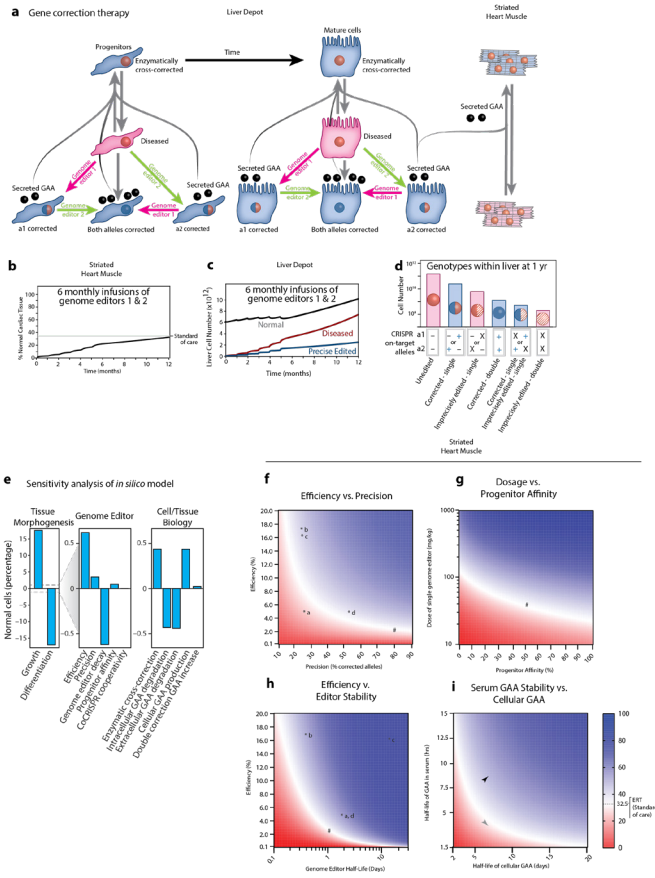

Figure 2: a. Model schematic combining both models from Figure 1 for biallelic editing tracking precise and imprecise edits at each allele b. Percentage of phenotypically normal cells after 6 administrations of the gene therapy and 6 months of growth c. Numbers of diseased, enzymatically corrected and precise hepatocytes d. Genomes present in the liver after 1 year of therapy e. Sensitivity analysis of the model f. Heatmaps showing genome editor selection and delivery trade-offs. (a: Plasmid spCas9, b. Nanoparticle spCas9 c. AAV saCas9 and d. Base Editor) Through this modeling, we uncover that using precise genome editors, engineering genome editors to have more stability in vivo, using adjuvants to stabilize GAA. For cell therapies we uncover that the lifespan and genotype of the cells being dosed is critical. Normal hepatocyte lifespan is 300 days, if proliferative progenitors have <100 day lifespans they fail to provide a sustained rescue of the Pompe disease phenotype. We hope that this model will help generate a framework to evaluate multiple gene therapies in silico.

639. Multiplexed Bioluminescent Reporter Enables Non-Invasive Tracking of DNA Double Strand Break Repair Dynamics *In Vitro* and *In Vivo*

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Repairing DNA damage plays a key role in maintaining genome integrity and cell viability. Two major pathways are responsible for DNA double strand break (DSB) repair: error prone non-homologous end joining (NHEJ), and template-dependent homology directed repair (HDR). Importantly, cellular preference of the repair pathways can significantly affect the choice of sensitizer for cancer treatment, as well as the efficiency of introducing therapeutic gene. Studying DSB repair outcomes, however, is time-consuming and typically requires disruption of cells for subsequent DNA sequence analyses. Here we describe a multiplexed reporter for non-invasive monitoring of DSB repair pathways in living cells and animals, termed bioluminescent repair reporter (BLRR). The reporter employs secreted Gaussia and Vargula luciferases to simultaneously detect HDR and NHEJ, respectively, from a few microliters of medium or blood. BLRR assay demonstrated a strong correlation with next-generation sequencing (NGS) results in reporting HDR ($R^2 = 0.9722$) and NHEJ events ($R^2 = 0.919$). In addition, BLRR reports the DSB repairs with a high specificity and sensitivity by identifying a significant difference in HDR and NHEJ activities in CRISPR-Cas9 edited cells which were guided by six different gRNAs within 40-nucleotides of target site. This finding positively correlates with in silico Benchling and CHOPCHOP on-target scores, as well as in vitro cleavage assays(1,2). Moreover, BLRR allows longitudinal tracking of NHEJ and HDR activities in cells, and further enabled detection of the DSB repairs in subcutaneous tumor xenograft in vivo. Using BLRR, we accurately monitored changes in DSB repair dynamic induced by small molecule modulators, and identified a novel function of tumor-suppressing cardiac glycosides which inhibits RAD51 to suppress HDR in human glioblastoma cancer stem-like cells. Altogether, BLRR provides a highly sensitive and versatile platform to monitor DSB repair dynamics in cells and animals. References: 1.J. G. Doench *et al.*, Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nat Biotechnol 34, 184-191 (2016). 2.K. Labun *et al.*, CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic Acids Res 44, W272-276 (2016).

640. Proteomic and Genomic Analysis of CRISPR/Cas9 Engineered Cells and Their Stability

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Objective: CRISPR/Cas9, one of the most commonly used genome editing systems, has huge potential, and is being used for improving protein, cell and gene therapies. While an exciting tool, there is not a lot of long-term data about its genomic and phenotypic stability and off-target effects that may arise during the editing process. Additionally, there are many options for editing processes that may result in edited cells behaving differently. Since whole cells can be used in the patient with cell and gene therapies, cell characterization is essential for safety. In support of the production of safe and effective CRISPR/Cas9 engineered protein, cell and gene therapies, the objective of this work is to study both on-target and off-target effects of CRISPR/Cas9 using flow cytometry on a B-lymphoblast cell line, GM24385, whose genome sequence has been well characterized. **Methods:** The cultured B cells were characterized with a B cell panel using flow cytometry. The CD19 marker was knocked out using CRISPR/Cas9 ribonucleoproteins with a nucleofection method using four different gRNAs. The resulted pools were then evaluated with sanger sequencing and a flow cytometry B cell panel and sorted using either a FACSAria II flow sorter or magnetic beads via negative selection. **Results:** The $CD19^-$ B cells were created from nucleofection of cultured GM24385 cells with CRISPR/Cas9 ribonucleoproteins. The characteristics obtained from the B cell subtyping panel showed different efficiencies of CD19 knockout and different B cell subtypes. The growth rate of $CD19^-$ B cells was dependent of sorting methods and unpredictable over time post transfection. Additionally, different gRNAs against CD19 resulted in different editing efficiencies when evaluated at genomic and proteomic levels. The expression levels of surface and cytosolic CD19 on edited cells were evaluated for obtaining proteomic editing efficiency. **Conclusions:** Using CRISPR/Cas9 with different gRNAs to knock out CD19 in B lymphoblasts, GM24385, exhibits different editing efficiency and influences the B cell subtype profile. The work with well sequenced GM24385 enables the identification of off-target effects of the editing method through whole genome sequencing, the next step of the study.

641. CRISPR-SCReT : Stop Codon Read Through Method

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CRISPR/Cas9 has paved the way for the development of therapies that permanently correct genetic mutations. However, constitutive expression of the Cas9 gene can increase off-targets and induce an immune response against the Cas9 protein. Our project aims to control the expression of nuclease by induction with a molecule. The approach consisted of introducing a premature termination codon (PTC) at the beginning of the Cas9 gene and subsequently using readthrough drugs. First, we co-transfected into HeLa cells plasmid PX458 with a TGA codon at the beginning of the SpCas9 gene (SpCas9*) with two sgRNAs targeting the dystrophin gene (exon50 and exon54). We treated 3 wells with different doses of geneticin (G418). 72 hours after the transfection we evaluated the rate of genome editing by PCR. Wells not treated with the molecule were used as a negative control and wells treated with SpCas9 without a PTC as a positive control. The hybrid exon 50-54 due to the deletion of part of the dystrophin gene was detected in the

wells treated with G418 despite of the PTC presence (SpCas9*). We carried out a similar experiment with a plasmid coding for CjCas9 with a TGA stop codon at the beginning of CjCas9 (CjCas9*) and two sgRNAs able to delete the GAA expansion present in the frataxin gene of people with Friedreich's ataxia. This plasmid was transfected into HeLa cell. 72 hours after transfection, we noted an absence of editing in the wells not treated with G418 and an editing in the wells treated with G418 comparable to the edition generated at CjCas9 without the stop codon. Our results show that is possible to control SpCas9 and CjCas9 expression by Stop Codon Read Through method.

642. NoveSlice: A Novel Chromatin Context-Sensitive Gene-Editing Endonuclease

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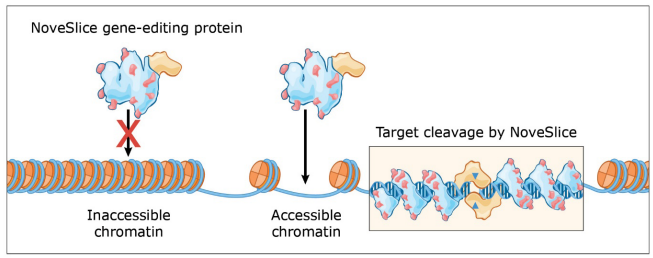
Genome-editing endonucleases are currently undergoing early clinical evaluation for the treatment of a wide range of diseases. However, *in vivo* use of gene-editing endonucleases is limited by the risk of potentially harmful off-target effects. It has been previously shown that gene-editing endonucleases are blocked by heterochromatin and show reduced efficiency in nucleosome-associated targets. We hypothesized that a gene-editing endonuclease with increased sensitivity to chromatin context could offer reduced off-target cutting without sacrificing on-target efficiency. Here we present NoveSlice, a novel heterodimeric gene-editing endonuclease comprising an engineered DNA-binding domain assembled from non-tandem repeat sequences connected by flexible linkers. We show that while both NoveSlice and TALENs are able to cleave a variety of targets with comparable efficiency in a cell-free amplicon-cutting assay, the efficiency with which NoveSlice cleaves genomic DNA depends more strongly on the chromatin context of the target than an equivalent TALEN pair. We constructed NoveSlice pairs targeting sequences within various human genes and the AAVS1 safe-harbor locus using both Golden Gate assembly and direct gene synthesis. We tested the effect of pair spacing and binding-site length on cutting efficiency both in a cell-free amplicon-cutting assay and, using a novel minimally immunogenic mRNA, in primary human epidermal keratinocytes, fibroblasts, induced pluripotent stem cells (iPSCs), and a cutaneous squamous cell carcinoma cell line. Both NoveSlice and TALENs efficiently cut a variety of targets in the AAVS1 safe-harbor locus in both the cell-free amplicon-cutting assay and in primary human cells. Also, co-transfection with a donor plasmid containing AAVS1 homology arms resulted in insertion of the donor into the AAVS1 locus. Similarly, both NoveSlice and TALENs efficiently cut a variety of targets in amplicons generated from genomic regions with reduced chromatin accessibility. However, while TALENs also efficiently cut these targets in primary human cells, NoveSlice showed no cutting of these targets in cells, suggesting that NoveSlice activity is more strongly dependent on the chromatin context of the target than an equivalent TALEN pair. The risk of potentially harmful off-target effects has restricted the clinical translation of gene-editing technologies. Here we present a novel gene-editing endonuclease that exhibits sensitivity to the chromatin context of the target. NoveSlice could thus serve as an important tool for the development of new precision medicines, including *in vivo* gene-editing therapies.



643. CRISPAltRations an Accurate, Validated Analysis Pipeline for Targeted Resequencing of CRISPR Genome Editing Events

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CRISPR systems enable targeted genome editing in a wide variety of organisms by introducing single- or double-strand DNA breaks, which are repaired using native molecular pathways. For precision editing needs, such as during preclinical therapeutic development work, we recommend first performing empiric characterization of the on- and off-target activity of all guide RNAs (gRNAs) under study. Once sites at risk for off-target cleavage have been identified, subsequent studies using the same gRNAs can be more easily performed using NGS resequencing methods. Publicly available bioinformatics software to analyze targeted resequencing data lack thorough optimization of alignment/variant calling, validation of robust performance, usage recommendations, and easy-to-use interfaces. To further enable the genome editing community, we developed our own tool (CRISPAltRations). Our tool uses an optimized alignment module, which improves our ability to correctly resolve Cas9 and Cas12a (Cpf1) editing events. We validated our tool using two simulated rhAmpSeq™ panel datasets representing 11 Cas9 and 11 Cas12a gRNAs at 603 on- and off-target locations, each. For each target, we modeled expected noise profiles as observed on an Illumina MiSeq and indel repair profiles, which are unique to each enzyme. Using synthetic and real data, we demonstrate that CRISPAltRations outperforms other publicly available tools. In addition, we improve our pipeline's usefulness by adding: quantification of frame-shifting indels, a comparison of observed versus *in-silico* predicted indel profiles (*e.g.* FORECasT), detailed characterization/visualization of perfect vs imperfect homology directed repair (HDR) events, support for multi-guide-introduced deletions, and more.

644. Development of a Molecular Switch to Regulate Cas9 Expression

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Studies on therapeutic gene editing using nucleases such as mega-nucleases, zinc fingers, and CRISPR are being pursued widely to treat a large array of genetic diseases. Though the approach holds a huge potential for permanent genome correction, the constitutive expression

of foreign protein, and the risk of generating off-target effects when using AAV raises important safety concern. The ability to regulate the intracellular expression of Cas9 independently is essential to ensure the safety of the AAV mediated therapeutic strategy. In order to develop a molecular switch to regulate such approaches, we adapted the splice switching mechanism of IVS2-654 intron for AAV mediated nuclease delivery. The system uses IVS2-654 mutant intron and its corresponding antisense oligonucleotide (ASO) to turn "ON" or "OFF" the expression of a Cas9 cassette both in vitro and in vivo. IVS2 is the second intron of β-globin transcript, which contains consensus 5' and 3' splice site. During the normal splicing process, the intron is constitutively removed to produce functional Cas9. IVS2-654 mutant intron causes aberrant 5' splice site with cryptic 3' splice site and alternatively used exon (AUE) is incorporated in RNA. Use of cryptic splice sites results in the retention of AUE mRNA and shifts the downstream open reading frame generating truncated/non-functional Cas9. The aberrant splicing can be corrected by administration of ASO targeting oligo. We used AAV plasmid with and without *Staphylococcus aureus* Cas9 transgene and IVS2-654 mutant intron within the Cas9 sequence. Transfection of HEK293 suspension culture with the respective plasmids resulted in a significant reduction in the splice variant Cas9 expression. ASOs corresponding to the cryptic 5' splice site restored Cas9 expression. Viral vectors carrying reporter genes have validated this system in vitro and in vivo. Experiment testing AAV regulates ASO switching will be presented and the potential to advance AAV editing using molecular switches that respond to small molecules will be presented. The success of such system should allow the spatial and temporal control of the gene editing in target organs in vivo when the system is administered along with the guide RNAs targeting the desired genes.

645. Specific Knock-Down of P347S Dominant Mutation in Rhodopsin Gene by CRISPR/Cas9 System

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Rhodopsin (*RHO*) mutations represent a common cause of blindness, accounting for 25-30% of autosomal dominant Retinitis Pigmentosa (adRP) and 8-10% of all RPs. Developing CRISPR/Cas9 system as genome editing tool to specifically knock-down the defective *RHO* allele represents a valid gene therapy approach. Cas9-mediated DNA double strand breaks predominantly trigger non-homologous end joining repair that introduces insertions/deletions in the target region potentially leading to permanent knock-down. In this study, *S.pyogenes* Cas9 (SpCas9) and its high-fidelity variant VQR (VQR-HF1-SpCas9) were employed to inactivate the RHO P347S, a highly frequent C-terminal dominant mutation causative of adRP. Specific gRNAs were properly coupled to SpCas9 nucleases generating two effector vectors used *in vitro*and in transgenic P347S mice. For *in vitro* analyses, HeLa clones stably expressing P347S or wild-type (wt) *RHO* cDNA were established and transfected with effector plasmids. gRNA efficiency and specificity were analysed by next generation sequencing of PCR amplicons surrounding target sites. Genomic analysis clearly showed an efficient editing on P347S target site. To address the safety profile, the top ranked off-targets predicted by COSMID were investigated by NGS. Quantitative RT-PCR and western blot analysis on edited P347S HeLa clones demonstrated a strong reduction of *RHO* P347S mRNA and protein levels compared to cells transfected with SpCas9 nuclease alone or untreated. To address the possible outcomes resulting from unpredictable editing of RHO P347S cDNA, a comprehensive study on the most frequent RHO mutants generated by editing was performed. To translate this strategy to a preclinical *in vivo* model, P347S transgenic mice were subretinally co-injected with AAV2/8 vectors coding for SpCas9, or VQR-HF1 SpCas9, together with AAV2/8 vectors carrying the appropriate or scramble gRNA. Indels in the human P347S gene were detected by NGS analyses in the retinae treated with effector viruses compared to scramble gRNA. The *in vivo* data indicated a variable but still specific and significant targeting, up to 30%, of P347S mutation approximately 30 days post-injection. Our strategy specifically designed to target the human P347S RHO gene resulted in a significant reduction, up to 60%, of the P347S mRNA in the treated retinae expressing effector vectors compared to contralateral eyes injected with Cas9-gRNA scramble vectors. Electroretinogram performed on mutant P347S treated mice revealed a significant improvement of retinal function in mice eyes injected with effector vectors compared to controls. To conclude, this study shows a CRISPR/Cas9-mediated approach to target one of the most frequently mutated amino acids in the C-terminus domain of RHO protein leading to a severe form of adRP.

646. Optimization of *In Vitro* Gene Delivery Targeting Neurons Afflicted with Alzheimer's Disease for the Development of a Base Editing Strategy

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Introduction The Amyloid Precursor Protein (APP) is preferentially cut by the alpha-secretase enzyme in healthy individuals. However, an abnormal cleavage by beta-secretase leads to the accumulation of beta-amyloid peptides. Numerous APP genetic mutations cause Familial Alzheimer Disease (FAD). However, a rare variant of the APP gene (A673T) found in Icelanders drastically reduces cleavage via beta-secretase. We hypothesized that the insertion of this APPA673T mutation in a patient's genome using the CRISPR/Cas9 Base-Editing technology could be an effective and sustainable method to slow down the progression of familial and sporadic forms of Alzheimer's disease. The objective of this study was to compare the efficiency by which a lentiviral or a dual-AAV-based vector could deliver a previously optimized base-editing complex into neurons *in vitro*. The presence of Cas9 and the beta-amyloid peptides was quantified and used to compare the relative efficiencies of each viral vector. **Methods** Neurons were obtained using two different methods. The first method involved differentiating London V717I patient/mouse model to yield the neurons (Figure 1), while the second method involved isolating hippocampal neurons from prenatal NL/F/G mice. The dual AAV complex was designed using a base editor whose genetic material was separated by inteins. Hippocampal or induced neurons were transduced with lentivirus or AAV1. Cas9 detection was performed

by immunofluorescence and Western Blot. The beta-amyloid peptides in supernatant were characterized and quantified by Meso Scale Discovery's Abeta kit. **Results** We were able to detect the Cas9 protein in neurons by western blot and immunofluorescence (Figure 2). We succeeded in demonstrating a noticeable decrease in the presence of the beta-amyloid peptide with both viral vector types. **Conclusion** Our approach aims to attest to the protective effect the A673T mutation has against the development of familial and sporadic forms of Alzheimer's disease as well as develop an efficient delivery method for the base editing complex which will lead to an *in vivo* application. These results could be applicable for various other genetic diseases.

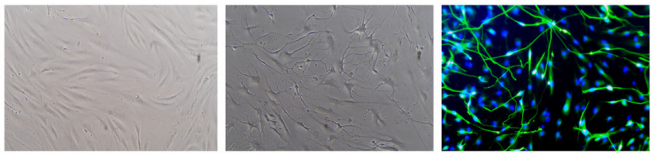

Figure 1: Evolution of the Induced neurons conversion from patient fibroblasts over time. PI: post induction.

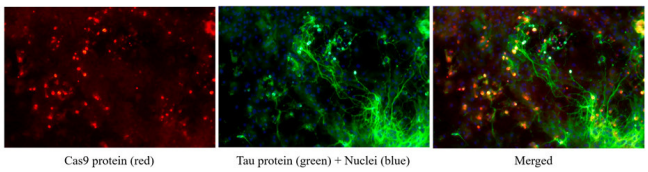

Figure 2: Cas9 delivery immunofluorescence results in hippocampal neurons from NL/F/G prenatal mice.

647. An Innovative Curriculum for the Instruction of Gene Editing for High School and College Students

Kristen M. Pisarcik[1], Amanda Hewes[1], Brett Sansbury[2], Olivia Tharp[3], Eric B. Kmiec[1]

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The field of CRISPR-Cas gene editing has greatly impacted biomedical research, but the incorporation of knowledge into undergraduate education appears to be lacking. Through funding from a National Science Foundation Advanced Technological Education (NSF-ATE) grant, and partnership with the Gene Editing Institute, an innovative laboratory exercise and curriculum on gene editing has been developed. An education kit based on *in vitro* gene editing will allow for the students to gain familiarity in CRISPR-Cas technology, as well as, the ability to see the global outcome of genetic events associated with the usage of CRISPR. The use of an *in vitro* gene editing system allows for students to recapitulate the methodology that research scientists use regularly when determining selection of CRISPR nuclease, cut site, and construction of the repair oligonucleotide. An *in vitro* system is novel in its ability to present the broad spectrum of gene editing reaction outcomes without the complexity of *in vivo* cell work. Through this experiment and curriculum, students will be able to obtain an understanding of CRISPR-Cas and the ethical concerns surrounding gene editing.

648. An Innovative, Highly Efficiency Method for Transfecting Large Quantities of Primary Human T-Cells

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With 18.1 million new cases and 9.6 million related deaths observed worldwide in 2018, cancer continues to pose a significant threat to health and well-being. Therefore, a strong need exists for improved cancer treatment options. One promising approach relies on genetically modified, patient-derived cells like T-cells or natural killer cells which express chimeric antigen receptors (CARs) targeting cancer cells. Primary human T-cells are difficult to genetically modify with chemical transfection reagents, just as with virtually all non-dividing primary cells. Current viral transduction methods require expensive, cumbersome viral vector production. Classical electroporation methods are often limited in throughput as well as volume and can result in cells with impaired viability and functionality. The study objective was to optimize transfection and culture protocols for primary human T-cells using the 4D-Nucleofector™ System to maximize transfection efficiency while maintaining cell viability and proliferation. Cryopreserved human peripheral blood mononuclear cells (PBMC) were transfected in the 4D-Nucleofector™ X-Unit with pmaxGFP™ Vector in 100 µl volume. Donor-dependent transfection efficiencies of up to 70% with high cell viability were achieved 24 hours after the Nucleofection™ Procedure. The optimized transfection conditions were applied to larger cell numbers using the 4D-Nucleofector™ LV Unit - a closed, scalable transfection system which supports sterile cell processing. In this study, we used up to 250 million PBMCs in 5 ml volume. Post transfection, T-cells were stimulated via anti-CD3/CD28 and cultured for up to 10 days during which the CD3+ T-cell population expanded. Comparable transfection efficiencies to the small-scale experiments were observed for both cryopreserved and fresh PBMCs. The number of GFP-positive T-cells peaked around day 6 with a decline thereafter, most likely caused by the transient loss of pmaxGFP™ Vector in the cells. Stable integration approaches (i.e. CRISPR) would presumably support stable gene of interest expression. Finally, we evaluated whether the 4D-Nucleofector™ System is suitable for CAR plasmid transfection. Indeed, up to 40% of T-Cells expressed this large, complex cell surface molecule 24 hours post transfection.In summary, we present an efficient and reliable transfection system which allows the processing of up to 1 billion PBMCs in a 20mL maximum volume. This method could help to advance various cell and gene therapies through development more quickly while shedding the need for expensive GMP vector lots.

649. Optimal Epigenetic Landscape Favors Enhanced TALEN® Activity

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Transcription activator-like effector nucleases (TALEN®) are a prominent tool for a large array of gene therapy applications. As TALEN® moves into clinical applications, high precision, specificity and efficacy are imperative features of this state-of-the-art gene editing technology. Our previous work demonstrated that TALEN® enables gene inactivation and integration with high precision and specificity. While methylated DNA poses a major interference to TALEN® efficiency, we showed that replacing conventional repeat variable di-residues (RVDs) with 5mC-insensitive N* (RVDs) provides efficient editing on methylated loci. Here, we focus on the role of the epigenetic landscape in TALEN® design and show that precise nuclease design based on whole genome methylation data delivers highly efficient TALEN® using classical RVDs. While facilitating TALEN® design around methylated areas, this strategy also informs of the need of using non-conventional N* RVDs to target specific loci where methylation residues cannot be avoided. Moreover, we demonstrate that high efficiency does not lead to undesired off-site cleavage. As DNA methylation is a key determinant of cellular identity, this approach has also the advantage of achieving efficient gene editing in a cell-specific manner. Thus, integrating cell-specific DNA-methylome data into TALEN® design can benefit the design of highly accurate and efficient gene editors for therapeutic use while preserving specificity.

650. Non-Viral Gene Delivery - Supporting the Next Generation of Gene-Edited Cell Therapies

Tristan R. Thwaites, Ilaria Santeramo, Roseanna Petrovic, Jay Kearney, Mustafa Munye, Marta Bagnati, Georgina Hermitage, Alexia Toufexi, Anusha Seneviratne, Camilla Farnetani, Hamza Bhatti, Damian Marshall

Industrialisation, Cell & Gene Therapy Catapult, London, United Kingdom

As the cell and gene therapy field evolves, therapeutic developers are aiming for higher levels of complexity in their cell engineered products. A diverse range of cargos (mRNA, proteins, DNA) and cells (T cells, NK cells, HSCs and IPS cells) require increasing levels of sophistication in the choice of gene editing and gene transfer delivery technology. Viral vector delivery is currently the most widely used technology for GMP based gene transfer. However, there are limitations in the carrying capacity of viral vectors and challenges in cost and supply chain. CAR-T manufacture, for example, relies on viral-mediated *ex-vivo* gene transfer into the T-cells, which can be 30-50% of the overall cost-of-goods. Non-viral delivery technologies are attractive alternatives for developers, but they are currently inferior to viral vectors for gene transfer. However, these technologies may be better suited to delivery of gene editing cargoes, which must be delivered transiently to ensure reduced the risk of off-target edits. In this presentation we will show how different non-viral gene delivery technologies can be used for highly efficient delivery of therapeutic targets using a primary Regulatory T-cells (Treg) immunotherapy model. The data, which is part of a H2020-funded project (ReSHAPE), shows how a range of GMP-ready non-viral platforms impact on the immunosuppressive functionality of Tregs and their proliferation capacity. We will also demonstrate how these technologies can be used to support functional delivery of therapeutically-relevant cargos such as CRISPR/cas9 and an exemplar Chimeric Antigen Receptor (CAR). For the latter, we have also successfully developed of a robust, automated and closed manufacturing process to produce an mRNA-based CAR-T product. This process has a reproducible transfection efficiency of 77-98% with a cell viability greater than 80%. In summary, gene editing is expanding the application of genome surgery, providing exciting opportunities to further functionalize cells and offering new avenues to address limitations with gene augmentation therapies, such as the treatment of gain-of-function mutations. However, delivery remains a major obstacle. Our ability to converge non-viral delivery methods with gene editing systems is therefore key to ensure future clinical adoption.

651. Engineered B Cells as a Novel and Sustainable Cell-Based Enzyme Replacement Therapy for Hurler/Scheie Syndrome

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Hurler/Scheie syndrome or Mucopolysaccharidosis type I (MPS I) is a rare inherited lysosomal storage disorder caused by mutations in the *IDUA* gene, leading to α-L-iduronidase (IDUA) deficiency. Lacking IDUA resulted in accumulation of its substrate in the lysosome, Glycosaminoglycans (GAGs) - a metabolic waste of cells. Clinical severity ranges from Scheie (mild) to Hurler syndrome (Severe) and most of the patients succumb to the disease at young ages. Currently, enzyme replacement therapy (ERT) and bone marrow transplantation (BMT) are the standard of care for this disease. However, due to the fluctuations of the IDUA enzyme from ERT and the low levels of the enzyme produced by BMT-derived cells alternative therapeutic approaches are desperately needed. To overcome these challenges, a method to produce high and sustainable IDUA levels is required. B cells are a small subgroup of adaptive immune cells responsible for producing large quantity antibodies (or Immunoglobulins) against foreign antigens or pathogens. Upon activations (or immunizations), B cells have the ability to differentiate into a long-lived plasma cells, producing a large quantity of protein (aka antibody) for many years, and even decades. This makes B cells a perfect candidate for use as cell-based enzyme replacement therapy to treat MPS I, and potentially many other enzymopathies. Thus, we aim to establish a universal engineering platform of B cells to treat enzymopathies. We previously demonstrated that B cells can be efficiently edited using CRISPR/Cas9 system for gene knockout and targeted integration. In our current work, a therapeutic *IDUA* transgene cassette was designed to co-express *IDUA* and *RQR8* under the regulation of MND promoter within a targeting vector harboring homology arms to a locus of interest, either AAVS1 or near the eMu enhanced of the B cell receptor heavy chain locus. The IDUA functions as a therapeutic enzyme and the RQR8 serves as a marker of the

engineered cells. Here, we utilized a combination of CRISPR/Cas9 and rAAV vector to mediate up to 20% RQR8+ B cells, indicating successful targeted integration of the transgene (Figure 1A). Furthermore, insertion of IDUA expression cassette into the B cell genome resulted in a high level of expression and secretion of IDUA enzyme (up to 200 nmol/hr/mL) in the culture medium of engineered B cells (Figure 1B). Transplantation of the engineered B cells (via intraperitoneal injection) into NSG resulted in high level of plasma IDUA enzyme when compared to of untreated littermate (150 nmol/hr/mL and 2 nmol/hr/mL, respectively; Figure 2). This preliminary data indicates that engineered B cells can be transplanted into an NSG-MPS mice.

Figure 1.

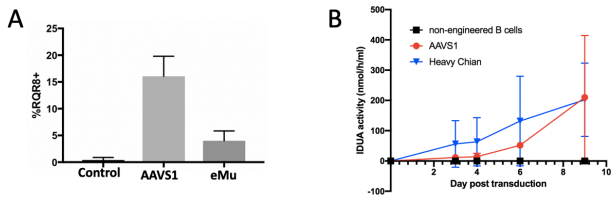
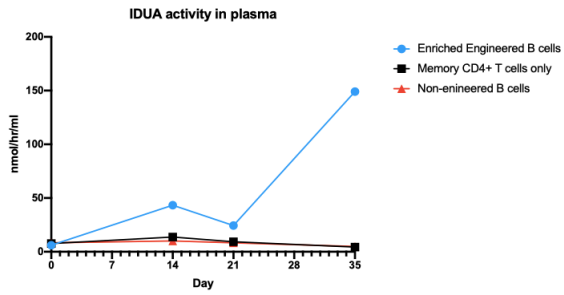


Figure 2.



652. Efficient Nuclease-Based Correction of HPS1 Associated Microduplication

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The development of improved and efficient programmable nucleases such as CRISPR-Cas9 has greatly enhanced our ability to precisely correct pathogenic mutations. Current correction strategies that rely on the Homology Directed Repair (HDR) pathway have several limitations including the requirement for co-delivery of an exogenous DNA donor and the inefficiency of HDR in many cell types. Recently, we demonstrated that disease-causing frameshift mutations resulting from microduplications can be efficiently reverted to the wild-type sequence simply by generating a double-strand break (DSB) near the center of the duplication. We are currently developing a nuclease-based therapeutic for Hermansky-Pudlak Syndrome Type 1 (HPS1), as the most common pathogenic allele is the result of a 16 bp microduplication. We have demonstrated efficient repair (>75% of edited alleles) of the pathogenic microduplication in a homozygous HPS1 patient-derived B-lymphoblastoid cell line. HPS1 microduplication collapse can also be achieved in other types of patient-derived cell lines. We have evidence that nuclease-mediated collapse of the microduplication is mediated by the MMEJ (microhomology-mediated end joining) repair pathway based on inhibition of PARP-1 (poly (ADP-ribose)) polymerase. We are continuing to examine the impact of other factors involved in the MMEJ and NHEJ (non-homologous end joining) repair pathways to better understand mechanisms favoring microduplication collapse. In preparation for *in-vivo* experiments in HPS1 animal models, we are investigating parameters that influence nuclease activity and examining alternate cleavage positions within the microduplication to optimize the efficiency of duplication collapse. Our on-going analysis of potential off-target sites for different HPS1 microduplication targeting guide RNAs will allow fine-tuning of the specificity of nuclease components and facilitate clinical applications of this strategy. Insights learned from the development of an MMEJ-based therapeutic strategy for HPS1 should permit the development of nuclease-based gene correction therapies for a variety of other diseases that are associated with microduplications.

653. Transcriptional and Position Effect Contributions to rAAV-Mediated Gene Targeting

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Recombinant adeno-associated viral (AAV) vectors constitute one of the most promising tools for gene transfer. While the majority of AAV transduction events are episomal, our laboratory exploits the vector's ability to induce homologous recombination for targeted integration of therapeutic sequences into the host genome downstream of an endogenous promoter (AAV-HR). Not only do nuclease-free, promoterless AAV-HR systems offer the permanence of gene transfer associated with integration, but a vector lacking a promoter reduces the chance for oncogene activation from off-target vector integration. AAV-HR corrects only about 0.1-1% of cells, so we hypothesize that choosing an optimal target site could improve upon that rate. Targeting rate may be positively influenced by factors thought to predispose transcribed regions to homology directed repair such as exposure of single stranded DNA in R loops, collision of transcription and replication forks leading to genomic instability, and the transcription elongation histone mark H3K36me3. Negative influences may include nucleosome compaction and propagation of heterochromatin by position-effect variegation. Yet, it is unclear if transcription itself or other factors that cooperate with transcription, such as chromatin state, are directly linked to AAV-HR. To investigate this link, we developed a high-throughput strategy to map and quantify precision AAV-HR genome wide by exploiting an engineered locus whose transcriptional rates are controlled by drug administration. We used lentiviral vectors to generate a polyclonal population of HAP1 cells each harboring a single-copy, doxycycline-

inducible genomic site expressing eGFP, which are mapped by ligation-mediated PCR and NGS. The population is subsequently infected with a library of AAV serotype DJ vectors designed to integrate mScarlet and a unique genetic barcode, followed by enrichment of targeted cells based on gain of mScarlet and loss of eGFP expression. The barcode is detectable in both genomic DNA and RNA transcripts originating from the inducible promoter; barcode heterogeneity is a measure of the targeting rate at a given site. We targeted a cell population comprising more than 1000 target sites, with and without transcriptional induction. Inverse PCR was used to map integrated barcodes to target genomic sites and transcriptional level was estimated by amplifying barcodes from transcripts. The system uniquely allows us to investigate AAV-HR efficiency at more than 1000 sites with and without transcription through the target site. In a preliminary analysis, we find a significant increase in barcode heterogeneity at a given site if the site is transcribed upon transduction, yet the heterogeneity—a measure of AAV-HR efficiency—is still highly correlated between paired sites (Pearson R=0.98), suggesting that transcription alone may play a minor role in AAV-HR. We find an increased likelihood of AAV-HR at transcribed target sites if they intersect a protein-coding gene and are investigating the effect of repeat elements. We will discuss our finding that promoter-associated chromatin states are predictive of categorical targeting only when the target site is transcribed, while heterochromatin is negatively predictive of categorical targeting regardless of transcriptional state. However, upon narrowing the regression analysis to sites that are targeted, we find a different set of features important for predicting barcode heterogeneity. Finally, we will discuss correlations between barcode heterogeneity and DNase-seq, ATAC-Seq, and ChIP-Seq peak intensities at targeted genomic sites.

654. Engineering a Self-Inactivating Adeno-Associated Virus (AAV) Vector for ARCUS Nuclease Delivery

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Significant advances in engineering sequence specific nucleases have enabled a broad range of biomedical applications, particularly when combined with AAV, a versatile viral vector for *in vivo* post-mitotic cell gene delivery. The long-term expression of nuclease mediated by AAV delivery, however, raises concerns about specificity and immunogenicity. Persistent expression of the nuclease can increase the likelihood of off-target cleavage which can induce genotoxicity. Expression of an exogenous nuclease has the potential to elicit an immune response against transduced cells. Thus, it would be advantageous to limit the duration of nuclease expression following delivery. Here, we reported a self-inactivating AAV system using our proprietary ARCUS nuclease technology, which consists of a tissue-specific promoter, a nuclease open reading frame (ORF), and adjacent sites targeted by the nuclease. We demonstrate *in vitro* that this system can progressively reduce nuclease expression over time, and the decrease in nuclease expression can be impacted by the locations and copy numbers of the target site insertions. We further showed *in vivo* that this system can eliminate ~80% of nuclease expression within 6 weeks in mouse liver, while enabling ~70% of on-target cleavage efficiency. In addition, off-target cutting and AAV indels were measured to fully evaluate the efficiency of the system. We believe that our self-inactivating system has the potential to improve the therapeutic applications using engineered nucleases.

655. Modulation of the DNA Damage Response to Enhance Targeted Integration at CRISPR/Cas9-Mediated DNA Double Strand Breaks

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Clustered regularly interspaced short palindromic repeats (CRISPR) - CRISPR-associated protein 9 (Cas9) has revolutionized the field of genome engineering. However, low efficiency of targeted gene integration represents one of the major obstacles in therapeutic genome editing. Previous reports have established that small molecules can enhance gene insertions in various cell types. However, most studies using compounds to increase knock-in efficiency have been limited to a small number of tested substances and to HDR pathway for integration. We first tested 19,516 well annotated compounds to enhance the efficiency of genome editing in a high throughput screening. Successful hits were then validated using a novel assay, *RIMA[1] for knock-in*, to evaluate different knock-in strategies (like ObLiGaRe, HITI, PITCh, SSDR) in various genomic loci and cell types. The results contributed to our understanding of the interplay between DNA Damage Repair (DDR) and CRISPR-mediated knock-ins, but also reveal insights into the role of DDR in off-target mutagenesis induced by Cas9. These findings pave the way for gene therapy and cell line generation where high knock-in efficiencies are needed.

[1] Taheri-Ghahfarokhi, A. et al. Decoding non-random mutational signatures at Cas9 targeted sites. Nucleic Acids Res (2018).

656. Engineered Nuclease Off-Target Assay with Improved Sensitivity

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Engineered nucleases are genome-editing reagents that can function with high efficiency in all cell types. Characterization of their off-target activity is a critical aspect of evaluating performance and this is especially important for therapeutic applications where potential off-target genome modifications may drive oncogenesis. Given the high numbers of patient cells edited within each therapeutic application, extremely sensitive methods are required to detect off-target activity.

Utilizing Illumina high-throughput DNA sequencing to measure the insertions and deletions (indels) that occur when the cell's DNA repair machinery acts upon cleaved genomic DNA is the most commonly used method, but the intrinsic error rate of the standard Illumina platform imposes a limit of detection of approximately 0.1% alleles. Here we employ much higher-depth sequencing, multiple technical replicates, and new data processing methods to increase the assay sensitivity. Our method eliminated common sequencing artifacts present in both treated and control samples as well as rare sequencing artifacts occurring less frequently than expected for the number of sequence reads obtained for each input allele. An initial titration experiment starting with 5% off-target indels demonstrated good linearity over a 1,000-fold range of input indel levels. Next we treated a pool of human K562 cells with a pair of zinc finger nucleases (ZFN) containing a Q481A mutation in the FokI DNA cleavage domain known to improve nuclease specificity. This yielded 85.5% indels at the target locus with no detectable off-target activity using the standard indel assay. However, when a total of 1.44 million input haploid genomes from either ZFN treated cells or control cells with an average of 15 sequence reads per input haploid genome were used, we were able to measure an off-target indel signal of 0.0024% for the ZFN treated cells vs. a background signal of 0.00004% for control cells ($P = 0.00014$, one-tailed t-test). The occurrence of unique indel sequences in the technical replicates for the Q481A ZFN treated cells was consistent with Poisson statistics as would be expected if individual input alleles were being accurately measured. The key to this approach - oversampling of input genomes without the use of a unique molecular identifier - can be implemented using existing methodologies and sequencing platforms. We anticipate therefore that this approach will have general utility in the quantification of indels under conditions requiring especially high sensitivity, such as the de-risking of cancer-relevant loci in therapeutic contexts.

Synthetic/Molecular Conjugates and Physical Methods for Delivery

657. Optimization of Non-Viral Gene Delivery (e.g., Lipofectamine and Electroporation) to T Cells for CAR-T Cell Therapy

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CAR-T cell therapy has shown promising results in the treatment of hematological cancers that use retroviral transduction to deliver the CAR gene. However, viral vectors are expensive, pose risks of insertional mutagenesis, and vary in transduction efficiency. In contrast, non-viral delivery methods could potentially lower treatment costs and avoid some of the safety concerns associated with viruses. In this study, we compared several different cationic vehicles (e.g., PEI, Lipofectamine, etc.), promoters (EF1α, CMV, & CAG), and culture conditions to optimize the non-viral transfection of T cells (Figure 1). Overall, our results show that Lipofectamine LTX provides the highest transfection efficiency for Jurkat T cells (63.0±10.9% @ 48 hours post-transfection) when an EF1α promoter is used and the cells are cultured in X-VIVO™ 15 serum free media. However, application of this strategy to primary T cells yields a much lower transfection efficiency (5-10%), despite optimization of various parameters (e.g., timing of primary T cell transfection relative to activation with CD3/28 antibodies). Meanwhile, optimization of multiple parameters (e.g., shock buffer, cell number, etc) involved in electroporation of Jurkats yielded higher transfection efficiencies than Lipofectamine (95±0.81% @ 48 hours post-electroporation). These results motivated us to analyze the Jurkat and primary T cells for potential changes in host cell gene expression that might explain these disparities in transfection efficiency. Most notably, mRNA-sequencing experiments showed that Jurkat cells have low levels of HSPGs (heparan sulfate proteoglycans), which are involved in regulating endocytosis for gene delivery. In contrast, other adherent cell lines with higher transfection efficiency (e.g., HEK-293T and PC3) express HSPGs at significantly higher levels (Figure 2). Interestingly, our mRNA-sequencing experiments also show that Jurkat cells exhibit high constitutive levels of RNase and DNase activity compared to other cell lines that are relatively easy to transfect (e.g., PC3 & HEK-293T cells). Comparison of electroporated and Lipofected Jurkat cells also revealed lower levels of DNase and RNase in the electroporated cells. Taken together, this decrease in HSPG expression and increase in RNase/DNase expression could explain the significantly lower transfection efficiencies provided by Lipofectamine in T cells.

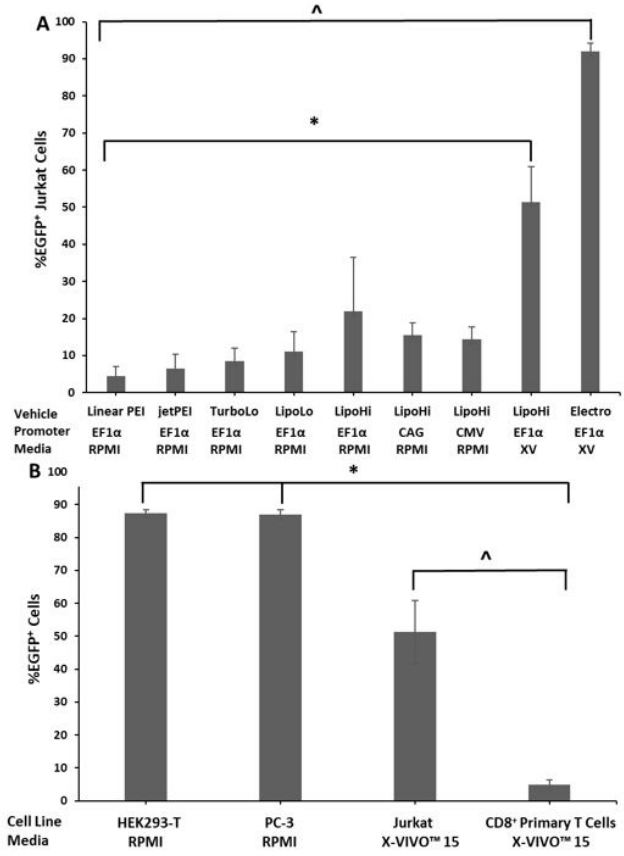

Figure 1. (A) Comparison of the effect of different cationic vehicles, promoters, and culture media on Jurkat transfection efficiency at 24 hours post-transfection. Linear PEI involved a 10:1 and jetPEI involved a 5:1 nitrogen:phosphate (N:P) ratio of positively charged polymer to negatively charged DNA. (^) denotes significant differences of electroporated sample compared to all other samples. (*) denotes

significant differences of X-VIVO™ 15 LipoHi samples compared to all other samples. RPMI denotes RPMI SCM media, and XV denotes X-VIVO™ 15. (B) Comparison of transfection efficiency for Lipofectamine-mediated delivery of pEF-GFP across multiple cell lines. (*) denotes significant differences of HEK293-T and PC-3 cells compared to Jurkats and primary T cells. (^) denotes significant difference of Jurkats compared to primary T cells. All significant differences defined as $p \leq 0.05$.

658. Exosome Delivery of a CFTR Activating Zinc Finger Protein

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Cystic fibrosis (CF) is a genetic disorder that results in a multi-organ disease with progressive respiratory decline that leads to premature death. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene disrupt the capacity of the protein to function as a channel for transporting chloride ions and water across cell membranes causing abnormally thick and sticky mucus. Small molecule treatments targeted at potentiating or correcting CFTR have shown clinical benefits by increasing the amount of mature CFTR protein or enhance channel function at the cell surface, but they are limited to a few specific CFTR mutations. To overcome this limitation, specific up-regulation of CFTR promoter in the airway epithelia can increase CFTR mRNA and protein levels in patients with CF. To activate CFTR expression, we designed a Zinc Finger Protein fusion with potent activation domains VP64, P65 and Rta (ZFP-VPR) to target the CFTR promoter. Here, we demonstrate robust activation and function of the CFTR transcription in Human Bronchial Epithelial cells (HuBEC) from patients with cystic fibrosis after treatment with ZFP-VPR. A major barrier to treat patients with CF is the lack of efficient vectors to deliver functional therapies without immunological complications. To overcome this issue, we used cellular derived exosomes packaged with the ZFP-VPR. Exosomes are emerging as an ideal delivery vehicle due to their being a natural material, excellent biocompatibility and their inherent versatility to deliver proteins and nucleic acids. We find here that exosomes packaged with ZFP-VPR protein and mRNA can functionally activate CFTR expression in HuBEC. Collectively, the novel approach outlined here, to generate exosomes carrying transcriptional regulator ZFP-VPR, offers a potentially valuable clinical tool for more effective treatment strategies of patient with Cystic Fibrosis. Funding: NIDDK R01 DK104681-01 to KVM.

659. Microfluidic-Enabled Delivery of mRNA in Human NK and Gamma Delta T Cells

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Natural killer cells (NK cell) and gamma delta T cells (γδ T cell) are both promising therapeutic cell carriers because they can be used in allogeneic chimeric antigen receptor (CAR) therapy. In contrast to autologous CAR T cell therapy, allogeneic approaches can employ cells from any donor to produce an "off the shelf product", that can in turn be administered to multiple patients, in a more readily accessible and cost-efficient manner. Currently the two main methods routinely used to generate oncology gene therapies are lentiviral vector transduction and non-viral transfection by means of electroporation. Lentiviral transduction has shown to be time-consuming and economically prohibitive at large scale. In addition, human NK-cell are resistant to transduction by commonplace lentiviral vectors, thus making it particularly problematic to provide adequate NK-cell immunotherapy. Non-viral transfection utilising electroporation typically shows good delivery efficiency, but destroys a high percentage of cells, and literature suggests it disrupts normal T cell function. We have developed a microfluidic device capable of inducing transient volume exchange in cells, resulting in cell transfection with payloads of interest. Here we share proof of concept results generated by this new microfluidic device, showing successful transfection of nucleic acids (i.e. mRNA) into NK-Cell and γδ T cells.

660. Ultrasound and Cationic Microbubble Assisted Gene Delivery in the Brain for Fragile X Syndrome Therapy

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Fragile X syndrome (FXS) is neurodevelopmental genetic disease caused by the mutation of FMR1 gene leading to intellectual disabilities. At present, there is no cure of this pathology and current pharmacotherapy consists exclusively of symptomatic drug treatment (stimulants, antidepressants, antipsychotics,..). Gene therapy could be a promising therapeutic strategy for this pathology to restore the expression of normal gene. However, it is crucial to set up an efficient, noninvasive and safe gene transfer method. The goal of this study is to evaluate the feasibility of delivering FMR1-gene in mouse brain of *fmr1KO* mice by focused ultrasound (US) combined with original cationic microbubbles (cMB). We developed a custom motorized ultrasound platform that consists of a focused transducer coupled to a positioning system for *in vivo* brain sonoporation. We determined an optimal condition consisting of a pulsed US at 1 MHz, 145 kPa, 5% duty cycle during 1 sec period that efficiently opened the blood-brain barrier (BBB) in presence of cationic MB intravenously. A significant echo signal from cMB that persisted over at least 2 minutes was measured in the US-treated brain area (fig 2). BBB permeabilization was confirmed by magnetic resonance imaging (MRI) with DOTAREM® contrast agent and T2* MRI images did not show any bleeding or damage in the brain after treatment. When cMB were complexed with plasmid DNA encoding luciferase gene or FMR1 gene, an efficient transgene expression restricted to the left hypothalamus area, which was sonoporated. Interestingly, FMR1 protein was expressed in neurons and astrocytes two important target cells in FXS as confirmed by immunohistochemistry of brain slices from treated mice. Overall, our data indicate that this FUS assisted cationic microbubbles gene delivery could be an alternative to viral vectors to restore normal *fmr1*

gene expression. Experiments are in progress in our lab to evaluate the impact of the FMRP expression restoration on its molecular targets and the behavioral of treated mice.

661. Safe and Highly Efficient Plasmid DNA and mRNA Delivery Platform Based on Polyelectrolyte Nanocapsules for Hard to Transfect Clinically Relevant Cell Types

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Introduction. The delivery of genetic material remains a major problem and an obstacle to the introduction of gene therapy into clinical practice. The most commonly used viral methods at the moment have relatively high efficiency, but they have serious limitations, such as immunogenicity, genotoxicity, and immune response, as well as extremely high cost due to the complex production process. Introduction of a novel, non-viral delivery methods can overcome these limitations. Polyelectrolyte nanocapsules have attracted attention as one of the promising platforms for gene delivery. Compared to solid nanoparticles, nanocapsules have higher loading capacity, optimal cargo/carrier polymer ratio, low toxicity, and allows to combine various types of cargo in different compartments of the carrier. Control of the size, thickness and number of layers, which will determine the biological distribution and rate of release of the cargo can also be attributed to the features of polyelectrolyte nanocapsules. Therefore, the aim of this work was to demonstrate the potential of a fundamentally new synthesis of polyelectrolyte nanocapsules as a platform for the delivery of genetic material to clinically relevant groups of cells, such as T-lymphocytes and hematopoietic stem cells. Methods and results. First, a new synthesis of vaterite (CaCO3) nanoparticles with a diameter of about 50 nm were developed. Nanocapsules were obtained by layering oppositely charged polyelectrolytes using layer-by-layer technologies on calcium carbonate nanoparticles with the subsequent dissolution of the core. eGFP plasmid DNA and mRNA were delivered to K562 and Jurkat cell lines, as well as to primary human T-lymphocytes. The uptake was assessed with confocal scanning microscopy, the transfection efficiency, as well as cell viability after the procedure, were assessed using flow cytometry. The developed protocol allows the reproducible and robust synthesis of nanocapsules with the desired size in the range of 50-100 nm. Importantly, mRNA- and DNA-loaded capsules of both types exerted minimal cytotoxicity (viability ~94%). mRNA loaded polyelectrolyte nanocapsules exhibit high transfection efficiencies (~80%). This efficiency was higher compared with previously published data. We also performed experiments with pDNA loaded nanocapsules which demonstrate ~50% transfection rate, comparably lower to mRNA loaded carriers for all cell types. Conclusions. We have created a GMP-compatible protocol for the preparation of nanocapsules for the efficient nucleic acid delivery. Low toxicity, high transfection rate open up new prospects for the development and implementation of this technology in gene therapy approaches. The study was supported by the RFBR as part of a scientific project № 19-015-00098. We would also like to thank RFBR for the grant support (No. 19-29-04025).

662. Further Development and Intra-Laboratory Validation of a Novel Pyro-Drive Jet Injector (PJI)

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Vaccinations have been developed to prevent and cure several kinds of diseases, such as infection or cancer. DNA-based vaccines (DNA vaccine) are a type of vaccine and were developed as an alternative to traditional protein-based vaccines. Even though DNA vaccination could be applied in different fields, DNA vaccine delivery technology still needs to be improved, especially as intradermal delivery is expected to induce an efficient immune response. To resolve this issue, we investigated the potential of pyro-drive jet injector (PJI). The PJI could control injection depth by adjusting the combined amounts of gunpowder and smokeless powder. In this study, we focused on the safety after intradermal injection and on the reproducibility for gene expression and immune response. First, we determined the suitable injection conditions to deliver DNA to the intradermal region in both rat and murine models, and assessed for resultant skin damage. The optimum gunpowder amount for rats was larger than that for mice; this was dependent on the difference in skin thickness between the different species. In optimized intradermal injection conditions, no remarkable difference in inflammation was observed compared with a traditional needle. Next, we investigated luciferase gene expression in rat and mouse, and ovalbumin (OVA) gene expression in rats at the injection site. In all cases, intradermal gene expression was observed and peak expression was obtained 24 h after injection. Expression decreased to less than one tenth of the maximum level after 72 h. These results indicate that the PJI could induce efficient gene expression at the intradermal area where antigen-presenting cells are expected to be localized. To examine the potential of the DNA vaccination device, we used an OVA expression plasmid as an antibody production model. When the OVA plasmid was injected into the intradermal region of the mouse and rat models using PJI, OVA-specific antibody was detected in both animal species. The experiments were repeated three times, gaining similar results and certifying the reproducibility of this method in optimized conditions. Furthermore, in the immunoglobulin subclass analysis, PJI induced both IgG1 and IgG2a antibodies. In contrast, the traditional needle syringe method did not result in robust antibody production in both animal models. These results suggest

that PJI is a reliable method of DNA delivery, resulting in subsequent protein expression and exhibiting potential as a new device for DNA vaccination.

663. Hepotocyte-Directed Gene Delivery Using Targeted Poly-L-Lysine Coated Selenium Nanoparticles

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Cancer is a term referring to a collection of diseases in which cells divide uncontrollably, and spread to surrounding tissue by the processes of invasion and metastasis. The liver is a central and vital metabolic organ in the human body and an attractive target for gene therapy. Selenium nanoparticles (SeNPs), are Se species with powerful anticancer activity and lower toxicity compared to other Se species, can be beneficial nanocarriers of drugs or therapeutic genes. This study aimed to design, functionalize and characterize SeNPs for efficient binding, protection and targeted delivery of pCMV-*Luc* DNA to hepatocytes (HepG2 cells). These nanoparticles were also assessed in the human embryonic kidney (HEK-293) and cervical carcinoma (HeLa) cells. SeNPs synthesized by ascorbic acid reduction was functionalised with poly-L-lysine (PLL), and a targeting ligand, lactobionic acid (LA) for hepatocyte-specific delivery. HepG2 cells possess abundant asialoglycoprotein receptors (ASGP-R) on their surface which bind terminal galactose sugars on the targeting moiety (LA). All nanoparticles were physicochemical and morphologically characterized by UV-vis spectroscopy, transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and Fourier- transform infrared spectroscopy (FTIR). The band shift, ethidium bromide intercalation and serum nuclease protection assay, was used to assess the nanocomplex formation. All nanoparticles displayed spherical morphologies, with an increase in size upon functionalization. Untargeted PLL-SeNP had the smallest hydrodynamic size (84.7 nm), while the targeted LA-PLL-SeNP had the largest hydrodynamic size of 118.7 nm. Nanoparticle:DNA nanocomplexes were also characterized by an increase in size. Both targeted and untargeted SeNPs were stable with zeta potentials of 28.6 mV and 32.7 mV, respectively. These nanoparticles were able to successfully bind, compact and protect the pDNA, with LA-PLL-SeNP showing a higher compaction potential than the PLL-SeNP. *In vitro* cytotoxicity assessed by the MTT assay, revealed negligible cytotoxicity in all cell lines. Transfection monitored by the luciferase reporter gene assay showed significant transgene expression for LA-PLL-SeNPs in the HepG2 cells compared to the HeLa and HEK293 cells, which was due to receptor-mediated endocytosis as confirmed by a receptor competition binding assay. Overall, this study has confirmed the safe and efficient targeted delivery of pDNA delivery to the hepatocytes *in vitro*. These results warrant further investigation in an *in vivo* system.

664. Engineered Multifunctional Exosomes as a Therapeutic Platform for Immune Oncology

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Introduction: Codiak BioSciences has leveraged natural exosome biology to develop a therapeutic platform based on precisely engineered exosomes. Our engEx™ platform utilizes the unique exosome scaffold PTGFRN that allows surface display of an array of structurally and biologically different proteins. Here we sought to expand the capabilities of our engEx platform for the precise engineering of multifunctional exosomes by constructing combinatorial extracellular vesicles (EVs) derived from our lead therapeutic candidate exoIL-12. **Methods:** IL-12 combinatorial screening with recombinant cytokines and TNF family members in a mouse tumor model identified CD40L and FLT3L as potential synergistic agents. Thus, we constructed single, double and triple exosomes of IL-12, CD40L and FLT3L fused to PTGFRN by engineering EV producer cells using a combination of random integration and safe harbor targeting. Purified EVs were tested for the co-expression of recombinant proteins by co-IP, western blot, ELISA and in vitro activity assays. We tested cellular stability, EV titers, protein expression levels and biological activity of the engineered multifunctional EVs and compared them to the mixture of single exosomes. **Results:** Double and triple exosomes composed of IL-12/CD40L/FLT3L demonstrated comparable EV titers, expression levels and activity to their single EV counterparts, demonstrating that PTFGRN is capable of efficiently co-expressing multiple proteins on EV surface without compromising individual protein expression. Different safe harbor sites resulted in different expression levels, thus allowing the precise control of protein EV levels. In vitro biological activities of multifunctional EVs were correlated with protein EV levels and were similar to the mixture of single ligand exosomes. Future in vivo studies will investigate the potential synergistic activity of these multifunctional exosomes when applied as combinatorial therapeutic agents in mouse cancer models. **Summary/Conclusion:** This POC study demonstrates the potential of our engEx™ platform to engineer multifunctional exosomes and has broad therapeutic applications for immuno-oncology, vaccines development and targeted functional delivery.

665. mRNA/LNP-Based Engineering of Erythroid Progenitors to Generate Allogeneic Red Cell Therapeutics™ (RCTs)

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Considerable effort has gone into developing non-viral methods for intracellular delivery of genes to express proteins in order to generate cellular therapies. We previously reported that long-lasting protein

expression can be achieved by introducing mRNA via electroporation into *ex vivo* generated primary human erythroid precursor cells to derive novel Red Cell Therapeutics™ (RCTs). Though electroporation is an efficient process, the ability to scale remains challenging. To achieve consistent and scalable non-viral gene delivery into erythroid precursor cells, we developed a method that takes advantage of active transferrin receptor (CD71) internalization to target lipid nanoparticles (LNPs) containing mRNA. mRNA encapsulated LNPs (mRNA/LNPs) were generated containing chemically modified polyethylene glycol (PEG) lipids that permit bio-conjugation of the mRNA/LNPs to a variety of targeting moieties that bind the CD71. mRNAs that encode various therapeutic proteins were packaged into CD71-targeting mRNA/LNPs and applied to *ex vivo* cultured primary human erythroid precursors in various states of differentiation, resulting in expression of these therapeutic proteins. Using CD71-targeted LNPs, we generated RCTs expressing functional 4-1BBL at high efficiencies (>90%) in a tunable fashion, demonstrating the ability to titer protein expression levels using various mRNA concentrations to meet the desired therapeutic activity. Utilizing this strategy, efficient expression was achieved of both single and multiple intracellular proteins as well as extracellular / membrane-bound proteins. In addition, we demonstrate that mRNA/LNP can be readily added to bioreactors to generate genetically modified RCTs at scale. An mRNA/LNP-based platform provides advantages over traditional viral/DNA-based methods: 1) there is no genomic integration; 2) no exogenous DNA; 3) the platform can be used to deliver single or multiple mRNA species simultaneously into cells; 4) is titratable; and 5) should, therefore, simplify and enhance the manufacturing of genetically modified RCTs at scale.

666. Surgical Technique for Bilateral Intrathalamic Infusion of rAAVrh8-HEXA/HEXB Gene Therapy in Infant With Tay-Sachs Disease

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Tay-Sachs disease (TSD) is a monogenic rapidly fatal, ultra-rare neurodegenerative disorder due to Hexosaminidase A (HexA) enzyme deficiency. Intrathalamic infusion of investigational rAAVrh8-*HEXA*/*HEXB* in animal models demonstrated efficacy and safety. We report the surgical technique used in the first human bilateral intrathalamic infusion of this gene therapy in a 6-month old with TSD. Neurosurgeons face challenges in convection enhanced delivery (CED) procedures to obtain optimal coverage and containment of drug infusion without compromising patient safety. Available tools and stereotactic techniques for CED are not fully applicable to infants due to small head size and thin skull. There is currently no published data regarding feasibility of CED based intraparenchymal drug infusion in infants. We performed stereotactic infusion in our patient using a robotic platform (ROSA® Surgical Robot, Medtech, Montpellier, France). Operative trajectories including entry point, target and trajectories for infusion cannula placement were planned before the procedure using the robotic surgical navigation software. Both trajectories were selected via trans-frontal route ensuring sulci, ventricle, cisternal spaces and vessels were avoided. Following general anesthesia induction in the operating room, the head was fixed with a pediatric multi-purpose skull clamp (DORO®, Pro-Med Instruments, Freiburg, Germany) with gel head ring and 2 dual pin holders as side clamps. The head was placed on gel head holder and positioned without side pins ensuring the entire weight of the head was resting on the gel head holder. The head holder was attached to the ROSA and side pins with dual pin holders were gently clamped to skull on each side applying <10 lbs of torque. Pins were placed on the mastoid bones and posterior parietal bones to stabilize the head without carrying any weight. The ROSA robot registered the head via facial scanning software and validated once the precision was <1 mm. Both skull entry points were marked on frontal region using ROSA laser pointer. The entry points were approximately 1 cm anterior to coronal sutures and 5 cm lateral to midline on both sides. The planned trajectory was selected and confirmed on a touch screen interface and the robot arm movement was initiated to take position for the pre-determined entry sites. The infusion cannula (SmartFlow® cannula, MRI Interventions, Irvine, CA, USA) depth was calculated via ROSA software, marked and then inserted through the guide tube of robotic arm to target through small drill holes. Cannula and robotic arm were locked and secured in this position. The O-Arm™ imaging system was placed around the table (Medtronic, Minneapolis, MN, USA) and 3D imaging of the head was obtained. 3D imaging data was transferred to ROSA platform and O-Arm images with cannula were merged with pre-operative MRI. Accuracy of insertion was analyzed on merged images by comparing the position of the actual cannula trajectory with the planned trajectories. Error measurements between cannula and planned target were obtained in all 3 planes and saw a perfect correlation. Then thalami were infused sequentially with 180 µl of rAAVrh8-*HEXA*/*HEXB* vectors (1.55E12 vg/thalamus) using a micro-infusion pump (Harvard Apparatus, Holliston, MA, USA) at 3µL/min. The cannulas were removed after completion of the infusion and patient underwent MRI prior to transfer to the ICU where she was extubated. Post-infusion MRI study demonstrated that T2 MRI distribution volumes were 457 and 499 mm^3, corresponding to Vd/Vi = 2.6 and the infant thalami had a volume of 5.4 cm^3. The patient tolerated the procedure very well.

667. Immune Checkpoint Inhibitor Nanocomposites for Pulmonary Metastatic Colorectal Cancer

So Young Yoo

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Here we demonstrate theranostic immune checkpoint inhibitor nanocomposites (ICI NC) having an improved tumor targeting ability in pulmonary metastatic colon cancer model. Atezolizumab, a PD-L1 antibody, was conjugated with methoxy poly(ethylene glycol) (MePEG) and chlorin e6 (Ce6) via cathepsin-B-sensitive peptide as a linkage (named as ICI nanocomposites, ICI NC). This ICI NC is delivered to tumor sites enriched with tumor-specific enzymes such as cathepsin B, whereas undesired ICI exposure to normal tissue is avoided. When ICI NC were incubated with cathepsin B, Ce6 was released from ICI NC with increased fluorescence intensity in cathepsin B dose-dependent manner, which was by degradation of the peptide and then liberated Ce6 was activated in the aqueous solution. In

animal pulmonary metastasis model using CT26 cells, ICI NC showed superior tumor targetability, i.e., fluorescence intensity was significantly strong in the mouse lung having metastatic tumor. On the contrary, cathepsin-B-deficient carriers such as atezolizumab-Ce6 conjugates or atezolizumab-Ce6/MePEG conjugates showed strong fluorescence intensity in the liver as well as lung. Our proposed ICI NC may be used for theranostic cancer therapy with superior tumor specificity of releasing ICI and Ce6 into tumor microenvironment, thereby showing an efficient inhibitory effect on pulmonary metastasis of CT26 cells.

668. Robust Gene Expression Introduced by an Amino-Lipid-Based Nanoparticle Platform Comparable to AAV2 Vector

Da Sun, Wenyu Sun, Songqi Gao, Cheng Wei, Jonathan Lehrer, Zheng-Rong Lu
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Robust Gene Expression Introduced by an Amino-lipid-based Nanoparticle Platform Comparable to AAV2 Vector Da Sun, Wenyu Sun, Songqi Gao, Cheng Wei, Jonathan Lehrer, and Zheng-Rong Lu**Introduction:** Gene replacement therapy (GRT) has received increasing attention because of the success of adeno-associated virus (AAV2)-based GRT for Leber's congenital amaurosis (LCA2). However, due to the limitation of AAV2's 4.7 kB loading capacity, this platform is restricted from applications in therapies that require delivery of large genes such as Stargardt's disease (STGD). Here, we developed an amino-lipid (ECO)-based nanoparticle GRT platform with unlimited cargo capacity. The transfection efficiency of the ECO nanoparticle system in comparison with AAV2 was evaluated using a GFP reporter gene. **Methods:** Subretinal treatments were performed using retinal pigment epithelium (RPE) targeted ACU-PEG-HZ-ECO/*pCMV-GFP* nanoparticles and AAV2-*CMV-GFP* vectors with the same doses to BALB/c mice. GFP expression was examined using a scanning laser ophthalmoscopy (SLO) featuring a fluorescent mode with an argon wavelengths of 488/514 nm 14 days after injection and using a fluorescent slide scanner 30 days after injection.**Results:** Subretinal delivery of both ACU-PEG-HZ-ECO/*pCMV-GFP* nanoparticles and AAV2-*CMV-GFP* vectors induced significant GFP expression comparing to untreated control (**Figure 1A**). Both the nanoparticle and AAV2 demonstrated GFP expression across the examined retinal area demonstrated by SLO 14 days after injection. This robust GFP expression maintained high level demonstrated by the green fluorescence 30 days after injection for both ECO nanoparticle and AAV2 (**Figure 1B,C**). Targeted ECO-based nanoparticle platform demonstrated at least same level or better GFP expression than AAV2 viral platform. **Conclusion:** Both ACU-PEG-HZ-ECO/*pCMV-GFP* nanoparticles and AAV2-*CMV-GFP* vectors demonstrated well distributed GFP expression in BALB/c mice 14 days and 30 days after subretinal injections. ECO has shown better or at least the same transfection efficiency as AAV2. The non-viral ECO-based nanoparticle platform with no cargo size limitation can be a promising GRT strategy for a broad range of monogenic retinal disorders. **Acknowledgements:** This project was supported by the Gund-Harrington Scholars Award.

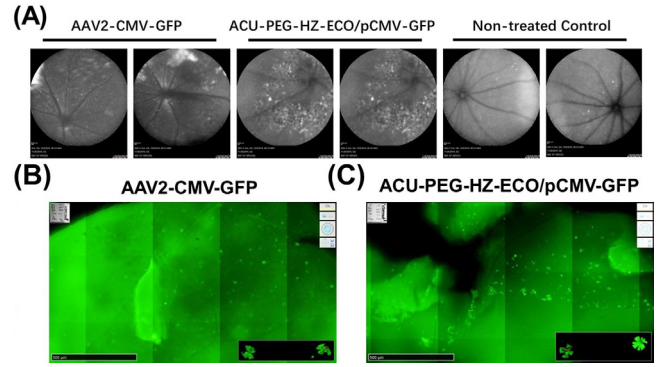

Figure 1. GFP expression in the eyes of BALB/c mice treated with ACU-PEG-HZ-ECO/pCMV-GFP nanoparticles and AAV2-CMV-GFP. Scanning laser ophthalmoscope (SLO) images of GFP expression 14 days after treatments (white dots) in the retina **(A)**. Fluorescent microscopic images of GFP expression in the retina flatmounts treated by AAV2-CMV-GFP nanoparticles **(B)** and ACU-PEG-HZ-ECO/pCMV-GFP **(C)** 30 days after injection.

669. Use of Oligopeptide-ModifiedPoly(beta-amino esters) (OM-PBAE) Polymers as Universal and Cross-Species In Vivo Delivery Agents of Lentiviral Vectors for Gene Therapy

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Intracellular delivery of nucleic acids to mammalian cells using non-viral gene delivery remains a challenge both *in vitro* and *in vivo*, with transfections often suffering from variable efficacy and limited stability. aratinga.bio TNP has developed a new technology that combines the versatility of negative and positive oligopeptide-modified poly(beta-amino ester)s (OM-PBAEs) as biodegradable and safe transfection material together with the gene transfer efficiency of lentiviral vectors (LVs). Here we report on a method to consistently manufacture LV particles encapsulated in OM-PBAE polymers able to transduce mammalian cells with high efficacy and without any pronounced cell toxicity. Essential biochemical parameters to prepare functional LV nanoparticles will be presented. This technology has been successfully applied to human and mouse immortalized and primary cells to trigger the expression of various reporter genes and Chimeric Antigen Receptors. The biodistribution of formulated LV nanoparticles encoding Green Fluorescent Protein or Luciferase has been investigated in Balb/c mice after intravenous administrations and we could show a different distribution profile compared to pseudotyped LVs, with a pronounced tropism for blood cells when compared to other viral vectors. Multiple intravenous injections or perfusions of these LV nanoparticles were safe and well tolerated in Balb/C mice as no obvious sign of distress, body weight loss, change in blood cell count or cytokine levels were reported. These encouraging results indicate that OM-PBAE encapsulated LVs have highly promising properties with a great potential for further preclinical and clinical development of gene-based therapeutics.

670. NF1 Gene Rescue via Polymeric Nanoparticles in Translational Animal Models

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To assess new potential treatment strategies for neurofibromatosis type I, we aim to restore the function of defective NF1 alleles through two approaches using polymeric nanoparticles as a delivery vehicle. Gene therapy for NF1 mutations poses unique problems as it is not yet clear which cell populations must be edited and when in order to prevent disease manifestations in numerous tissues. First, we are exploring CRISPR/Cas technologies for somatic gene editing. Secondly, we are developing gene replacement therapy through targeted delivery of a full-length mouse NF1 cDNA plasmid using nanoparticles. Currently, the most effective CRISPR/Cas9 editing system for homology driven repair is a three-component system including Cas9 protein and synthetic guide RNA complexed as a ribonucleoprotein (RNP) and a single stranded DNA repair template. To maximize gene editing efficiency and reduce off-target effects, we will deliver a combination of RNP and repair templates using engineered nanoparticles. We will treat rodents harboring patient-specific mutations in Nf1 (c.2041C>T; p.R681X in exon 18, c.5425C>T; p.Arg1809Cys in exon 38, and c.2446C>T; p.Arg816X in exon 22) with nanoparticle delivery of CRISPR/Cas reagents to correct the mutations at the genome level to prevent or mitigate NF1 pathologies. To optimize efficiency of conversion strategies ex vivo before packaging with nanoparticles, heterozygous mouse embryos were directly injected with RNPs and repair templates. Direct sequencing of PCR products from multiple c.5425C>T/+ embryos post-injection showed efficient restoration of the mutant allele to wild type. These validated complexes are being encapsulated into nanoparticles for in vivo delivery into rodents for assessment of a) rescue of lethality due to loss of NF1 gene function, and b) rescue and/or treatment of developing tumors. As a gene replacement therapy for NF1, we will deliver a full-length mouse NF1 cDNA plasmid using nanoparticles in the same patient-specific mutation models as above as well as a conditional loss of Nf1 system with multiple tissue-specific Cre drivers. We have established successful nanoparticle delivery of mouse Nf1 full length cDNA through both intracerebroventricular and intravenous administration in wild-type mice, with confirmation of excess neurofibromin in brain and liver, respectively. This nanoparticle system will now be assessed in vivo in mutant mice for ability to restore NF1 activity to: a) rescue lethality due to loss of NF1 gene function, b) rescue and/or treat developing plexiform tumors, and c) rescue and/or treat developing optic gliomas.
Funding: Gilbert Family Foundation Gene Therapy Initiative

671. Varying Ultrasound Parameters Allowed Specific Targeting in Liver Hepatocytes and Sinusoidal Endothelial Cells Respectively for Ultrasound Mediated Gene Delivery in Mice

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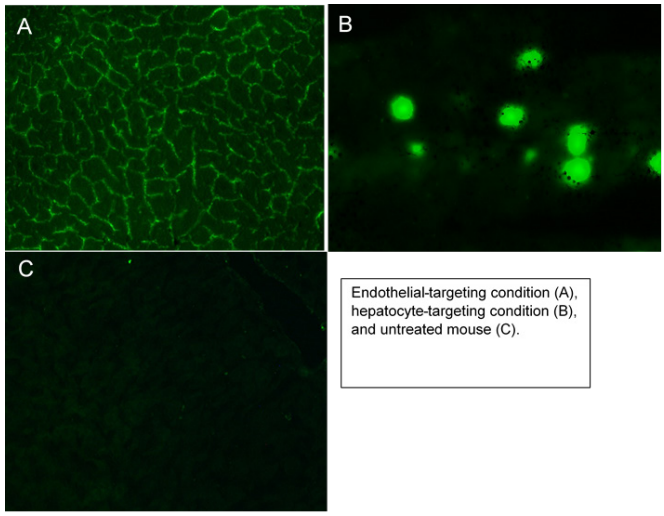
Ultrasound (US) mediated gene delivery (UMGD) in combination with microbubbles (MBs) has been shown to be an effective method for non-viral gene delivery. UMGD is an especially promising strategy for treating genetic diseases, including Hemophilia A and other diseases with a basis in the liver. In this study we show that it is possible to vary therapeutic US conditions to target gene therapy to two different types of liver cells: hepatocytes and liver sinusoidal endothelial cells (LSECs). A GFP plasmid driven by a ubiquitous cytomegalovirus (CMV) promoter (pCMV-GFP) in combination with RN18 microbubbles were injected via portal vein into normal BL6 mouse liver. Simultaneously, custom-designed therapeutic ultrasound transducer (H158) was applied to the surface of the liver to induce MB cavitation using a one-minute pulsed US treatment. In our previous work, a matrix of US conditions from 0.5-2.5 MPa peak negative pressure (PNP) and 18 μs to 22 ms pulse duration (PD) in combination with 1.1MHz frequency and 14 Hz PRF were tested to examine transgene expression following UMGD in mice. From the resulting plot of PNP versus PD we selected conditions falling on an energy curve that generated high level gene expression in hepatocytes. Furthermore, US conditions below the hepatocyte-targeting energy curve were selected for LSEC targeting based on the hypothesis that lower US energy may be sufficient for efficient UMGD since no endothelial membrane destruction is required to allow extravasation of pDNA/MBs to the extravascular space. Livers were harvested on day one, sectioned at 7 μm, and imaged with a Leica DM6000 fluorescent microscope to assess GFP expression. Furthermore, an endothelial-specific GFP construct driven by an intercellular adhesion molecule 2 (ICAM2) promoter, (pICAM2-GFP) was constructed and examined for its specific transgene expression in LSECs using hydrodynamic injection of the plasmid into mice. Assessment of GFP images following UMGD showed that by varying US parameters we were able to target different cell types within the liver (Figure 1 A-C). From the matrix tested, one selected condition (1.1 MPa, 150 μs PD) produced GFP expression predominantly in LSECs (Figure 1A), whereas our previously established hepatocyte-targeting condition (1.5 MPa, 150 μs PD) generated GFP expression largely in hepatocytes (Figure 1B). Images of liver sections from mice treated with hydrodynamic injection pICAM2-GFP showed a nearly identical pattern to that obtained from mice treated with UMGD of pCMV-GFP under the endothelial-specific US condition. This strategy can be further optimized to allow gene therapy targeting in liver sinusoidal endothelial cells only, and the ability to use ultrasound to target different cell types has broad implications for treating many different diseases.



672. Abstract Withdrawn

673. Conjugation of an Adenoviral Junction Opener Protein to Liposomal Doxorubicin and its Efficacy in Tumor Models

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Although the field of cancer therapeutics has advanced significantly due to the advent of revolutionary new therapies such as checkpoint inhibitor immunotherapy, several key problems remain for effective treatment of tumors. One such example is the physical barriers which block entry of therapeutics and mediate drug and/or immune resistance of solid tumors. Desmoglein-2 (DSG2) is part of the desmosome complex and has been previously observed to be upregulated in multiple cancers. This upregulation results in a physical barrier that may exclude the entry of small molecule drugs, biotherapeutics, or immune cells. An adenovirus-3-derived recombinant protein, termed Junction Opener (JO), aims to solve this problem. JO binds DSG2, initiating a cascade which ultimately results in the transient opening of tight junctions. This has implications for solid tumor therapy in that JO-mediated opening of tight junctions can allow better penetration of drugs or immune cells. JO can also act as a tumor-specific homing molecule as DSG2 is expressed on the surface of tumor cells whereas normal cells sequester it. We explored conjugation of a conjugatable JO (JOC) to a liposomal doxorubicin formulation via maleimide chemistry. A conjugatable form of JO was formed by removal of endogenous cysteines and the addition of a C-terminal cysteine residue. The conjugation proved successful with evidence of JO's retention on the surface of the liposome and retention of DSG2-binding and cytotoxic activity. *In vivo* testing of JOC-Liposomal doxorubicin in a human colon cancer xenograft mouse model indicated superior survival to liposomal doxorubicin alone (p=0.041, survival of 41 vs 51 days respectively). A conjugated form of JOC and liposomal doxorubicin has the advantages of ease of administration and decreased likelihood of systemic effects, due to JOC's ability to home to tumors. The conjugate presents a promising cancer therapeutic which may be used in conjunction with conventional chemotherapeutics and immunotherapy alike.

674. In Vitro Biochemical Stability and Transfection Efficiency of Receptor-Targeted Nanoparticles

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Nanoparticles are widely used in diagnostic techniques and being researched as carriers of drugs and therapeutic agents. However, more work is needed on developing nanoparticles that are biophysically stable over time whilst maintaining their transfection efficiency, which are highly desirable qualities for pharmaceutical industries. Here, we investigate the variables affecting the stability of targeted nanoparticles by studying the physicochemical characteristics and transfection efficiencies in human HepG2 and mouse Neuro2A cells. Nanoparticles were made through self-assembly by mixing liposomes, targeting peptides and luciferase DNA or mRNA and were made in water or PBS and stored at different temperatures (room temperature, 4°C, -80°C) for 2-4 weeks. In some cases, different cryoprotectants were used (sucrose, trehalose, Histidine/Sucrose). The nanocomplexes were more stable in water compared to PBS in terms of size and charge. Transfections in HepG2 cells showed that there was no significant difference in enzymatic activity over 14 days for almost all nanoparticles that contained mRNA (irrespective of whether they were made in water or PBS), whereas the DNA-containing nanoparticles were stable in terms of transfection efficiency for 14 days when made in PBS and 7 days when made in water. Transfections in Neuro2A cells showed that at room temperature and 4°C all formulations maintained enzymatic activity over time, whereas at -80°C only the formulations with cryoprotectants showed no decrease in transfection efficiency. Cytotoxicity was not detected with any of the nanocomplexes. This study provides insights of different ways of formulation and nanoparticle stability. The stability of nanoparticles is one of the most important barriers to successful clinical applications of non-viral delivery vectors and this study addressed this key question.

675. Flowfect™: A Scalable Non-Viral Process for T Cell Engineering

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The cell therapy industry has recently demonstrated the curative potential of engineered CAR T cells for treating patients with specific forms of leukemia and lymphoma. The manufacturing process of these treatments predominantly use viruses as the vehicle to deliver the genetic information into the patients' cells. However, viral engineering processes are hampered by timelines that takes weeks, cost millions of dollars, and require significant infrastructure and downstream regulatory testing. As an alternative, Kytopen is pioneering a novel, non-viral process known as *Flowfect*™ to engineer human cells for

therapeutic applications in immuno-oncology and genetic disorders. The *Flowfect*™ technology combines continuous fluid flow with electric fields to transfect primary T cells while maintaining high efficiency. This process eliminates the harmful effects of traditional delivery via electroporation by rapidly dissipating the heat associated with the applied electrical field. *Flowfect*™ transfection results in viable cells which can be expanded or activated as needed. We have successfully transfected mRNA into naïve $CD4^+$ primary T cells with 95% efficiency, which maintain 100% $CD45RA^+$ and 0% $CD45RO^+$ cell populations after *Flowfect*™ treatment. Additionally, we observed no induced expression of activation markers when expanded primary T cells are transfected with *Flowfect*™, suggesting that the *Flowfect*™ technology does not negatively impact functional state of input cells. The *FlowfectT*™ technology can be implemented on an automated liquid handling system, facilitating high throughout transfection optimization. The ability to rapidly optimize has been validated in high efficiency delivery of multiple payloads, including mRNA and CRISPR/Cas9 reagents. Using a commercial automated liquid handling platform augmented with the *Flowfect*™ technology, we were able to rapidly optimize payload delivery into primary T cells from multiple donors. Moreover, the optimized delivery parameters transitioned seamlessly to therapeutically-relevant cell numbers in the large volume clinical scale *Flowfect*™ Tx platform. Compelling transfection data at multiple scales suggest that the *FlowfectT*™ technology will enable rapid translation of therapeutic innovations from the bench to the clinic.

676. A System for Active Loading of miRNAs into Exosomes

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Introduction: Exosomes are nanoscale extracellular vesicles that are under intense research for therapeutic applications due to their ability to deliver biomolecules to recipient cells and modulate many pathophysiologic processes. Recent studies suggest that many therapeutic effects of mesenchymal stem cells (MSCs), a cell type that has been investigated in >1000 clinical trials, are actually mediated by the exosomes secreted by MSCs, which make MSC exosomes attractive candidates for off-the-shelf therapies, that could avoid manufacturing and safety challenges associated with whole cells. Many therapeutic effects of exosomes are associated with microRNAs (miRNAs) contained within the vesicles. This work aimed to engineer a system to actively load mature miRNA into exosomes using cellular machinery in order to potentially produce exosomes with enhanced therapeutic properties. The system uses expression of a minimal scaffold of the vesicular stomatitis virus glycoprotein (mVSVG) [1], a transmembrane protein that inserts into exosomes. mVSVG was fused at the C-terminus to RNA aptamer-binding domains to localize these fusions inside exosomes, and facilitate loading of precursor miRNA (premiR) containing 3' RNA aptamers. **Methods:** Three plasmids were produced to test the exosomal miRNA loading system (**Figure 1**).

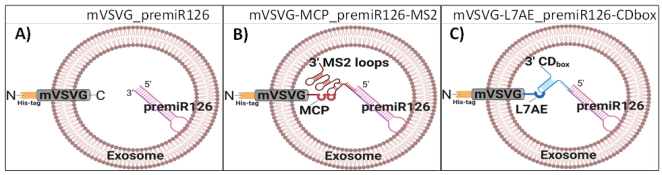

Figure 1: **A)** *mVSVG_premiR126* expresses mVSVG with no C-terminal fusion and premiR126 with no 3' aptamer **B)** *mVSVG-MCP_premiR126-MS2* expresses mVSVG with C-terminal fusion to MS2 coat protein (MCP) and premiR126 with 3' MS2 loop aptamer. **C)** *mVSVG-L7AE_premiR126-CDbox* expresses mVSVG with C-terminal L7AE fusion and premiR126 with 3' CDbox aptamer. Figure created with BioRender.com

Plasmid expressing a fusion of EGFP and luciferase (EGFP-Luc) was used as a control. HEK293T cells and human adipose MSCs derived from three different donors were transfected in 96-well plates with the above plasmids, using Turbofect reagent. 48 hrs later, exosomes were isolated from media and lysed to extract RNA. Exosomal miRNA was reverse transcribed and amplified with TaqMan Advanced miRNA cDNA Synthesis Kit prior to quantification of relative mature miR126 by qRT-PCR normalized to mature miR100, using TaqMan Advanced miRNA Assays. All experiments were performed in triplicate (n=3) and on duplicate days. **Results:** In HEK293Ts, transfection with *mVSVG_premiR126*, resulted in about 10-fold more mature miR126 in exosomes than EGFP-Luc control, indicating that increased transcription of a precursor miRNA can increase the amount of the mature miRNA in secreted HEK293T exosomes passively. However, transfection of HEK293Ts with *mVSVG-MCP_premiR126-MS2* and *mVSVG-L7AE_premiR126-CDbox* resulted in 19- and 15-fold increases in mature miR126 contained in exosomes, respectively, relative to passive loading with *mVSVG_premiR126*. In MSCs, transfection with *mVSVG_premiR126*, also resulted in about 10-fold more mature miR126 in exosomes by passive loading, relative to EGFP-Luc control. All MSCs also displayed an increase in exosomal mature miR126 when transfected with active loading systems (i.e. *mVSVG-MCP_premiR126-MS2* or *mVSVG-L7AE_premiR126-CDbox*), relative to passive loading with *mVSVG_premiR126*, but increased loading varied widely between donor and system (i.e. 3- to 36-fold), presumably due to transfection efficiency variability in MSCs derived from different donors. **Conclusion:** The described system is able to actively load a miRNA of interest into exosomes of transfected cells, while also allowing normal processing to yield the functional mature miRNA within exosomes, suggesting the system could be used to produce exosomes with enhanced therapeutic properties. **References:**
[1] Meyer C, et al. *Int J Nanomedicine*. 2017.

677. In Vivo Exosome Based Therapy for Liver Inherited Metabolic Disease: ASL Deficiency

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Background: Argininosuccinate lyase (ASL) is a cytosolic enzyme, which converts argininosuccinic acid (ASA) into L-arginine and fumarate. This reaction is essential for the urea cycle, which detoxifies neurotoxic ammonia produced by protein breakdown. ASL deficiency causes argininosuccinic aciduria, an autosomal recessive liver inherited metabolic disease and is the second most common urea cycle defect. Argininosuccinic aciduria causes hyperammonaemic decompensation and neurocognitive impairment. Standard of care aims to normalise ammonia with protein restricted diet, ammonia scavengers and in more severe cases liver transplantation. Exosomes are microvesicles secreted by most cell types, playing a critical role in cell-cell communication by proteins and nucleic acids transfer. Exosomes are not immunogenic, which makes them an appealing strategy to deliver therapeutic compounds. **Methods**: We investigated exosomes as a therapeutic strategy to deliver functional ASL protein in a knock-in $Asl^{Neo/Neo}$ mouse. This hypomorphic mouse recapitulates the phenotype of the human disease with increased ammonia and ASA levels and early death at 3 weeks of age. We assessed an ASL protein-loaded exosome manufactured by a proprietary method. Efficacy of these constructs delivered by single intraperitoneal ($2x10^{11}$ particles per mouse) were tested *in vivo* versus empty exosomes at 24-hours post-injection. **Results**: After a single intra-peritoneal injection of ASL-expressing exosomes in 2-week old mice, we observed a dramatic 65% and 48% reduction in ammonia and ASA levels, respectively at 24h post-injection. Further exploratory studies revealed that intravenous injection of ASL-expressing exosomes also resulted in significant reduction in disease biomarkers and that repeated administration of these exosomes was not only well tolerated but resulted in a marked improvement in animal survival. **Discussion**: Here, we demonstrate proof of concept of the therapeutic potential of exosomes in ASL deficiency. To our knowledge, this is the first attempt to use exosomes in treatment of an inherited metabolic disease *in vivo*. Future work will explore the effect of these exosomes on other measures of disease as well as further dose response studies and comparing the efficacy of protein-loaded exosomes with mRNA-loaded exosomes.

678. Explicating the Cellular Uptake, Trafficking, and Biodistribution Mechanism of Cell Derived Vesicles, a Unique Therapeutic Carrier

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Introduction: Cell derived vesicles (CDVs) are membranous, nanosized vesicles, which have recently emerged as an important carrier in many therapeutic areas. CDVs are produced by extruding cells through a series of membrane filters with different pore sizes. Increasing number of studies have demonstrated the potential role of CDVs in cell to cell communication. In addition, CDVs produced from certain types of immune cells carry molecules crucial for recognizing specific target cells, such as tumors, on their surface and were proposed as a novel carrier for anticancer medicine. However, the exact mechanism and route of cellular uptake remain largely unresolved, becoming one of the most challenging hurdles in the development and therapeutic applications of CDVs. In this study, we aim to investigate the cellular uptake mechanism of CDVs produced from natural killer (NK) cells using a manufacturing platform recently developed. Both *in vitro* uptake assay and *in vivo* distribution analyses were performed to provide precise insights into how CDVs exert its effect at the cellular level. **Methods**: CDVs extruded from NK cells were mainly used in this study. Two different types of cells (breast cancer cells, BT549, and human/rodent endothelial cells) with varying degree of intracellular adhesion molecules 1 (ICAM-1) expression were used to examine the interaction of ICAM-1 and integrin expressed on NK-CDVs. The role of ICAM1-1/integrin binding in the cellular uptake was assessed by FACS and confocal imaging analysis. Then, various inhibitors for uptake pathways, such as phagocytosis, dynamin dependent endocytosis, and receptor mediated endocytosis, were used to comprehend the underlying mechanism of cellular uptake of CDVs. Next, the biodistribution profile of NK-CDVs were characterized using both normal and tumor xenograft models by IVIS imaging. **Results**: NK-CDVs produced from the manufacturing process we established show efficient uptake into the target cells. The cellular uptake of NK-CDVs depends on the molecular interaction between ICAM-1 and lymphocyte function-associated antigen-1 (LFA-1), an integrin highly expressed on many types of immune cells (Figure 1). We also present a cellular uptake mechanism involved in the entrance of CDVs into the target cells. Furthermore, *in vivo* distribution profile of NK-CDVs are also assessed using various tumor models.

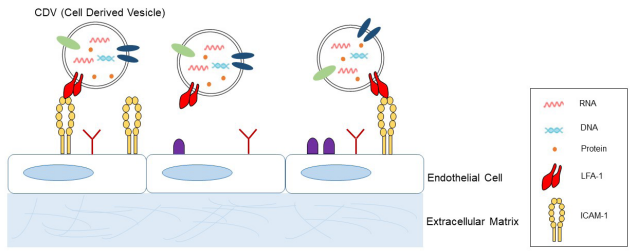

Figure 1. Cellular uptake mechanism of CDV

Summary/Conclusion: This study demonstrates that the CDVs produced at the manufacturing scale can be easily taken up by cells via specific cellular pathways. This finding will facilitate the development of more efficient therapeutics for cancer and other debilitating diseases.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

679. Abstract Withdrawn

680. Building Gene Therapy Clinical Trial Readiness for CLN8 Batten Disease

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The neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease, are a group of autosomal recessive neurodegenerative, lysosomal

storage disorders characterized by seizures, behavioral difficulties and progressive decline in cognitive, motor, visual and language abilities. CLN8 disease is one of 14 forms of Batten disease that results from biallelic variants in the *CLN8* gene. The CLN8 transmembrane protein assists in shuttling lysosomal enzymes from the endoplasmic reticulum to the Golgi apparatus and is critical for lysosomal biogenesis. Clinical presentations of CLN8 disease range from late infantile variant NCL with rapid progression to a more attenuated phenotype of juvenile onset without notable vision changes or myoclonus, known as Northern Epilepsy. There are no known treatments for CLN8 Batten disease, but may be amenable to AAV-mediated gene replacement. We report an illustrative case of a boy with compound heterozygous nonsense variants in *CLN8* (c.295C>T, p.Q99*; c.766C>T, p.Q256*) with onset of disease around 24 months initially characterized by language regression, and later development of myoclonic, astatic and generalized tonic-clonic seizures, behavioral dysregulation, vision loss, hypotonia, ataxia, spasticity and feeding difficulties, and death at age 6 years 8 months. We outline a comprehensive natural history study of CLN8 Batten disease in order to assess mean rates of decline in functioning using standardized Batten disease rating scales, as well as determine clinical assessments that show sensitivity to progression over a 12-month period. This dataset will be critical to assess efficacy of emerging interventions, including CLN8 gene therapy.

CLN8 Natural History Study Procedures	Baseline		Month 3	Month 6	Month 9	Month 12	
	Day 1	Day 2	Day 1	Day 1	Day 1	Day 1	Day 2
Consent (re-consent if needed)	X		X*	X*	X*	X	
Medical history, including medications	X		X*	X*	X*	X	
Physical exam	X		X*	X*	X*	X	
UBDRS (Batten disease rating scale)	X			X*		X	
Hamburg (Batten disease rating scale)	X		X*	X*	X*	X	
Cognitive assessment	X					X	
Language assessment	X					X	
Child Behavior Checklist (behavior measure)	X					X	
Social Responsiveness Scale (autism symptom scale)	X					X	
Vineland III (adaptive behavior)	X					X	
PedsQL (quality of life measure)	X			X*		X	
10 Meter Walk/Run Test	X					X	
Peabody Developmental Motor Scales	X					X	
Pediatric Eval. of Disability Inventory (PEDI) - Functional Mobility	X			X*		X	
Routine EEG	X					X	
Blood draw (see Labs on next sheet)	X					X	
Anesthesia evaluation	X					X	
Brain MRI (volumetrics and DTI) with sedation		X					X
Lumbar puncture with sedation, including CSF biobanking		X					X
Ophthalmology (OCT and Exam) with sedation		X					X
Nerve conduction studies (EMG) with sedation		X					X
Blood draw for biobanking		X					X
Skin biopsy		X					

*Via teleconference

681. Abstract Withdrawn

682. Nuclease-Free Glucose-6-Phosphatase-á Gene Integration Ameliorates Hypoglycemia in Glycogen Storage Disease Type Ia

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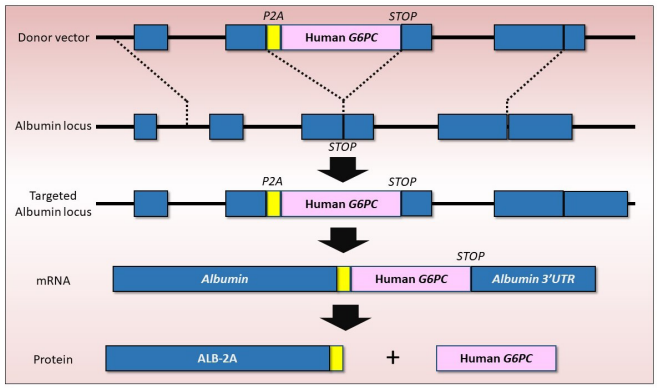
Background: Glycogen storage disease type Ia (GSD-Ia) is caused by a deficiency in glucose-6-phosphatase-α (G6Pase-α or G6PC), a key enzyme in maintaining blood glucose homeostasis. Patients affected by GSD-Ia manifest life-threatening fasting hypoglycemia along with hepatic abnormalities including excessive accumulation of glycogen and triglycerides. Recombinant adeno-associated virus (rAAV)-mediated gene therapy efficiently restored hepatic G6Pase-α and corrected fasting hypoglycemia in model animals of GSD-Ia. However, studies have shown that the transduced rAAV vectors are gradually diluted out or lost particularly in the livers of young animals due to high rate of hepatocytes proliferation associated with liver growth. Recently, gene editing technologies using nucleases such as CRISPR/Cas9 (CRISPR-associated protein 9), TALEN (transcription activator-like effector nucleases), and zinc fingers have been used to insert a target gene into the genome. However, off-target effects have been reported in nuclease-mediated gene editing, restricting the application of these technologies in human patients. **Method:** We examined the effect of a novel rAAV-based, promoterless, nuclease-free, genome editing technology (GeneRide™) on blood glucose homeostasis in liver-specific *G6pc*-deficient (LS-*G6pc*-/-) mouse.



Results: Here we show homologous recombination-based integration of human *G6PC* gene into *albumin* locus of mouse genome, without the use of nuclease, in LS-*G6pc*-/- mouse. Neonatal administration with the rAAV vector containing the cassette for homologous recombinant ameliorated fasting hypoglycemia in LS-*G6pc*-/- mice. We confirmed in-frame integration of *G6PC* gene between the penultimate and the stop codons of *albumin* locus in the livers of LS-*G6pc*-/- mice treated with the vector. Moreover, the P2A sequence-mediated ribosomal skipping enables G6Pase-α and albumin tagged with 2A (ALB-2A) to be produced as two separate proteins. Notably, plasma levels of ALB-2A positively correlated with the expression of *G6PC* gene as well as higher fasting glucose levels in LS-*G6pc*-/- mice treated with the vector, suggesting that plasma ALB-2A can be used for a surrogate marker of *G6PC* gene integration. Importantly, plasma levels of ALB-2A maintained from age 4 to 12 weeks in LS-*G6pc*-/- mice treated with the vector, indicating that G6Pase-α can be continuously expressed by the vector integrated in genome of the livers. **Conclusion:** Our results suggest that nuclease-free *G6PC* genome editing mediated by the homologous recombination may represent a potential strategy to constantly improve hypoglycemia by persistent hepatic expression of G6Pase-α in GSD-Ia.

683. Keratanase Treatment was Able to Decrease the Bone Keratan Sulfate Content in Mucopolysaccharidosis IVA Mice

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Background: Thermostable keratanase (TSK), endo-β-*N*-acetylglucosaminidase, is a keratan sulfate (KS)-degrading enzyme of *Bacillus* origin. In a previous study, we have shown that twice every 4 weeks TSK administration to the Mucopolysaccharidosis (MPS) IVA KO mice from immediately after birth improved the bone pathological condition (e.g. vacuolated chondrocytes) compared with that of untreated mice. The aim of the present study is to evaluate the therapeutic efficacy of TSK in terms of the tissue KS content in IVA KO mice. Results: TSK was administered intravenously to the mice at a dose of 2 or 20 U/kg twice every 2 weeks from immediately after birth. At 4 weeks of age, blood and various tissues were collected to prepare serum and protease-digested tissue extract samples. KS polysaccharide was partially purified from these samples by an anion exchange chromatography, desalted by gel filtration chromatography, and evaporated. After digestion of KS polysaccharide into oligosaccharides with keratanase II, KS oligosaccharide concentration was determined by LC-MS analysis, which was optimized for highly-sensitive detection of KS oligosaccharide using an alkaline buffer including the appropriate concentration of ammonium bicarbonate. The blood KS level was decreased by TSK treatment almost to the level of normal mice. Also, the KS contents of the trachea, spleen, and heart were significantly decreased. We found the KS contents of the lower leg bone and kidney were decreased in a dose-dependent manner. Conclusion: The tissue KS contents could be monitored with the improved method. TSK treatment was able to decrease the tissue KS contents, especially in the leg bone and trachea. These data support the potential of TSK therapy for MPS IVA.

684. Preclinical Development of SPK-3006, an Investigational Liver-Directed AAV Gene Therapy for the Treatment of Pompe Disease

Christopher R. Riling[1], Jayme M. L. Nordin[1], Helena Costa Verdera[2], Pauline Sellier[2], George Michael Preston[1], Daniel M. Cohen[1], Fanny Collaud[2], Marco Crosariol[1], William J. Quinn[1], Hayley Hanby[1], Umut Cagin[2], Francesco Puzzo[2], Virginia Haurigot[1], Giuseppe Ronzitti[2], Pasqualina Colella[1], Xavier Anguela[1], Federico Mingozzi[1], Sean M. Armour[1]

[1]Liver Research & Development, Spark Therapeutics, Inc, Philadelphia, PA,[2]Genethon, Inserm, Integrare, France

Pompe disease is a lysosomal storage disease caused by loss-of-function mutations in the acid alpha-glucosidase (GAA) gene, which lead to significant accumulation of glycogen in tissues resulting in multi-system pathology. Enzyme replacement therapy (ERT) increases survival, slows disease progression, and is the current standard of care for Pompe patients. However, ERT has several potential drawbacks such as limited biodistribution and insufficient uptake in certain affected tissues, immunogenicity of recombinant GAA, and high treatment burden. We have shown that investigational liver-directed adeno-associated viral (AAV) gene therapy expressing secretable GAA results in decreased glycogen accumulation, increased survival, and improvement of cardiac, respiratory, and muscle phenotypes in the Gaa / mouse model of Pompe disease. Secretable GAA vectors demonstrated greater efficacy in restoring muscle strength, morphology and autophagic defects in Gaa / mice when compared to the standard of care regimen of biweekly 20 mg/kg ERT. Pharmacokinetic analysis of GAA tissue uptake in Gaa-/- mice suggests improved uptake when comparing the sustained plasma GAA levels achieved with secretable GAA vectors with the peak and trough observed following ERT administration. Further optimization of the expression cassette and selection of a highly hepatotropic capsid led to the development of the clinical candidate, SPK-3006, an investigational liver-targeted gene therapy for the treatment of Pompe disease. A single infusion of SPK-3006 in non-human primates at three ascending doses demonstrated dose-dependent expression of GAA in plasma, evidence of associated uptake in peripheral tissues, but no adverse histopathological findings or significant changes in clinical pathology parameters. Additional in vitro and in vivo studies are ongoing to further characterize SPK-3006 and to determine the biochemical properties of the transgene-derived protein product following liver expression of secretable GAA. Overall, these studies demonstrate the potential of an investigational liver-directed gene therapy approach with secretable GAA and support initiation of clinical studies utilizing SPK-3006.

685. AAV-Mediated Gene Therapy Rescues GALT Activity and Reduces ER Stress in Classic Galactosemia

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Classic galactosemia, a recessive autosomal disease, is caused by point mutations in the *GALT* gene that encodes the galactose-1-phosphate uridyltransferase enzyme (GALT), which is essential in galactose metabolism. Without dietary intervention, newborns present with liver inflammation, cataracts, sepsis, and premature death. Long term consequences with dietary intervention include ataxia, speech and intellectual problems, and subfertility. Recombinant adeno-associated virus (AAV)-mediated gene therapy to restore GALT activity offers the potential to address the unmet medical needs of galactosemia patients. We are utilizing fibroblasts derived from classic galactosemia patients and developing a novel mouse model of disease to better understand the pathophysiological consequences resulting from loss of GALT activity. We established a GALT enzyme activity assay and showed fibroblasts derived from galactosemic patients exhibited significant reductions in enzyme activity compared to control fibroblasts. Further analysis revealed that this reduction was likely due to a decrease in GALT protein levels as opposed to mRNA levels. We also confirmed that high galactose conditions induce ER stress in patient fibroblasts. Transduction of classic galactosemia patient fibroblasts with a

recombinant AAV vector encoding GALT reconstituted GALT activity and reduced galactose-induced ER stress. We have also developed a novel mouse model. Initial phenotypic characterization of this mouse demonstrated a reduction in GALT protein in the liver, reduced enzymatic activity in RBCs and liver, and signs of liver inflammation based on immunohistochemical assessment. Additional studies will determine if these mice have cerebellar defects, ataxia, and/or reduced fertility and if these aberrant characteristics could be rescued via gene replacement therapy. These cellular and mouse models of galactosemia will advance our understanding of the underlying pathological mechanisms associated with classic galactosemia. AAV-GALT gene therapy holds promise in addressing the unmet medical needs in classic galactosemia patients.

686. mRNA Therapy for Inherited Disorders of Glycogen Metabolism

Paloma H. Giangrande[1], Jingsong Cao[1], Minjung Choi[1], Eleonora Guadagnin[1], Marine Silva[2], Erwann Philippe[2], Arianna Markel[1], Jenny Zhuo[1], Shi Liang[1], Andrea Frassetto[1], Lisa Rice[1], Tatiana Ketova[3], Cosmin Mihai[3], David Reid[3], Maria Abbasi[3], Anne-Renee Graham[3], Kristine Burke[3], Zach Zalinger[3], Giles Besin[3], Meredith Wolfrom[3], Athanasios Dousis[3], Vladimir Presnyak[3], Christopher Pepin[1], Wei Zheng[3], Lei Ci[3], Marjie Hard[3], Edward Miracco[3], Christine Lukacs[1], Patrick Finn[1], Lin Guey[1], Giles Mithieux[2], Fabienne Rajas[2], Paolo Martini[1]

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Storage Disease 1a (GSD1a) is a rare inherited metabolic disorder caused by deficiency of glucose 6-phosphatase (G6Pase) encoded by G6PC gene. G6Pase is a membrane protein, expressed predominantly in liver and kidney, required for maintaining interprandial euglycemia. GSD1a patients exhibit life-threatening hypoglycemia, metabolic complications, and liver/renal disease. There is no FDA-approved treatment for GSD1a and the current standard-of-care for managing hypoglycemia (uncooked cornstarch supplementation) fails to prevent long-term complications and presents with compliance issues. Enzyme replacement and gene therapy-based approaches are not an option for some patients due to drug-delivery and efficacy/safety considerations. To develop a new treatment for GSD1a, we used lipid nanoparticle (LNP) encapsulated mRNAs encoding for human G6Pase. We demonstrate robust therapeutic efficacy of hG6Pase-mRNA-LNP therapy in human cells and in a mouse model of GSD1a with liver-specific deletion of G6PC (L.G6PC-/-) that resembles the human condition. Of note, single intravenous (i.v.) administration of the hG6Pase mRNA-LNPs resulted in marked improvement in fasting blood glucose levels for up to 10 days post-dose, along with dose-dependent improvement in serum and hepatic biomarkers. Moreover, we observed reproducible rescue in blood glucoses level and hepatic biomarkers even after 5 consecutive doses. Taken together, our results support the notion that mRNA-LNP therapy could have a therapeutic impact on the treatment of GSD1a and other enzymopathies with limited treatment options.

687. A Single Combination Gene Therapy Treats Multiple Age-Related Diseases

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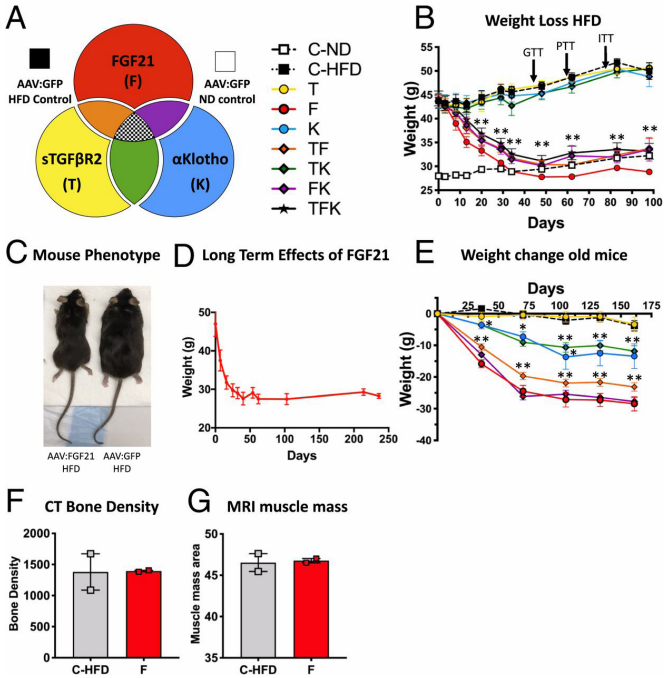
Comorbidity is common as age increases, and currently prescribed treatments often ignore the interconnectedness of the involved age-related diseases. The presence of any one such disease usually increases the risk of having others, and new approaches will be more effective at increasing an individual's health span by taking this systems-level view into account. In this study, we developed gene therapies based on 3 longevity associated genes (fibroblast growth factor 21 [FGF21], αKlotho, soluble form of mouse transforming growth factor-β receptor 2 [sTGFβR2]) delivered using adeno-associated viruses and explored their ability to mitigate 4 age-related diseases: obesity, type II diabetes, heart failure, and renal failure. Individually and combinatorially, we applied these therapies to disease-specific mouse models and found that this set of diverse pathologies could be effectively treated and in some cases, even reversed with a single dose. We observed a 58% increase in heart function in ascending aortic constriction ensuing heart failure, a 38% reduction in α-smooth muscle actin (αSMA) expression, and a 75% reduction in renal medullary atrophy in mice subjected to unilateral ureteral obstruction and a complete reversal of obesity and diabetes phenotypes in mice fed a constant high-fat diet. Crucially, we discovered that a single formulation combining 2 separate therapies into 1 was able to treat all 4 diseases. These results emphasize the promise of gene therapy for treating diverse age-related ailments and demonstrate the potential of combination gene therapy that may improve health span and longevity by addressing multiple diseases at once.



688. Determining the Benefit of CNS Correction in Gene Therapy for Methylmalonic Acidemia

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[1]Organic Acid Research Section, NHGRI, National Institutes of Health, Bethesda, MD,[2]NIH Medical Research Scholars Program, National Institutes of Health, Bethesda, MD

Background: Methylmalonic acidemia (MMA) is a heterogenous genetic disorder most commonly caused by a mutation in the *MMUT* gene, which codes for the enzyme methylmalonyl-CoA mutase. Patients with MMA can experience metabolic strokes in the basal ganglia even after liver transplantation, raising the possibility that for AAV gene therapy delivery of *MMUT* to be fully curative, targeting of the basal ganglia (e.g. striatum) would be required. For this reason, we believe that systemic AAV delivery of *MMUT* with a capsid that can cross the blood brain barrier and transduce cells in the central nervous system (CNS) will be more beneficial in treating MMA than vectors that do not support CNS transduction. As a first step to develop a vector to restore CNS expression, we explored the cellular expression pattern of endogenous *Mmut* in wildtype mice, then tested a variety of report vectors, and finally, prepared a CNS-trophic therapeutic AAV to treat MMA mice. **Methods:** Tissue specific expression of methylmalonyl-CoA mutase in wildtype mice was investigated by RNA *in situ* hybridization. Vectors containing the *MMUT* gene or *GFP* driven by the truncated *CaMKII* (Ca2+/calmodulin-dependent protein kinase) promoter, or the ubiquitous *CBA* promoter, were pseudoserotyped with either AAV9 or PHP.eB capsids. Viral vectors were delivered by retro-orbital systemic injection at a dose of 1e11 GC/pup on day of life 1. MMA mice were compared to control littermates at 3 weeks post-treatment. **Results:** RNA *in situ* hybridization showed expression of native *Mmut* throughout the brain, including the striatum, and was 2-fold increased in the cerebellum relative to other brain regions (cerebrum, pons, hippocampus, thalamus, striatum, and midbrain). Brain sections were evaluated by both manual H-score and algorithmic positive cell counting using QuPath. Expression of GFP after treatment with reporter vectors was variable between PHP.eB and AAV9 serotypes. AAV transduction was quantified by GFP expression in select brain regions and revealed pronounced GFP expression in the choroid plexus. The neuron-specific *CaMKII* PHP.eB and AAV9 vectors enabled expression of GFP in the striatum, in cells which co-stained with the neuronal marker, NeuN. Based on favorable CNS transduction by PHP.eB and AAV9 *CaMKII*-GFP vectors, PHP.eB and AAV9 *CaMKII-MMUT*therapeutic vectors were prepared in an identical fashion and used to treat MMA mice in the neonatal period. The analysis of CNS *MMUT* expression, biodistribution and effects on mortality, weight gain, metabolites, and phenotype in the MMA mice compared to controls is in progress. **Conclusions:** *Mmut* expression was found to be widespread in the CNS of wildtype mice, including in the striatum, an area that corresponds to the basal ganglia in humans. Reporter studies with CNS-trophic AAV vectors demonstrated variable performance between capsid serotypes, with high transduction of the choroid plexus. As a key site of small molecule transport and metabolism between the brain parenchyma and the CSF, targeting the choroid plexus could, in theory, assist in reduction of toxic metabolite accumulation in the CNS. In addition, expression in the striatum, a common site of metabolic stroke in MMA patients, was effectively targeted by PHP.eB and AAV9 vectors. These results support the idea that restoring expression of *MMUT* in the CNS, either by using a vector that corrects expression in multiple tissues including the CNS, or by combining a second CNS-specific vector with a liver-directed vector, may benefit MMA patients.

689. Oligodendrocyte Dysfunction and Global CNS Dysmyelination Occur in Arginase Deficiency That is Prevented with AAV-Based Hepatic Gene Therapy

Jenna Lambert[1], Xiao-Bo Liu[1], Suhail Khoja[1], Matt Nitzahn[1], Adam Eliav[1], Steve Cederbaum[2], Gerald S. Lipshutz[1]

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In exploring the cause of the unique neuromotor manifestations in Arginase (Arg1) Deficiency, we have recently described evidence of a leukodystrophy occurring in the corticospinal tract (CST) of a murine model of hyperargininemia. This finding is also supported by imaging data from a patient with ARG1 deficiency corroborating the conclusion that dysmyelination is the likely cause of spastic diplegia in afflicted patients. In the current investigation we have now sought to understand further the abnormalities of myelination, uncover its extent and cause, and examine the durability that gene therapy may have in its prevention. Methods: Constitutive biallelic arginase 1 knockout mice (KO) die between postnatal days (P) 12 and 14. KO mice were administered IV 1x10e14 gc/kg of AAV RH10 expressing murine arginase 1 under a liver-specific promoter at P2. Untreated KO, AAV-treated KO and wild type (WT) mice were all studied. Time course of myelination of the subcortical white matter and the pyramidal tract were determined in KO and WT mice with myelination quantified by electron microscopy at P2, P6, P10, and P14 comparing untreated KOs with wild type mice; myelin-basic protein (MBP), oligodendrocyte (OL) transcription factor 2 (Olig2), and neurofilament protein expression were also examined. Treated KO and wild type mice were also studied at 4 months to assess the durability and effectiveness of single AAV administration in the neonatal period. Results: Normal myelination (WT controls) of the pyramidal tract begins at P6 with little detection of myelination at P2; myelinated axon density increases markedly by P14. While delayed, myelination in the subcortical white matter begins around P10 and increases further, albeit at reduced density, by P14 (end of study as untreated KO mice typically perish by P14). In contrast, the untreated Arg1 KO demonstrated markedly reduced myelination in the CST with almost no myelination in the subcortical white matter and little in the pyramidal tract by P14; when found, OLs appeared to be inactive. Furthermore, western blot analysis of untreated KO mice suggests the frontal cortex has decreased expression of heavy chain neurofilament, MBP, and Olig2 at P12 when compared to wild type mice of the same age. Dramatically, AAV-treated KO mice show expression of neurofilament and Olig2 at comparable levels to the WT. CLARITY tissue-clearing of P12 whole brains of Arg1 mice bred with a fluorochrome-labelled myelin proteolipid protein (PLP) mouse suggests dysmyelination is not limited to the CST but is throughout the CNS; this globalized decreased myelin expression is restored with P2

hepatic AAV gene therapy. Furthermore, myelination of the CST at P4 months in P2 treated KOs is similar to wild type controls. Conclusions: The biallelic Arg1 KO results in widespread CNS dysmyelination that is not limited to the CST. Quantitatively, the subcortical white matter and pyramidal tract are severely affected during development. Olig2, which has been shown to be a necessary regulatory of OL development, and MBP, a differentiation and maturation marker for OLs (normally increasing during development as an indicator of myelination), are both reduced in Arg1 deficiency along with neurofilament, an axonal marker. In the short term, early postnatal hepatic Arg1 gene therapy appears to restore OL function and axonal injury appears to be prevented (as suggested by recovery of near WT level of neurofilament expression). Long-term and into adulthood, normal myelinated density of the CST is maintained. These findings further support our previous studies that suggest early postnatal gene therapy is an effective treatment in preventing the CNS dysfunction in arginase deficiency.

690. Effects of Dysfunctional Sulfatide Metabolism in Schwann and Mesenchymal Stromal Cells in Metachromatic Leukodystrophy

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Metachromatic leukodystrophy (MLD) is an inherited lysosomal storage disease caused by deficient arylsulfatase A (ARSA) activity, resulting in intra-lysosomal accumulation of sulfatide. Sulfatide is integral for the proper maintenance of myelin in the central and peripheral nervous systems. The progressive loss of myelin-producing Schwann cells (SCs) due to sulfatide accumulation in MLD causes severe demyelination and neuropathy. Though previous studies have shown that sulfatide levels are directly proportional to the severity of peripheral nerve abnormalities, disease pathology at a cellular level remains poorly understood. Mitochondria and phagocytosis play important roles in cellular signaling, maintenance of homeostasis in peripheral nerve glia and in multipotent cells used in numerous cell-based therapies, such as SCs and mesenchymal stromal cells (MSCs). To delineate different cellular contributors to disease pathology, this study characterized mitochondrial morphology, cytokine secretion and phagocytic activity in SCs and MSCs isolated from *ARSA*$^{-/-}$ knock out (KO) mice. Cells were treated with different concentrations (0-25µg/ml) of sulfatide for a period of 24 hours. MitoTracker Red CMRos was used to monitor and quantify live mitochondrial morphology and transmembrane potential through fluorescence (Ex./Em. 479nm; 499nm) imaging and intensity measurements. MiNa (ImageJ tool) and MicroP (MATLAB tool) analyses of mitochondrial morphology demonstrated KO cells presented with persistent, increased ($P < 0.0001$) fragmented mitochondrial structures suggestive of prolonged mitochondrial fission within 6-hours following sulfatide exposure. Investigation of the immune response to sulfatide treatments revealed increased ($P < 0.05$) secretion of pro-inflammatory cytokines TNF-α and IL-1β in KO cells at 12- and 24-hours. No differences were noted in the cellular phagocytic capacities following sulfatide treatment. These findings suggest that mitochondrial function is impacted in MLD cells. Further characterization of *in vivo* mitochondrial function in MLD tissues can provide a clearer pathological picture.

691. Treatment of Krabbe Disease Using AAV and Bone Marrow Transplantation

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Krabbe disease is a rare lysosomal storage disorder caused by a deficiency in the enzyme galactocerebrosidase (GALC). Infantile Krabbe disease presents with symptoms within the first year of life, including irritability, muscle weakness, feeding difficulties, progressive spasticity, and death by 2 years. The current standard of care for Krabbe disease, hematopoietic stem cell transplantation (HSCT), improves outcomes for presymptomatic or minimally symptomatic patients. In spite of improved survival and cognitive outcomes, all treated children experience worsening of peripheral nerve disease. A better treatment is needed to prevent respiratory failure and death. AAV vectors have been shown to correct GALC deficiency in pre-clinical studies. Combination of HSCT and AAV gene therapy has been shown to be synergistic in improving both central and peripheral nerve disease in mice and dogs with Krabbe disease. Studies in the twitcher mouse model have shown that the combination therapy drastically extended the life-span twitcher mice from <40 days to an average of 500 days. The combined treatment corrected brain demyelination and peripheral neuropathy and improved the behavioral phenotype. These results also showed gene dose dependent effects. In a canine model of Krabbe disease, combination treatment extended the average lifespan from 20 weeks to as long as 2 years, at which point the dog that received the highest dose was euthanized per study protocol. As in the mice, the combination treatment improved both central and peripheral nerve disease. Rats in the study received a combination of BMT and one intravenous dose of AAV vector expressing human GALC cDNA, either at a low, middle, or high dose. Controls received either BMT or high dose of the vector. Of all tissues examined, changes related to AAV administration were noted in the liver, heart, and adrenal gland. Most observations were only minimal or mild in nature, and severe issues were not observed in any tissues at any timepoints. Abnormalities were most commonly observed in the AAV-only group, and the addition of BMT appeared to lessen the severity and frequency of these observations. AAV vectors can increase GALC expression in the small and large animal models, but only AAV combined with HSCT results in full correction of pathology of both CNS and PNS. The combination therapy with a reduced toxicity HSCT shows a safe profile in the pre-clinical studies which warrants advancement into human trials.

692. Haematopoietic Stem Cell Gene Therapy with IL-1Ra Prevents Cognitive Loss in Mucopolysaccharidosis IIIA Mice

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Mucopolysaccharidosis IIIA is a neuropathic paediatric lysosomal storage disease, characterised by the accumulation of heparan sulphate and other substrates in the brain. Progressive behavioural disturbances and cognitive decline are a hallmark of the disease, a possible consequence of neuroinflammation and abnormal substrate accumulation. In order to understand the potential neuroinflammatory pathways involved in disease progression we initially characterised cytokine expression in mouse models of MPSIIIA and patients with MPSIII. Both Interleukin (IL)-1β and interleukin-1 receptor antagonist (IL-1Ra) expression (amongst several other cytokines) were significantly increased in murine models of MPSIIIA as well as in human MPSIII patients, suggesting inflammasome activation. We initially hypothesised that abnormal substrates accumulated in disease may be eliciting inflammasome responses and identified pathogenic mechanisms of inflammasome activation, including that disease specific 2-O-sulphated heparan sulphate was essential for priming an IL-1β response via the toll-like receptor 4 complex. However, mucopolysaccharidosis IIIA primary and secondary storage substrates, such as amyloid beta, were both required to activate the NLRP3 inflammasome and initiate IL-1β secretion. IL-1 blockade in mucopolysaccharidosis IIIA mice using either IL-1 receptor type 1 knockout or haematopoietic stem cell gene therapy over-expressing IL-1Ra, reduced gliosis and completely prevented behavioural phenotypes. In conclusion, we demonstrate that IL-1 drives neuroinflammation, behavioural abnormality and cognitive decline in mucopolysaccharidosis IIIA, highlighting haematopoietic stem cell gene therapy treatment with IL-1Ra as a potential neuropathic lysosomal disease treatment.

693. Combinatorial Knock-Down and Knock-Out Strategies to Eliminate Persisting Hepatitis B Virus cccDNA and Hepatitis D Virus RNA

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Over 257 million people worldwide are chronically infected with hepatitis B virus (HBV) leading to the death of several hundred thousand people each year, caused by the development of liver cirrhosis and hepatocellular carcinoma. Deposition of HBV covalently closed circular DNA (cccDNA) is responsible for viral persistence and rebound after cessation of antiviral therapy. In addition, the excessive secretion of HBV antigens and their prolonged exposure to the host immune system cause functional alteration and depletion of HBV-specific B and T cells, thus contributing to HBV persistence. The co- or superinfection with hepatitis D virus (HDV), a satellite virus of HBV that is dependent on the HBV surface antigen (HBsAg), accounts for the most severe progression of viral induced liver disease. Although a vaccination for HBV is available that is also effective against HDV, some individuals are non-responders and unable to develop anti-HBV antibodies. Moreover, there is no curative treatment available for HBV and HBV/HDV co-infections, and cessation of conventional interferon- and nucleos(t)ide analogues-based therapy is often associated with viral relapse and rebound of the disease. Therefore, and given the global burden of viral hepatitis, new curative therapy approaches are urgently needed, in particular for hepatitis B and D co-infection. To this end, we developed a new generation of recombinant Adeno-associated viral (rAAV) vectors that enable the combination of the powerful CRISPR/Cas9 and RNAi technologies. First, we investigated several approaches to co-express small RNAs from AAV vectors in the same target cell, including i) the separate production and subsequent mixing of each vector encoding a single small RNA, ii) their pooled production in the same dish or iii) the multiplexing of several expression cassettes on a single AAV vector. We show that the latter approach, named TRISPR, clearly outperforms the other two when three single guide (sg)RNAs were multiplexed to knock-out HBsAg. Accordingly, we further engineered TRISPR to co-express sgRNAs and shRNAs against HBV and show that only the combination of RNAi and CRISPR/Cas9 can reduce HBV infection to undetectable levels in HepG2-NTCP cells. Next, we employed our TRISPR system to direct the RNAi machinery against the HDV RNA genome and CRISPR/Cas9 against HBV cccDNA. In a complex immunization and curation setting, we show that the juxtaposition of both technologies reduces both infections to background levels in HepG2-hNTCP cells with integrated HBsAg. To our knowledge, this provides the first evidence that CRISPR/Cas9 and RNAi can be combined to target HBV and HDV co-infections. Taken together, our study exemplifies the successful combination of very powerful tools for gene therapy—rAAV vectors, RNAi and CRISPR/Cas9—and implies its great potential for treatment of numerous diseases that have a DNA and/or RNA component. To facilitate the broader use of the TRISPR toolbox, we provide a set of standardized cloning plasmids and a step-by-step manual for multiplexing of sgRNAs, compatible with Cas9 from *Streptococcus pyogenes* and *Staphylococcus aureus*, and/or short-hairpin (sh) RNAs in a single AAV vector. Finally, our results inform and encourage future *in vivo* studies in HBV or HBV/HDV animal models, to test the effect of our AAV-based combinatorial knock-down and knock-out strategy on the host immune response and the possible synergistic impact on viral clearance.

694. AAV-Mediated Gene Therapy for Glycogen Storage Disease Type III Using a Bacterial Glycogen Debranching Enzyme

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Background: Deficiency of glycogen debranching enzyme (GDE) causes glycogen storage disease III (GSD III) resulting in progressive accumulation of abnormal glycogen (limit dextrin) in the liver, heart, and skeletal muscles. Most patients have both muscle and liver involvements (type IIIa) while others have the disease limited to the liver (type IIIb). Currently, there is no curative treatment for this disease. Gene therapy with adeno-associated virus (AAV) vector provides an optimal treatment approach for GSD III. However, the 4.6-kb human GDE cDNA cannot be packaged into a single AAV vector due to its small carrying capacity. Previously, we demonstrated the ability of AAV-mediated expression of a smaller bacterial GDE, Pullulanase, to reduce glycogen storage in the liver of GSD IIIa mice. In this study, we tested the efficacy of this treatment approach in GSD IIIa mice. **Methods**: AAV vectors containing a 2.2-kb codon-optimized coding sequence of Pullulanase under the control of the CMV enhancer/chicken β-actin promoter (AAV-CB-Pull) and a liver-specific promoter (AAV-LSP-Pull) were packaged into AAV9 and AAV8, respectively. To minimize transgene-induced cytotoxic T cell response, two-week-old infant GSD IIIa mice (n=15) were intravenously injected with the AAV9-CB-Pull at a dose of 1×10^{13} vg/kg. After 10 weeks, eight mice were sacrificed to collect tissues and blood. The remaining seven mice were subsequently treated with the same dose of the AAV8-LSP-Pull for another 10 weeks to correct liver abnormalities. Age-matched untreated GSD IIIa mice were used as controls. AAV vector biodistribution, Pullulanase activity, and glycogen content were analyzed in tissues. Skeletal muscle functional tests including treadmill, inverted grid hang, and Rota-rod were performed during the periods of treatment. **Results**: Ten weeks after the AAV9-CB-Pull (CB) treatment, AAV copy numbers were high in the heart (8.69±2.22 vg/genome) and low in the quadriceps (0.68±0.15) and liver (0.40±0.32). Consistent with this result, Pullulanase enzyme activity was profoundly high in the heart (33.77±8.51 mU/mg), readily detectable in the quadriceps (6.64±2.11 mU/mg), but undetectable in the liver (Figure 1A). Glycogen content was markedly reduced in the heart (-80%) and skeletal muscle (quadriceps, -80%), but remained unchanged in the liver, accompanied by the significant improvement of muscle functions. Periodic acid-Schiff (PAS)-staining of tissue sections confirmed the clearance of glycogen accumulation in muscle tissues (Figure 1B). The lack of efficacy in the liver by the AAV9-CB-Pull treatment is likely a result of vector dilution caused by the rapid growth of the liver from infant to adult. Additional treatment with the AAV8-LSP-Pull vector (CB+LSP) reduced liver glycogen content by 75%, normalized liver size and plasma alanine aminotransferase (ALT) activity, and reversed hepatic fibrosis (Figure 1C) while maintaining the effect of AAV9-CB-Pull treatment on the heart and skeletal muscle. **Summary:** Our data suggest that early treatment with an AAV9 vector ubiquitously expressing Pullulanase followed by the additional treatment with a liver-restricted AAV8 vector is a feasible treatment approach for infant patients with GSD IIIa. To our knowledge, this is the first report of gene therapy to replace the defective human gene with a microbial analog.

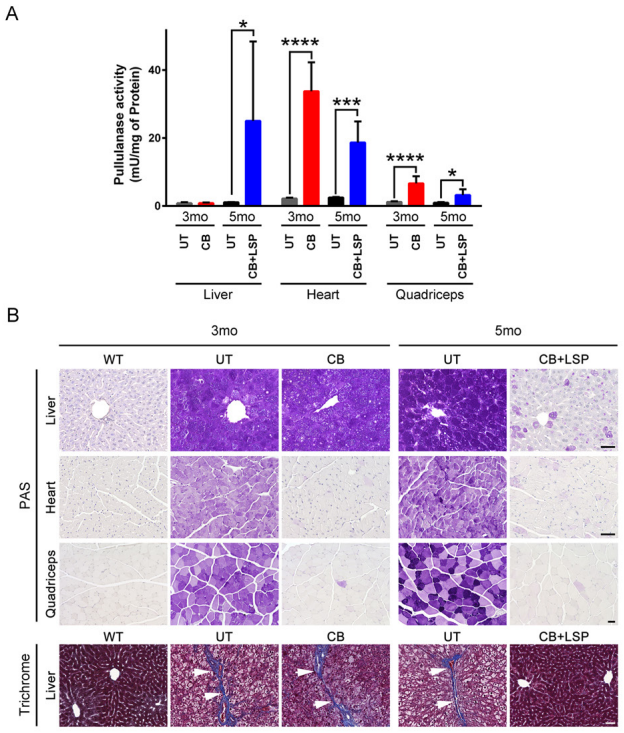

Figure 1. Pullulanase reduced glycogen accumulation in the liver and muscles and reversed liver fibrosis in GSD IIIa mice. (A) Pullulanase activities were significantly elevated in the heart and quadriceps of both CB and CB+LSP treated mice compared to UT. The enzyme activity was not detectable in the CB treated liver but drastically increased in the CB+LSP treated liver. The graph represents the mean ± SD. UT (n=5), CB (n=8), and CB+LSP (n=7); Student's test. *p<0.05, ***p<0.001, and ****p<0.0001. (B) Periodic acid–Schiff (PAS) staining was used for detection of glycogen accumulation in tissues. Untreated GSD IIIa mice showed intense PAS-positive glycogen accumulation (purple) in the liver, heart, and skeletal muscle. Glycogen buildup was profoundly cleared in the heart and skeletal muscle by both CB and CB+LSP treatments. CB treatment had no effect on liver glycogen accumulation; in contrast, CB+LSP treatment markedly reduced glycogen accumulation in the liver. Trichrome staining was used for detection of liver fibrosis. The blue staining (arrows) indicates the presence of fibrotic tissues in the liver of UT and CB treated GSD IIIa mice. CB+LSP treatment successfully reversed liver fibrosis. At least 3 mice were examined in each group, and representative images are shown. Scale bar=50 μm.

695. Macrophage Tunneling Nanotube-Mediated HSPC Transplantation Therapy for the Lysosomal Storage Disorder Cystinosis Remains Effective in Preclinical Mouse Study Following Shpk Elimination

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Cystinosis is a lysosomal storage disorder caused by loss-of-function mutations in the CTNS gene. CTNS encodes the ubiquitously expressed lysosomal transporter cystinosin, whose functional absence leads to cystine accumulation and crystallization. Symptoms progressively manifest due to damage of the kidneys, thyroid, cornea, muscle and neurological systems and ultimately cystinosis leads to multi-organ failure and lethality. Current treatment can slow but not halt disease progression, so our group has developed a new investigational therapy based on transplantation of hematopoietic stem and progenitor cells (HSPCs). In mice, a single transplantation of heathy HSPCs into irradiated Ctns-/- recipients prevents tissue degeneration and restores biochemical function via delivery of functional cysitnosin-bearing lysosomes from HSPC-derived macrophages to diseased cells through tunneling nanotubes (TNTs). A Phase I/II clinical trial investigating the use of autologous transplantation of patients' HSPCs ex vivo transduced

with functional CTNS is currently being conducted at UC San Diego. The most common mutation in cystinosis is a large 57-kb deletion eliminating not only the CTNS gene but also the neighboring SHPK (a.k.a. CARKL) locus. SHPK influences macrophage polarization and differentiation through regulation of glucose metabolism. Due to the central role of macrophages in HSPC therapy for cystinosis, and the fact that roughly 40% of all human cystinosis patients carry the 57-kb deletion at the homozygote state, we investigated if Shpk expression was necessary for effective transplantation therapy. Such studies will indicate if patients without SHPK are likely to benefit from ex vivo replacement of CTNS alone. We generated the first two Shpk knockout (KO) models by introducing genomic deletions using CRISPR-Cas9. We confirmed elimination of Shpk expression at the mRNA and protein levels across multiple tissues, and also observed perturbations in the Pentose Phosphate Pathway, the metabolic shunt regulated by Shpk. Shpk-/- HSPCs along with WT and Ctns-/- controls were then transplanted into lethally irradiated 2-month-old Ctns-/- mice, and analyzed 6 months post-transplantation. Shpk-/- HSPC recipients showed significant reduction in tissue cystine content, restoration of Ctns expression and improvements in renal function comparable to WT HSPC recipients, demonstrating that in vivo macrophage-mediated transplantation therapy remains effective despite elimination of Shpk in donor HSPCs. Furthermore, we investigated the phenotype of in vitro bone marrow derived macrophages (BMDMs) isolated Shpk-/- and note that despite loss of Shpk mRNA expression, they normally form TNTs in a co-culture system with cystinotic fibroblasts. These data strongly suggest that cystinosis patients lacking Shpk will benefit from ex vivo gene therapy that restores only CTNS expression and can be included in the ongoing clinical trial.

696. Comparing the Efficacy of Constitutive vs Liver-Targeted AAV8-*MMUT* Gene Therapy in a Knock-In Mouse Model of Methylmalonic Acidemia

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Methylmalonic acidemia (MMA) is caused by pathogenic variants in the gene coding for the mitochondrial enzyme methylmalonyl-CoA mutase (*MMUT*) and results in multi-organ complications including chronic kidney disease. Patients with variants resulting in partial enzymatic deficiency (mut^-) have a milder phenotype than those with little to no enzyme activity (mut^0). Currently, liver transplantation is employed for the fragile mut^0 patients, but, despite improved metabolic control and quality of life, patients remain at risk for kidney disease and other multisystem complications. In this study, we sought to explore clinical, metabolic, and biomarker responses to AAV gene therapy in a transgene-free MMA mouse model. Given that full knock-out murine models of MMA display early neonatal lethality, we created a CRISPR-edited knock-in mouse to recapitulate a known mut^- enzymatic variant common in individuals of African descent (*Mmut*: p.Gly715Val). $Mmut^{G715V/G715V}$ mice manifest a milder MMA phenotype, are growth impaired, display elevated levels of plasma methylmalonic acid, and exhibit weight loss/growth failure when exposed to a high protein diet. These mice, like MMA patients, also have significant elevations of plasma fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15), biomarkers of hepatic and muscle mitochondrial dysfunction, respectively. Our previous mouse studies showed superior efficacy of the constitutive (AAV8-CBA-*Mmut*) vs liver-targeted vector (AAV8-TBG-*Mmut*), but with high genotoxicity attributed to the strong promoter-enhancer combinations. In this study, we aimed to compare AAV8 vectors expressing *MMUT* under the control of a truncated EF1α (AAV8-EF1α-MMUT-HPRE) or a liver-targeted hAAT (AAV8-hAAT-MMUT) promoter. At 2-3 months of age, AAV8 vectors were delivered by retro-orbital injection into homozygous mice ($Mmut^{G715V/G715V}$) at a dose of 5×10^{12}VG/kg. Survival, growth, metabolites (methylmalonic acid), and biomarkers (FGF21 and GDF15) were monitored after gene therapy, and after a dietary high protein challenge. As expected, both vectors produced significant therapeutic effects in the treated MMA mice. Twelve days post-injection, plasma methylmalonic acid levels decreased from 906.8±295.6 μM and 808.4±126.3 μM to 196.8±42.99 μM and 203±76.86 μM in EF1α-MMUT-HPRE and AAV8-hAAT-MMUT treated mutants respectively ($p<0.0001$ for both). Levels remained stable in untreated mutant controls, averaging 947±87.19 μM. The biomarker response was more distinct, with only EF1α-MMUT-HPRE treated mutants exhibiting decreased levels of FGF21 and GDF15 ($p<0.0001$ and $p=0.006$, respectively). After three weeks, the same groups had a 34% ($p=0.0027$) or 27% ($p=0.03$) increase in weights, while the untreated mutants showed no change. When challenged with high protein chow for 18 days, the untreated mutants lost 23% ($p=0.009$) of their starting weight while EF1α-MMUT-HPRE and hAAT-MMUT treated mutants maintained a 15% ($p=0.002$) and 10% ($p=0.0098$) weight gain from baseline. Methylmalonic acid levels increased under dietary stress in both EF1α-MMUT-HPRE (479.6±59.75 μM) and hAAT-MMUT (489.4±89.35 μM) treated mutants but remained lower than untreated mutants. Furthermore, EF1α-MMUT-HPRE, but not hAAT-MMUT, treated mutants sustained low levels of GDF15 ($p=0.0002$, compared to baseline) after 18 days of dietary stress. In this mouse model, both AAV vectors protected the mice after a high protein challenge with no apparent difference in clinical outcomes. At early points, however, only the constitutively expressed vector uniformly reduced non-metabolic biomarkers. Long term follow-up of these mice will allow further evaluation of the effects on other organs, especially the kidney, with a parallel longitudinal survey of biomarkers.

697. Circadian-Triggered Gene Therapy for Hypoparathyroidism: A Step Toward Physiological Gene Switches

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As the horizons of possibility continue to expand in gene therapeutics, developing a gene therapy for secretion of a therapeutic protein or hormone becomes more plausible. Here, we show that exogenous cell types can be engineered to secrete functional and potent PTH (parathyroid hormone) as a hypoparathyroidism therapeutic, with the potential for regulated release based on physiological cues. Hypoparathyroidism is a rare endocrine disorder resulting in PTH,

calcium and vitamin D deficiencies. The current FDA-approved treatment, recombinant hormone therapy, does not alleviate the need for additional mineral and vitamin supplementation. In addition to having a very short-half life, as most recombinant proteins do, this treatment results in excessively high PTH levels and risk for osteosarcoma. A therapy which could deliver the native form of the peptide with the correct dosing and timing could drastically reduce the risk of side effects, while improving drug efficacy. Using AAV vectors, we demonstrate secretion of functional PTH from liver cells *in vitro* and detection of hPTH in the serum from mice injected with PTH-encoding AAV vectors. A complicating factor in this approach is the dynamic effect of PTH on bone metabolism. Endogenously, serum PTH levels follow a circadian pattern, which when disrupted can cause catabolic bone metabolism and receptor desensitization. We are developing a novel type of gene therapeutic in which PTH expression and release is guided by the patient's individual body clock through the direction of DNA response elements, resulting in a secretion pattern that matches natural circadian rhythms. To ensure this PTH gene therapy is a robust improvement, we utilized osteoblast assays to study the effects of PTH on bone metabolism. Initial studies show that the PTH gene therapy can elicit the same proliferative response in osteoblasts as recombinant hormone *in vitro*, as well as causing an expected spike in intracellular cAMP. Experiments to assess efficiency and toxicity in disease model mice are ongoing. PTH could represent the first of many peptide hormones to be delivered via a physiologically-triggered gene therapy.

698. rAAV Vector Encoding a Novel Engineered Human PAH Shows Improved Efficacy Over Endogenous Human PAH in the PAH^{enu2}Mouse Model for Phenylketonuria

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Phenylketonuria (PKU) is a genetic deficiency of human phenylalanine hydroxylase (hPAH) in liver and results in elevated levels of phenylalanine (Phe) in brain with subsequent neurotoxicity. Various strategies to reduce blood Phe levels are currently being tested in preclinical research and in the clinic. Among these, recombinant adeno-associated virus (rAAV) based gene therapy to correct the liver defect has now entered into the clinic and could provide a truly transformative long-term stable Phe control to the patients. To improve on the efficacy of rAAV-based gene therapy for PKU we have optimized the expression cassette to include a highly active liver promoter expressing an engineered form of hPAH. The codon-optimization increased PAH protein production 7-fold. The engineered hPAH variant-1 (V1) with four amino acid modifications was designed to increase the stability of the endogenous PAH. When tested by transient transfection into human Huh7 cells, hPAH-V1 provided 10-fold higher levels of PAH protein and activity as compared with unmodified hPAH. We then generated rAAV8 vectors with the optimized liver promoter for both the hPAH and hPAH-V1. Vector evaluation in PAH^{enu2} mice demonstrated that hPAH-V1 significantly reduced Phe levels in blood and brain after a single vector administration while little efficacy was seen with a vector encoding the unmodified hPAH dosed at comparably. Similarly, significant improvement in brain neurotransmitters dopamine and serotonin levels was achieved with hPAH-V1, while similar levels of brain amino acids tyrosine and tryptophan were observed for both treatment groups. In the liver, 10-fold higher levels of hPAH-V1 protein were detected and correlated to a comparable 10-fold increase in PAH activity compared to that obtained with hPAH. Liver PAH analysis by Western blot demonstrated significantly less breakdown of the hPAH-V1 suggesting enhanced in vivo stability of the variant as a potential mechanism for the improved efficacy. The rAAV vectors were subsequently evaluated in NHPs and demonstrated improved detection of hPAH-V1 over endogenous hPAH in NHP liver two weeks after comparable vector dosing. Taken together, our results demonstrate that the novel PAH-V1 can improve the potency of rAAV vectors and hence is likely to provide therapeutic efficacy in PKU patients with clinically feasible and safe vector doses.

699. Dose Response of *scAAV9-HexM* Gene Transfer in a Mouse Model of Sandhoff Disease

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Sandhoff disease (SD) belongs to a group of neurodegenerative diseases known collectively as GM2 gangliosidoses, which result from the excessive accumulation of GM2 gangliosides in the lysosomes of neuronal cells. Typically, these lipids are hydrolyzed by an enzyme, β-hexosaminidase A (Hex A), a heterodimer comprised of an α- and a β-subunit. Mutations in the genes encoding either of the subunits for Hex A can lead to improper functioning of the enzyme and the subsequent accumulation of GM2 gangliosides. SD is caused by a mutation in the *HEXB* gene. Ganglioside accumulation ultimately results in widespread cell death, and consequently, progressive symptoms and rapid neurological decline which culminate in death at approximately age 4 in the most prevalent, infantile, form. A homodimer formed by a hybrid μ-subunit called HexM, an isoenzyme to Hex A, has been recently developed to effectively hydrolyze GM2 gangliosides *in vivo*. Previous studies have determined the effectiveness of gene transfer with the gene, *HEXM,* packaged within a self-complementary adeno-associated viral vector, serotype 9 (scAAV9), through increased life span in a SD mouse model (*Hexb*$^{(-/-)}$). This study aims to determine the dose response of the *scAAV9-HEXM* treatment in the SD mouse model through a combination of treatment delivery via the intra-cisterna magna (ICM) and intravenous (IV) route, along

with the ancillary daily administration of immunosuppressant drugs, rapamycin and prednisone. Treatment for 10 long-term cohorts of 8 mice involves a combination of either vehicle, or a low, medium, or high dose of the *scAAV9-HEXM* vector delivered ICM and IV. Mice are randomly assigned to each cohort. The researcher conducting the procedure and subsequent testing is blinded to the dosage each animal is receiving. Biweekly behavioural testing and monthly blood collections are done until mice reach their humane endpoint as determined by specific UACC criteria. An additional 4 short-term cohorts of 4 mice reach their humane endpoint at 16 weeks of age. At termination, blood, gross organs, brain, and spinal cord are collected for analysis of GM2 ganglioside accumulation, vector copy number, enzyme activity, cellular and humoral immune response, rapamycin level and histology. It is hypothesized that mice administered the *scAAV9-HEXM* vector via both ICM and IV routes will have lower levels of accumulated GM2 gangliosides and higher HexM enzyme activity in the central nervous system, compared to mice administered with vector only through the IV route at the same total dose. The primary measure of efficacy for this study is length of survival. The short-term cohort data will be presented.

700. Functional Benefits of Systemic rAAV9-h*SGSH* Gene Delivery for Treating Mucopolysaccharidosis (MPS) IIIA

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Mucopolysaccharidosis (MPS) IIIA is a neuropathic lysosomal storage disease caused by autosomal recessive defects in *N*-Sulfoglucosamine Sulfohydrolase (SGSH), leading multisystem disorders with profound neurological impairments and high mortality. There is unmet medical needs for MPS IIIA and no treatment is currently available. Systemic gene delivery targeting the root causes using the trans-BBB-neurotropic AAV9 vector platform have been demonstrated to be effective and safe for treating neurogenetic diseases. In this study, we developed a new self-complementary (sc) AAV9 vector using a truncated miniature CMV (mCMV) promoter to drive the expression of human *SGSH* cDNA. The scAAV9-h*SGSH* vector was tested in MPS IIIA mice via an IV injection. MPS IIIA mice were treated with the vector at age 1 month, 3 months or 6 months. The vector treatments led to rapid and persistent SGSH expression, the clearance of lysosomal storage pathology throughout the CNS, PNS and periphery tissues, significant behavior improvement, and extension of survival (ongoing) in all treated MPS IIIA mice, indicating the prevention and reversal of the disease progression. Notably, 2e12vg/kg was identified as the minimal effective clinical dose. Furthermore, toxicology and biodistribution testing showed a safe profile. Our data demonstrate that this new scAAV9-h*SGSH* product via systemic delivery is safe and effective, with substantial functional benefits for treating MPS IIIA at early and advanced disease stages, supporting the clinical potential.

701. Adeno-Associated Viral Gene Therapy Rescues the Maple Syrup Urine Disease Phenotype in a Mouse Model

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Maple syrup urine disease (MSUD) is a rare, inherited metabolic disorder characterized by the dysfunction of the mitochondrial enzyme complex branched-chain alpha-keto acid dehydrogenase (BCKDH). BCKDH catabolizes branched-chain amino acids (BCAAs). Without BCKDH, BCAAs and their neurotoxic alpha-keto intermediates can build up in the blood and tissues; the distinctive sweet odor of MSUD patients' urine due to high levels of alpha-keto acids underlie this disease's name. There is currently no cure for MSUD and treatment is limited to a restrictive diet and liver transplantation. These limited approaches highlight the need to develop novel treatments for MSUD. We evaluated potential gene therapy applications for MSUD by utilizing the intermediate MSUD (iMSUD) mouse model, which harbors a mutation in the DBT subunit of BCKDH. Systemically delivering an AAV vector to the liver that expressed DBT from the liver-specific TBG promoter did not sufficiently ameliorate all aspects of the disease phenotype in the iMSUD mouse. A similar effect occurred when we used the ubiquitous CB7 promoter for expression following intravenous vector administration. These findings underscored the need for an alternative approach. In primates, muscle is a larger source of BCAA metabolism than liver. However, when we deployed a muscle-specific approach using the tMCK promoter for expression of DBT following intramuscular (IM) administration, this strategy only partially rescued the MSUD phenotype. When we combined the ubiquitous CB7 promoter with vector delivery by IM injection, survival drastically increased across all assessed doses. Additionally, serum BCAA levels remained near normal levels in the mid- and high-dose cohorts throughout the study duration. This gene therapy approach also protected iMSUD mice from lethal high protein diet challenge. Therefore, administering a gene therapy vector that expresses in both the muscle and liver may be a viable alternative treatment path for patients with MSUD.

702. Correction of Hyperglycemia Through Adenoviral Mediated Compartmentalized Liver Transduction of the Human Insulin Gene in a Type 1 Diabetes Mellitus Pig Model

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Type 1 diabetes mellitus (T1DM) is a complex metabolic disease characterized by elevated blood glucose levels caused by the autoimmune destruction of insulin producing pancreatic β-cells. Lifelong daily monitoring of blood glucose levels and administration of precise doses of exogenous insulin, with the goal of controlling glycemic fluctuations and therefore prevention of life-threatening complications, remains the cornerstone of T1DM management and a colossal burden for patients and physicians. Gene therapy strategies aim at ameliorating glycemic fluctuations by achieving the secretion of physiologic levels of insulin from non-pancreatic-β-cells. Using streptozotocin to induce T1DM in pigs, our group assessed the glycemic effect exerted by the secretion of human insulin dictated by an expression cassette driven by a carbohydrate response element (CRE) carried by E1-E3 deleted type 5 adenovirus and delivered through compartmentalized liver transduction (CLT). CLT is a novel mode of hepatic vector administration achieved by the intra-parenchymal injection of the vector into a blood flow isolated portion of the liver. At a recombinant viral vector dose of $2x10^{10}$ IFUs/kg ($2x10^{12}$ VPs/kg), fasting and 2h postprandial normoglycemia was achieved and insulin levels were restored and followed for three and a half years in 88-107kg male pigs (n=2). Glucose response curves depicted a return to normoglycemic levels 7h post glucose challenge. Additionally, insulin levels measured during glucose response curves depict an insulin secretion pattern consistent with a transgene carbohydrate response and not to an endogenous insulin pulse post glucose challenge. Our results further demonstrate that CLT is a safe and effective mode of vector delivery in large animal models. Finally, our results demonstrate that glycemic fluctuations are manageable through gene therapy strategies and support further preclinical investigation of CLT for the treatment of T1DM.

703. Use of Adenine Base Editors to Precisely Correct the Disease-Causing PiZ Mutation in Alpha-1 Antitrypsine Deficiency

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Alpha-1 Antitrypsin Deficiency (A1AD) is a rare genetic disease most commonly caused by a G>A transition mutation within the SERPINA1 gene referred to as the PiZ allele. The resulting PiZ protein containing a single amino acid substitute E342K is prone to polymerization within hepatocytes leading to fibrosis and cirrhosis in a subset of patients. The impaired secretion of alpha-1 antitrypsin (A1AT), an inhibitor of neutrophil elastase, leads to loss of lung elastin and progressive pulmonary disease. Adenine base editors (ABEs) enable the programmable conversion of A:T to G:C base pairs and in principle could be used to precisely correct the pathogenic PiZ mutation. After significant protein engineering of both the Cas9 and TadA deaminase domains that constitute ABE, we identified editors that yield high levels of editing in patient-derived cells. Chemical modification of the guide RNA (gRNA) led to further increases in base editing efficiencies. Although the predominant gene editing outcome was correction to a wild-type gene, we also observed editing of neighboring bystander adenines. The second most common allele contained two A>G edits simultaneously correcting the PiZ mutation and introducing a novel amino acid substitution D341G. We found the D341G protein to behave similarly to wild-type A1AT in cellular secretion assays and in elastase inhibition assays of purified proteins. Consequently, we postulate that both wild-type A1AT and D341G A1AT are beneficial alleles. We delivered optimized gRNA and mRNA encoding ABE using lipid nanoparticle formulations, to the livers of PiZ transgenic mice. In total liver genomic DNA, we observed considerable levels of beneficial editing. The percentage of beneficial alleles was correlated with a significant increase in serum A1AT concentration and a concurrent increase in serum elastase inhibitory capacity. These data indicate that base editing is a feasible approach for the treatment of A1AD lung and liver disease.

Neurologic Diseases

704. Minicep290 Gene Replacement Therapy: A Mutation-Independent Approach to Treat Cep290-Leber Congenital Amaurosis (LCA10)

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Leber congenital amaurosis (LCA) is a debilitating childhood blindness disorder and is considered one of the most severe forms of retinal degeneration. Mutations in *CEP290* (LCA10) account for > 26% of all LCA cases and are the most frequent cause of LCA. Adeno-associated viral (AAV) vectors are currently the most efficient vectors for gene delivery to the retina. However, the development of a gene therapy for LCA10 has been challenging because the size of the CEP290 gene is too large to be packaged into conventional AAV vectors. Mutation specific anti-sense oligo and gene editing therapies have been previously reported. Our goal is to design a mutation-independent gene therapy delivered with an AAV vector to treat LCA10. We designed shorter versions of CEP290 (miniCEP290) that are functional and can be delivered into the subretinal space using AAV vectors. We previously demonstrated that a CEP290 minigene [CEP290 amino acid 580-1180 domain] under the control of a ubiquitous promoter improved the function and survival of photoreceptors when delivered in neonatal *Cep290*-mutant mice (*Cep290*^{rd16}). However, the effect was short-lived with degeneration ensuing after 5 weeks of age. This current study was undertaken to further improve the efficacy of the miniCEP290 approach and optimize protein expression in the mutant mice. We show that the expression of CEP290-580-1180 under the control of the photoreceptor-specific rhodopsin kinase promoter improved the electroretinogram (ERG) response for both rod and cone photoreceptors by ~1.5 folds. Further, modification of the miniCEP290 gene construct by adding in different CEP290 domains improved

the photoreceptor structural and functional rescue by ~500% when delivered at postnatal day 10 in the *Cep290*^{rd16}mice. We also show that the expression of the new miniCEP290 prolonged the survival and improved the protein trafficking defects in the photoreceptors in the *Cep290*^{rd16} mice. Further studies are currently underway to optimize the expression of the identified lead miniCEP290 using different promoter combinations to potentially extend the treatment window and improve the efficacy. Our preliminary results indicate that miniCEP290 approach with conventional AAV vectors may have the potential for mutation-independent gene therapy in LCA10 patients. These studies may also pave the way to develop new minigene therapies for retinal degenerative diseases caused by mutations in other large genes including Stargardt disease and Usher Syndrome, which are not currently amenable to treatment using conventional AAVs.

705. Development and Characterization of a CRISPR/Cas9-Based Gene Therapy for Early-Onset Familial Forms of Parkinson's Disease

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The CRISPR/Cas9 gene editing tool allows selective disruption of mutated alleles as a novel therapeutic approach for CNS disorders. Previous work in our group focused on CRISPR/Cas9 against amyloid precursor protein (*APP*) mutations, which cause early-onset forms of Alzheimer's Disease. After the successful disruption of the *Swedish APP* mutation and the concomitant decrease in amyloid β secretion, we propose to adopt the same strategy for a familial form of Parkinson´s Disease (PD), caused by the *A53T* mutation in the α-synuclein gene (*SNCA*). To achieve this we have transfected human fibroblasts from patients carrying this mutation with plasmids encoding different Cas9 variants and guide RNAs, designed to disrupt the mutated site. The formation of insertions and deletions (indels) is being validated via Sanger sequencing. Previously, it has been reported that familial forms of PD, including the *SNCA A53T* mutation, may cause alterations in mitochondrial respiration and cellular ATP production. Thus, in ongoing studies we are characterizing cellular bioenergetics including fibroblast proliferation, respiration, ATP production and mitochondrial morphology and assessing how the disruption of the mutated α-synuclein allele is influencing these parameters. Bearing in mind functional differences between fibroblasts and neurons further model developments could include direct differentiation of fibroblasts into neurons and even more precise genetic alterations of the mutated site, such as prime editing.

706. OXB-203, a Lentiviral Vector Expressing Aflibercept as a Single Dose, Long-Term Treatment for Wet Age-Related Macular Degeneration

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Wet age-related macular degeneration (wet AMD) is a leading cause of blindness in the aging population. It is caused by abnormal blood vessel leakage leading to progressive degeneration of the central retina. Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that is known to have a key role in the development of wet AMD. The current standard of care for wet AMD consists of anti VEGF therapeutic strategies and includes aflibercept, ranibizumab and bevacizumab. A drawback of these treatments is that in many patients they are only effective for 1-2 months and as a result require frequent re-administration via intravitreal injection. This is associated with side effects which include inflammation, haemorrhage and retinal detachment. Therefore, there is a need for the development of new therapies to circumvent these side-effects and obviate the need for repeat administration. We have developed an alternative treatment that avoids the complications, discomfort and healthcare burden associated with repeat injections in which a Lentiviral vector is engineered to express the anti-VEGF protein, aflibercept directly in the eye following a single subretinal injection - designated OXB-203. Clinical data from OXB-201 (RetinoStat), our first-generation treatment for wet AMD (Lentiviral vector expressing endostatin and angiostatin) program (Campochiaro *et al*, 2017) demonstrated that following a single subretinal injection of the vector in patients, there was stable long term expression of the transgenes to 6 years (longest timepoint assessed) providing proof-of-principle for this type of single-injection gene therapy approach. OXB-203 is our second-generation wet AMD programme. Evaluation of OXB-203- produced aflibercept detected via anti-VEGF ELISAs and binding analysis demonstrated comparable binding characteristics to that of recombinant aflibercept. *In vitro* angiogenesis assays comparing OXB-203 derived and recombinant aflibercept demonstrated that both cell proliferation and tubule formation were significantly inhibited to a comparable extent. Additionally, in a preclinical study using a rat choroidal neovascularization (CNV) model of wet AMD, a single subretinal administration of OXB-203 vector demonstrated excellent expression of aflibercept in the vitreous and reduction in CNV lesion sizes comparable to recombinant aflibercept delivered intravitreally. Our data indicates that a single dose of OXB-203 is an effective anti-neovascularisation gene therapy product for wet AMD.

707. Retinal Degeneration in Mice Lacking Cyclic Nucleotide-Gated Channel Subunit CNGA1

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Purpose: Cyclic nucleotide-gated (CNG) channels are important mediators in the transduction pathways of rod and cone photoreceptors.

Native CNG channels are heterotetramers composed of homologous A and B subunits. To develop a mouse model that resembles human retinitis pigmentosa, we deleted the *CNGA1* gene in mice and investigated the course of retinal degeneration. Methods: We used CRISPR/Cas 9 technology to obtain *CNGA1*$^{-/-}$ mice and validated the genotype via PCR. And we performed ophthalmic examinations including fundus photograph, optical coherence tomography (OCT) and electroretinogram (ERG) to observe the morphological and functional change at different time points after birth. Immunofluorescence and TUNEL staining were performed to investigate the degeneration of retinal structure and detect the apoptosis of neuroretinal cells. Results: In the absence of CNGA1, retinal thickness of *CNGA1*$^{-/-}$ mice decreased gradually and reduced by half at 7 weeks old. But *CNGA1*$^{-/-}$ mice failed to respond to light and ERG showed no rod-mediated responses as early as 3 weeks. The rods showed a rapid progressing degeneration caused by apoptotic death and concurred by retinal gliosis. Apoptosis of rod cells showed significant difference from WT mice as early as 10 days. Cones were primarily unaffected and showed normal ERG responses up to 6 weeks, but they also started to degenerate in later stage. Conclusion: CNGA1 is a crucial determinant of native CNG channels. As a result of the lack of rod CNG channels, *CNGA1*$^{-/-}$ mice develop a rapid progressing retinal degeneration that resembles human retinitis pigmentosa, which can further serve as a favourable tool for gene therapy attempts.

708. Herpes Simplex Virus (HSV) Vector-Mediated Expression of Protein Phosphatase 1a (PP1a) Reduces Pain Behaviors in Rat Models of Interstitial Cystitis/Bladder (IC/BPS) Pain Syndrome and Post-Herpetic Neuralgia (PHN)

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We previously developed a screen for genes from a PC12 cell-derived cDNA library that could negatively regulate the vanilloid receptor TRPV1, a gene that is upregulated during various forms of neuropathic and inflammatory pain syndromes that represents an important drug target for many standard and novel pain therapeutics. Our selection system identified the protein phosphatase 1a gene (PP1a), a serine/threonine cellular phosphatase, that mimicked the activity of the poreless (PL) dominant-negative mutant of TRPV1, in capsaicin-induced calcium imaging studies. When PP1a was ectopically expressed from replication-defective HSV (rdHSV) vectors in rat dorsal route ganglia (DRG) neurons following footpad vector injection, PP1a was able to ameliorate capsaicin- and heat-induced thermal allodynia but had no effect on cold allodynia (TRPM8) or formalin (TRPA1) induced behavioral responses reaffirming the hypothesis that PP1a was acting via the heat- and capsaicin-sensitive vanilloid receptor (TRPV1). To determine whether this therapeutic vector expressing PP1a had more widespread applicability to relieve chronic pain in clinically relevant pain models, we evaluated the PP1a vector in both a rat model of interstitial cystitis/bladder pain syndrome (IC/BPS) and varicella zoster-virus (VZV)-induced post-herpetic neuralgia (PHN), models which our groups established and routinely employ. We showed that vectormediated PP1a reduced resiniferatoxin (RTx; TRPV1 agonist)-induced bladder dysfunction in cystometry assays and RTx-induced freezing behaviors following vector injection into the bladder wall. Using our rat PHN model, we demonstrated that VZV ORF47 Ser/Thr protein kinase-null virus, or viruses with specific point mutants that disrupt ORF47 kinase activity failed to induce pain responses when injected into the rat footpad compared to wild-type VZV that always induce a potent pain response. Furthermore, rdHSV expressing VZV ORF47 kinase developed chronic pain responses unlike rats receiving rdHSV empty vector alone or rdHSV expressing kinase-dead VZV ORF47. This led us to test the PP1a expression vector in the PHN model, as PP1a phosphatase activity targets phosphorylated Ser/Thr residues. We next evaluated injection of,the PP1a vector into the footpads of Sprague-Dawley rats that were previously inoculated into the same footpad with wild-type VZV-infected cells. This resulted in statistically significantly reduced mechanical hypersensitivity and thermal hyperalgesia from 2-8 weeks post vector injection. These behavioral results in the IC/BPS and PHN models made us postulate that cellular or viral kinases might influence host neuronal phosphorylation states and signaling pathways to ultimately induce pain. To test this hypothesis, we assessed the function of rdHSV vector-mediated PP1a phosphatase activity on a variety of potential molecular targets involved in pain signaling in both the IC/BPS and VZV-induced PHN models. We observed a reduction in pERK in both the IC/PBS and VZV PHN pain models. Both pTRPV1 and pIP3R were also reduced in the IC/BPS rats. We are currently assessing other targets such as pTRPV1, pMAPK, pIP3R, and pAKT in the PHN model.

709. Abstract Withdrawn

710. Molecular Consequences of APOE4, the Main Genetic Risk Factor for Alzheimer's Disease

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The E4 allele of apolipoprotein E (*APOE*) has been firmly established as a genetic risk factor for many diseases including cardiovascular diseases and Alzheimer's disease (AD), yet its mechanism of action remains poorly understood. APOE is best known to function as a lipid transport protein. Dysregulation of lipids has recently emerged as a key feature of several neurodegenerative diseases including AD, however it is unclear if and how *APOE4* perturbs the intracellular lipid state. In this work, we report that *APOE4* disrupts the cellular lipidome in human iPSC-derived astrocytes. We used combination of lipidomics, unbiased genome-wide screens, as well as functional and genetic characterization to uncover that *APOE4* induces widespread changes in intracellular lipid homeostasis. Additionally, we identified genetic and chemical modulators of these lipid disruptions. We propose

that *APOE4*'s status as a genetic risk factor for a number of distinct diseases originate from the pleiotropic consequences of perturbed lipid metabolism. Given the central role of lipid metabolism in physiology, our study sheds light on key molecular consequences that may underlie the disease risk linked to the *APOE4* genotype.

711. Novel Therapeutic Approach for Parkinson's Disease: Engineered Zinc Finger Protein Transcription Factors Efficiently and Specifically Reduce Alpha-Synuclein Expression

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Parkinson's disease (PD) is a progressive neurodegenerative disorder resulting in motor deficits such as bradykinesia, resting tremor, postural instability and axial rigidity, as well as non-motor symptoms including disordered sleep, neuropathic pain, anosmia, and dementia. Neuropathological hallmarks of PD include a loss of dopaminergic neurons in the substantia nigra and intraneuronal inclusions called Lewy bodies and Lewy neurites, which contain aggregates of the protein alpha-synuclein (aSyn). A large body of evidence implicates aSyn in the pathogenesis of PD. Due to its intrinsically disordered monomeric state, aSyn can misfold, assemble into toxic oligomers and eventually form fibrillar amyloid aggregates such as those seen in Lewy bodies and Lewy neurites. Furthermore, point mutations and duplication or triplication of the aSyn gene (*SNCA*) lead to early-onset familial PD. While familial cases of PD comprise only a small percentage of all PD cases, single nucleotide polymorphisms affecting the expression level of *SNCA* have been identified as risk factors for PD in genome-wide association studies. In addition, aSyn misfolding and aggregation have been shown to play a central role in the impairment of cellular degradation machinery, compromised mitochondrial function, and endoplasmic reticulum stress, as well as other cellular deficits described in PD and preclinical models of the disease. Reducing the expression of aSyn in the brain consequently has the potential to halt or slow the progression of PD. Zinc finger proteins (ZFPs) are the most abundant class of naturally occurring human DNA-binding proteins. ZFPs may be engineered to specifically bind virtually any genomic sequence and, when fused to transcriptional regulatory domains to form ZFP transcription factors (ZFP-TFs), they can be used to modulate the expression level of the targeted gene. Here we describe ZFP-TFs comprising a ZFP DNA-binding domain and the KRAB domain of the human Kox1 protein that repress human *SNCA* expression. These constructs were identified in a screen performed using human neuroepithelial cells transiently transfected with mRNA coding for each of 384 ZFP-TFs. The initial screen yielded a large number of hits, with *SNCA* repression activity ranging from ~40% to >99%. Representative ZFP-TFs were selected for AAV production and further evaluation. Experiments in human iPSC-derived neurons transduced with AAV expressing the ZFP-TFs confirmed the on-target activity of the selected constructs and the durability of the response for 30 days. Transcriptome-wide specificity analyses were performed in both human iPSC-derived neurons and mouse primary neurons transduced with selected AAV-ZFP-TFs. Consistent with the repression levels seen in the initial screen, we observed a range of *SCNA* repression activity in the human neurons. We identified several ZFP-TFs with minimal to no detectable off-target activity in both human and mouse neurons. Future work will include further optimization of the selectivity and on-target activity profiles of the ZFP-TFs, as well as validation of this approach *in vivo*. ZFP-TFs represent a potential one-time administration therapy targeting aSyn for the treatment of PD.

712. Loss of GSK-3beta Mediated Phosphorylation in an HtrA2 Variant Contributes to Parkinsonian Phenotype

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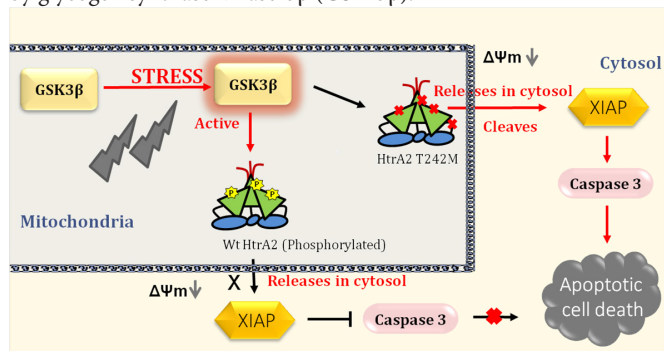
Recent advancements in neurodegeneration research have identified *HTRA2* as one of the important genes in the genesis of Parkinson's disease (PD). HtrA2, a proapoptotic mitochondrial serine protease, promotes cellular protection against oxidative damage and its deletion/mutations have shown severe neuropathologies in mice. Literature reports show positive correlation between loss of HtrA2 protease activity and PD susceptibility. Here, we report a novel pathogenic HTRA2 variant, c.725C-T (p.T242M) in Indian PD patients that exhibits no significant conformational changes compared to the wild-type. However, functional studies with HtrA2-T242M-transfected neurons reveal common features of PD pathogenesis such as dysfunction, altered morphology and membrane depolarization of the mitochondria. Despite exhibiting ~2-fold decrease in enzyme activity, observation of uncontrolled cell death in this mutant has been correlated with it being constitutively active. This gain-of-function has been attributed to the loss of phosphorylation-mediated regulatory checkpoint at the T242M mutation site that is otherwise controlled by glycogen synthase kinase-3β (GSK-3β).



Model of GSK-3β mediated phosphorylation of HtrA2 and its effect on apoptosis

713. RNA-Seq Shows Modulation of Immune Response and Extracellular Matrix (ECM) Organization Genes in Retinoschisis Knockout Mouse (*Rs1*-KO) Retina Before and After *In Vivo* Expression of Exogenously Delivered Transgene (AAV8-RS1)

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Purpose: To gain insight into RS1 gene interaction networks, we analyzed the transcriptome (RNA-Seq) of the Rs1-KO mouse retina before and after in vivo expression of the AAV8-delivered exogenous RS1 transgene. Methods: The transcriptome analysis (RNA-Seq) was performed between wild type (WT) and Rs1-KO retina at the postnatal days (P) 12 and 21. Rs1-KO retina also received subretinal injections of AAV8-RS1 or control AAV8-Null vectors (2e10 vector genomes/eye) on day P14 and the effects were evaluated 7 and 35 days after vector administration. The analyses of the transcriptomes and differentially expressed genes (DEG) are described in Stem Cell Reports. 2019 Nov 12;13(5):891-905). Protein-coding transcripts with > 1.5-fold change and a false discovery rate (FDR) of less than 5% were analyzed further. Results: Transcriptome analysis showed little differences in gene expression profiles between P12 WT and Rs1-KO retinas. However, the KO retina showed dramatic changes in gene profiles at P21. We identified 358 differentially expressed genes in Rs1-KO retina; of these, over half of the genes were associated with immune system including those involved in the development and immune effector processes. Our analysis also revealed upregulation of cell surface signaling receptors associated with cell adhesion. Immune-related transcriptional signatures evident in P21 Rs1-KO retina were entirely rescued to WT levels upon in vivo expression of the exogenous RS1 transgene. However, the Rs1-KO retinas expressing exogenous RS1 gene (AAV8-RS vs. AAV8 Null) at 7 days post-injection revealed downregulation of fibrillar collagen genes and upregulation of genes associated with the retinoid cycle in rods, oxygen transport, and antioxidant defense systems. Transcriptome analysis at post-injection day 35 demonstrated upregulation of gene networks necessary for stability of the basement membrane zone (network forming collagen IV, laminin 3 and integrin 6a), ECM-receptor interaction and photoreceptor function. Conclusions: Our studies suggest that the ECM disorganization caused by the loss of functional RS1 in Rs1-KO retina exacerbates aberrant ECM-immune cell interactions and upregulates pro-inflammatory pathways. Exogenous RS1 expression was able to largely rescue the expression of basement membrane components critical for matrix integrity, adhesion strength, and receptor-mediated signaling. In addition, exogenous RS1 gene induces Rs1-KO retina transcriptome transition from the inflammatory phase to recovery that is almost exclusively associated with remodeling of the extracellular matrix, anatomical structure formation, and visual phototransduction.

714. Potential Gene Therapy Approaches for SPG15

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Hereditary Spastic Paraplegia 15 (SPG15) is a motor neuron disease caused by biallelic pathogenic variants in the *ZFYVE26* gene. The two most common autosomal recessive hereditary spastic paraplegia gene products, the SPG15 protein spastizin and the SPG11 protein spatacsin, are pivotal for autophagic lysosome reformation (ALR), a pathway that generates new lysosomes. Loss of spastizin or spatacsin results in depletion of free lysosomes, which are competent to fuse with autophagosomes, and an accumulation of autolysosomes, reflecting a failure in ALR. In addition, spastizin and spatacsin are essential components for the initiation of lysosomal tubulation. Dysfunction of the autophagy/lysosomal biogenesis machinery is hence linked to neurodegeneration. SPG15 has been classified as a congenital disorder of autophagy, an inborn error of neuro-metabolism. Studying Spastic Paraplegia 15 can give insight into treatment approaches for similar lysosomal and neurodegenerative disorders. The exact number of SPG15 individuals is unknown, mostly due to lack of access to genetic testing in developing countries, but it is estimated that at least 50-100 individuals are living with this disorder worldwide. Cases have been reported in Germany, Italy, France, North Africa, Turkey, and Japan. The disease onset is typically in late childhood to early teen years. SPG15 has a complex phenotype that includes multiple progressive movement disorders and decline in cognitive function leading a severe physical and intellectual disabilities. Lifespan is shorter than normal. Currently no cure exists, and no treatment is effective at symptom relief. A joint effort between parents of affected children, charitable organizations, and institutions in the US and UK has been organized over the last two years to support SPG15 research. Progress has been made but funding remains an obstacle. Due to the size of the open reading frame (7,620 bp), gene replacement is not amenable to typical viral delivery approaches such as AAV. Other options are listed below: a) A strategy based on the use dual-vector approach to generate full-length therapeutic gene generated by co-infection of two separate AAV vectors encoding either the 5' or the 3' half of *ZFYVE2*6. SPG15 cDNA has been split in 2 separate fragments and cloned in dual AAV vectors. The vectors are being fully characterized for transgene expression in cell lines and neurons isolated from mouse embryos. b) Lentivirus has large packaging capacity and can be used to mediate ZFYVE26 expression. This viral vector has been validated in patient fibroblasts. c) Expression of a C-terminal protein fragment containing the 3 key protein domains is also an option. The advantages are that a higher percentage of cells in the brain and spinal cord are likely to receive the protein fragment if AAV is used as a delivery vehicle. The disadvantage is that introducing a new fragment in excess may have side-effects.

To determine the conservation of amino acid residues in the SPG15 protein across animals, an analysis has been performed to search for SPG15 in the genomes of 30 mammals and 18 additional multicellular animals. This will help identify the key domains for a mini-SPG15 gene. In addition to the above, research is underway to understand the function of spastizin and how it associates with spatacsin (SPG11) and AP-5. The next step is building a patient registry with a natural history study. It is important to develop a trial ready cohort to be able to take advantage of the development of therapeutic opportunities.

717. Transduction of Various Fragment Forms of Vectorized Anti-Tau Antibodies Using IV Dosing of a Blood Brain Barrier Penetrant AAV Capsid in Mice

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Anti-tau immunotherapy is being pursued as a promising therapy for tauopathies including Alzheimer's disease (AD), frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP). We have previously demonstrated broad distribution and expression of vectorized full-length anti-tau antibody in the mouse brain using a novel blood brain barrier penetrant AAV capsid administered intravenously (IV) with cell specific and ubiquitous promoters. This gene therapy based approach has potential advantages, including continuous expression of antibody in the central nervous system (CNS) after a single gene therapy administration, increased CNS levels of tau antibody relative to passive immunotherapy, and the potential to target intracellular tau aggregates. Antigen binding fragment (Fab, $F(ab')_2$) and single-chain fragment variable (scFv) antibody forms have also been studied as therapeutics, however their short serum half-life has made development via passive immunization challenging. Antibody fragments delivered via AAV following vectorization have the potential to express constitutive levels of these antibody forms in the brain, overcoming this pharmacokinetic challenge. Additionally, these antibody fragments could also offer improved tissue penetration, increased solubility, and the opportunity to engage epitopes that might otherwise be hidden. Here, we describe novel expression cassettes and characterization of vectorized Fab, $F(ab')_2$, and scFv fragment antibodies that are designed to optimize distribution and expression in the CNS following dosing of a blood brain penetrant AAV capsid in mice. AAV vectors comprising a novel capsid and a transgene encoding each form of anti-tau antibody fragments were constructed and administered by IV bolus to wild type mice. After one month, we assessed gene transfer and expression in the CNS. Biodistribution and cellular tropism were evaluated by ELISA and immunostaining, and vector genome levels were quantified with digital droplet PCR. Genome cassette-dependent transduction of different forms of vectorized antibody fragments in the mouse CNS was observed, including regions such as hippocampus, cortex, thalamus, brainstem and olfactory bulb. Furthermore, general or cell type specific expression of the anti-tau antibody fragments was achieved using ubiquitous or cell specific promoters, respectively. We were also able to detect secreted Fab, $F(ab')_2$, or scFv in the cerebrospinal fluid. Importantly, brain levels of anti-tau antibody fragments were substantially higher than those of full-length antibody when measured after passive immunization. Taken together, these results demonstrate expression of various forms of vectorized antibody fragments with specific AAV constructs, and suggest that IV dosing of vectorized anti-tau fragment antibody using a blood brain barrier penetrant AAV capsid can effectively deliver therapeutically relevant anti-tau antibodies to multiple brain region following a single dose.

718. Preclinical Safety and Efficacy of AAV9 Gene Replacement Therapy for SLC6A1 Disorder

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SLC6A1 gene encodes the highly conserved gamma-aminobutyric acid (GABA) transporter GAT-1, a protein crucial for GABAergic signaling in the central nervous system (CNS). A subset of patients presenting with rare forms of infantile encephalopathy with intellectual disability (ID) have been shown to be heterozygous for loss-of-function mutations in SLC6A1. Broad delivery of the SLC6A1 gene across the CNS using adeno-associated virus type 9 (AAV9) to restore normal GABAergic signaling may prove to be an effective strategy to treat patients with SLC6A1 genetic disorder. Two vector designs with different promoters were used; one is a weak universal promoter (P1), while the other is a stronger, neuronal-specific promoter (P2). To test safety, postnatal day (PND) 28-35 wild type C57BL/6 mice received the maximum feasible dose (7.5 and $7.0x10^{11}$ vector genomes (vg) for P1 and P2 constructs, respectively), four-fold lower dose, or vehicle control (PBS + 5% sorbitol) via a 5 µL intrathecal (IT) lumbar injection. Animals were weighed and observed weekly for body condition for four weeks, and monthly thereafter. Submandibular blood draw was taken at three weeks post-injection for serum chemistry analysis. No adverse reactions were observed post-injection in any animals. All treated animals appear healthy up to 12 weeks post-injection and have normal bodyweight, and serum chemistry results were normal. These results help support the safety of IT delivery of AAV9/hGAT1opt transgene with either promoter. These animals will be followed to a year post-injection, at which point tissue will be harvested and assessed by a trained pathologist. To test efficacy, the maximum feasible dose was tested in an animal model of the disease. Although SLC6A1 disorder occurs in patients with heterozygous loss-of-function mutations, GAT1 homozygous knock-out (KO) mice were used for the disease model as GAT1 heterozygous KO mice do not have an abnormal phenotype. GAT1 KO mice present with multiple symptoms and behavioral deficits relevant to the human disease, such as tremor, decreased motor coordination, and learning and memory deficits. Additionally, GAT KO mice are underweight, develop hind limb clasping, and are poor nest builders, a behavioral deficit linked to many genetic mouse models of autism spectrum disorder. KO mice were injected IT with the maximum feasible dose of either vector construct or vehicle control at PND 28-35 or received pre-symptomatic intervention at postnatal day 7-10. Mice were assessed in a behavioral battery consisting of rotarod

performance, open field, fear conditioning and nest building, behaviors known to be impaired in GAT1 KO mice. There was no apparent behavioral rescue of rotarod, open field or fear conditioning in treated animals. Hind limb clasping was also assessed on a 5 -point scale at weighing. Mice treated at PND 7-10 with either promoter construct had modest improvement in nest score at two months of age, and this change was statistically significant with the P1 promoter construct. Mice treated at PND 7-10 had significant improvement in hind limb clasping score at 20 weeks of age compared to vehicle controls. This modest change in behavioral outcomes could underscore the need for a true haploinsufficient mouse model that could better reflect achievable treatment outcomes. While additional safety and efficacy testing is needed to support further development of this gene therapy approach, the current data supports that IT administration of AAV9/SLC6A1 is well-tolerated and able to provide a partial rescue of behavioral abnormalities associated with SLC6A1 haploinsufficiency disorder.

719. *In Vivo* Proof of Concept for EDIT-102: A CRISPR/Cas9-Based Experimental Medicine for *USH2A*-Related Inherited Retinal Degeneration Caused by Mutations in Exon 13

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The most common cause of Usher syndrome type II (USH2) and nonsyndromic autosomal recessive retinitis pigmentosa (arRP) are mutations in the *USH2A* gene; these diseases are collectively known as *USH2A*-related inherited retinal degeneration (IRD). Within *USH2A* patients in the United States, a single nucleotide deletion in exon 13 (c.2299delG) in the *USH2A* gene is the most common mutation. The c.2299delG mutation results in a premature termination codon and a truncated Usherin protein, resulting in loss of protein function. Previous research showed that removal of exon 13 of *USH2A* creates a functional in-frame protein. For this reason, we developed EDIT-102, an experimental medicine for *USH2A*-related IRD through CRISPR/Cas9-mediated excision of *USH2A* exon 13. EDIT-102 is an AAV5 vector expressing *S. aureus* CRISPR/Cas9 driven by the photoreceptor-specific G protein-coupled receptor kinase 1 (GRK1) promoter and two U6 promoter-driven gRNAs that flank *hUSH2A* exon 13. To evaluate the ability of EDIT-102 to edit ≥10% of photoreceptors in animals, *in vivo* studies were performed in transgenic mice. In this humanized knock-in model (hUSH2A2299delG), mouse *Ush2a* exon 12 was replaced with the human *USH2A* exon 13 containing the 2299delG mutation and flanking introns. To understand the dose dependence of EDIT-102, we delivered 1E11, 3E11, 1E12, 3E12 and 1E13 vg/mL by subretinal injection to hUSH2A2299delG mice and measured percent editing at six weeks post dosing. To evaluate the editing kinetics and long-term stability, we injected 3E11, 1E12 and 3E12 vg/ml of EDIT-102 and measured the total editing at week 1, 3, 6 and 13 post dosing. On-target *USH2A* gene editing in the retinas was determined using the unidirectional targeted sequencing (UDiTaS) method. These results showed a dose-dependent increase in total editing from 1E11 to 3E12 vg/mL and reduced editing at the highest dose of 1E13 vg/mL. On-target editing profiles contained mostly deletions, inversions, small indels, and AAV integrations. Previous work showed that deletions and inversions were productive edits that led to removal of exon 13 from *USH2A* mRNA. These findings showed that productive edits were above the target therapeutic threshold of ≥10% for doses of 1E12 to 3E12 vg/ml, which supports the continued development of EDIT-102 for the treatment of *USH2A*-related IRD patients with mutations in exon 13.

720. Disease Specific Neurometabolic Pathways are Corrected in an Age-Dependent Manner Independent of rAAV Gene Delivery Efficacy

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Disorders of the Central Nervous System (CNS) are challenging to treat due to the CNS's limited capacity to regenerate. Particularly for genetic disorders where gene therapy allows for the correction of the disease-causing mutations, expectations of CNS recovery are high. We hypothesize that the CNS's ability to regenerate after rAAV-based gene therapy is limited by disease-specific molecular programs, which are either slow or resistant to reversal at later stages of disease progress, therefore preventing efficient disease recovery. N-acetylaspartate (NAA) is one of the most abundant metabolites in the mammalian CNS. It is commonly considered a neuronal viability marker and thus has potential implications in studying CNS recovery. NAA itself is hydrolyzed by aspartoacylase (ASPA) enzyme into acetate and L-aspartate. We, therefore, used an ASPA deficient mouse model, Nur7, which is known to suffer from white matter degeneration, ataxia, and seizures, and concomitantly demonstrates elevated NAA levels; in humans this condition is called Canavan disease (CD). First, we used rAAV to age-dependently express ASPA either ubiquitously or restricted to astrocytes. We found that injection of neonatal mice completely prevented the development of symptoms and neuropathology. Interestingly, symptomatic mice injected at juvenile age showed complete normalization of symptoms as well as neuropathology. In contrast, mice treated at adult age appeared to have normalized neuropathology, but recovery was limited in certain behavioral testing modalities. To rule out the possibility of rAAV transduction as a limit of therapeutic correction, we analyzed vector genome copies and ASPA mRNA in 11 different brain regions comparing WT, Nur7, and neonatal, juvenile, adult, and mature adult treatment groups using digital PCR. We found no significant difference between treatment groups, suggesting that rAAV gene delivery efficiency was not a limiting factor. We thus hypothesized that reversal of disease-characteristic metabolic programs was limited the further along the disease process. To test this hypothesis, we quantified the neurometabolome of n=8 mouse brains each of mice treated at juvenile or adult age with either ubiquitous or astrocyte-restricted ASPA expression and compared those to wild-type and control mice. We found that metabolic pathways of mice treated at juvenile age were

similar to that of WT mice. The juvenile treated group also showed complete disease rescue. In contrast, animals treated at adult age did not achieve the same level of metabolic correction. In light of our rAAV gene delivery data this might suggest that some factors, other than ASPA restoration, limit normalization of metabolic changes and thus limit the therapeutic outcome. We are currently investigating interactions between cellular metabolism and genetic programs that would allow for the extension of the treatment age to later time points.

721. Development of Novel Gene Therapy Paradigms to Treat a Form of Hereditary Blindness

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The RHODOPSIN (RHO) gene is the most common gene involved in autosomal dominant retinitis pigmentosa (ADRP-RHO). adRP-RHO is an inherited retinal dystrophies (IRD), which primarily affects the rod photoreceptors. ADRP-RHO leads to blindness and it is not yet curable. We have shown that 3 independent DNA-binding factors (natural transcription factor (TF) including synthetic TFs, and DNA-binding proteins) targeted to the same short regulatory DNA sequence of the Rho promoter, enable, when delivered by AAV vectors to the mouse and porcine retina, transcriptional repression of the Rho with remarkable efficiency and limited off targeting. These studies highlight novel properties on transcriptional regulation. In particular we discovered that a TF with sequence preference for a short regulatory DNA sequence of the Rho promoter when ectopically expressed in the target cell (where physiologically is not expressed) operates selectively on that regulatory sequence changing the transcriptional output of the associated Rho gene. Thus, supporting the yet unknown ability of TFs to induce gene-targeted changes of gene expression (Botta S, et al. JCI Insight. 2017 Dec 21;2(24). An additional novel property we discovered is that the sole binding to the same the Rho regulatory region by a synthetic DNA binding protein (ZF6-DB) without transcription repressor domains (which canonically in eukaryotes recruits co-repressor proteins) efficiently and selectively represses the target gene Rho (Botta S. et al. Elife. 2016 Mar 14;5). The limited off targeting of ZF6-DB (18 differentially expressed genes evaluated quantitatively by RNA-sequencing) supports its orthogonality with respect to the rod-photoreceptor-specific system, where ZF6-DBwas expressed by AAV gene transfer. Thus, the ZF6-DBprotein avoiding protein-protein interactions is "inert" to the system, while Rho promoter binding enables selective Rho transcriptional repression. Ultimately aimed at generating general tools to predict the consequences of gene therapy protocols to treat rod-photoreceptors, we analyzed the effects of DNA-binding factors on rod-photoreceptor-specific transcriptome and determined their impact on rod transcripts regulatory network based on a co-expression analysis. Finally, we generated a therapeutic with ZF6-DB for ADRP-RHO by a unique AAV system (AAV-ZF6-DB-hRHO, silencing of Rho endogenous alleles and Rho replacement), accommodating two independent expression cassettes enabling simultaneous and balanced Rho silencing (ZF6-DB) and RHO replacement with a human copy of RHO co-expressed in the same transduced photoreceptors, when delivered into the sub-retinal space of the pig eye. Currently, we are currying out dose effect studies to support AAV-ZF6-DB-hRHO clinical translation. Translation in therapeutics of these novel transcriptional regulation principles, modeled in the retinal gene therapy context, will be discussed.

722. Tyrosine Triple Mutated AAV2-BDNF Gene Therapy to Treat an Inner Retinal Injury Model Induced by Intravitreal Injection of *N*-methyl-D-Aspartate (NMDA)

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Purpose: Glaucoma is a group of chronic optic neuropathies characterized by the degeneration of retinal ganglion cells (RGCs) and their axons, which ultimately cause blindness. Previous attempts have been undertaken to protect RGCs by using neuroprotective substances. Among these, brain-derived neurotrophic factor (BDNF) especially resulted in beneficial effects. However, due to the very short half-life of the neurotrophic factors including BDNF, frequent administration of neurotrophic reagents is required clinically in order to maintain the therapeutic drug levels. Therefore, an effective gene transfer and stable expression of the neurotrophic factor within the target cells are necessary for safeguarding patient welfare. This study used the inner retinal injury mice model to evaluate the neuroprotective effect of the tyrosine-triple mutated and self-complementary adeno-associated virus (AAV)-based vector encoding BDNF (tm-scAAV2-BDNF). **Methods:** C57BL/6J mice were intravitreally injected with 1 μL of tm-scAAV2-BDNF and its control AAV vector at a titer of 6.6 E+13 genome copies/mL. Three weeks later, 1 μL of 2 mM *N*-methyl-D-aspartate (NMDA) was administered in the same way as the viral injection. Six days after the NMDA injection, we assessed the dark-adapted electroretinogram (ERG). Mice were sacrificed at one week after the NMDA injection, with RNA quantification, protein detection and histopathological analysis then performed. **Results:** RNA expression of BDNF in the retinas treated with tm-scAAV2-BDNF was about 300-fold higher than its control vector. The expression of recombinant BDNF protein increased in the retinas treated with tm-scAAV2-BDNF. Histological analysis revealed that tm-scAAV2-BDNF prevented thinning of the inner retina. Furthermore, b-wave amplitudes of the tm-scAAV2-BDNF group were significantly higher than those of the control vector group. Histopathological and electrophysiological evaluations showed that tm-scAAV2-BDNF treatment offered significant protection against NMDA toxicity. **Conclusions:** Results showed that the tm-scAAV2-BDNF-treated retinas were resistant to NMDA injury while the retinas treated with control vector exhibited histopathological and functional changes after the administration of NMDA. These results suggest that tm-scAAV2-BDNF is potentially effective against inner retinal injury, including normal tension glaucoma.

723. Abstract Withdrawn

724. *In-Vitro* Functional Validation of Missense Mutations in the *CHM* Gene in Choroideremia

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Purpose: Choroideremia is an X-linked progressive retinal degeneration caused by mutations in the *CHM* gene encoding Rab escort protein 1 (REP1). Phase II/III gene therapy trials are currently ongoing (Clinicaltrials.gov ID NCT02407678 and NCT03496012) using recombinant adenovirus-associated viral (AAV) vectors encoding the wild-type CHM coding sequence. REP1, in conjunction with Rab-geranylgeranyl transferases, catalyses post-translational lipid modification of Rab proteins. Impairment of this process appears to result in progressive photoreceptor and retinal pigment epithelium (RPE) dysfunction and eventual cell death. Most currently reported mutations result in loss of function in REP1 at a transcriptional level, secondary to promoter region mutations, intronic mutations, or exonic nonsense mutations. Missense mutations currently comprise approximately 5% of reported mutations (Human Gene Mutation Database, accessed January 2020); however, their pathogenicity is uncertain and consequently their prevalence may be underreported. This uncertainty presents challenges in genetic diagnosis and potentially subsequent clinical trial recruitment. Functional in-vitro assays are therefore required in readily available patient primary cell lines. An in-vitro prenylation assay has been previously used to validate the functional activity of a recombinant AAV2.REP1 vector in a primary human cell line. We detected a novel c.1413G>C (p.Gln471His) mutation in a 37 year old male patient with a typical choroideremia clinical phenotype and subjected the patient's peripheral blood mononuclear cells (PBMCs) to the in-vitro prenylation assay described below. **Methods:** PBMCs were isolated from blood samples from a choroideremia patient and a 27-year old healthy male control. Cell lysates were incubated with recombinant human Rab6A, biotin-labelled geranyl pyrophosphate (5 μM), guanosine 5'-diphosphate (20 μM) and DTT (1mM) for 2 hours at 37 degrees. An additional positive control was performed by spiking the patient cell lysate with fish His-REP1 protein. Reaction products were subject to Western blot with human anti-REP1 (MABN52), anti-beta-actin (AM4301) and subsequent horseradish peroxidase (HRP)- labeled secondary antibody. Prenylation with biotin-labelled lipid moieties was detected by incubation of the reaction with streptavidin-HRP. **Results:** As expected, there was no detection of REP1 protein in the PBMC cell lysate from *CHM* patients. There was no detection of biotin-labelled prenylation of Rab6A upon incubation with the patient cell lysate. Both the healthy control PBMC lysate and fish REP1 positive control resulted in incorporation of biotin-labelled prenyl groups in Rab6A. **Conclusion and significance:** Due to the ubiquitous expression of the REP1 protein, PBMCs represent a readily available patient primary cell line in which functional in-vitro assays may be developed. Defective prenylation in this assay may provide additional evidence to support the pathogenicity of equivocal genetic test results in the presence of a typical choroideremia phenotype.

725. AAV Mediated RNA Replacement as Therapy in a Mouse Model of Autosomal Dominant Retinitis Pigmentosa

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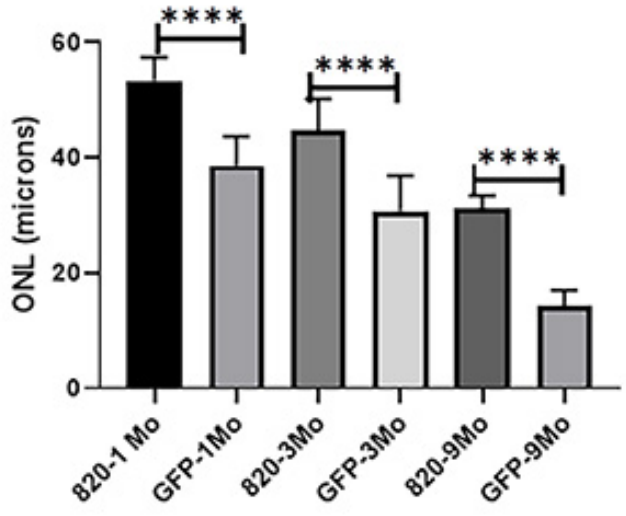
In previous experiments in dogs with the T4R mutation in *RHO*, the gene for rhodopsin, we demonstrated that an AAV2/5 vector expressing both shRNA820, directed to human and dog *RHO* mRNA, and an shRNA-resistant human *RHO* cDNA (*RHO820*), prevented retinal degeneration and preserved retinal function for more than 8 months following subretinal injection. To confirm that this approach to therapy for *RHO* adRP could protect the retinas of animals bearing a different *RHO* mutation, we subretinally injected mice transgenic for human P23H rhodopsin at postnatal day 30 and monitored their retinal structure using spectral domain optical coherence tomography (SD-OCT) and retinal function using electroretinography (ERG) for 9 months. At the final time point mice were euthanized, and their eyes prepared for histology. These samples were compared to P23H *RHO* transgenic mice injected with AAV-GFP to control for the impact of subretinal injection. Though retinas continued to thin over the time course, compared to control injected eyes, AAV-*RHO820-shRNA820* injection led to a statistically significant improvement in the thickness of the outer nuclear layer, indicating survival of photoreceptor cells starting at one month post injection and extending throughout the time course. An increase in ERG amplitudes in AAV-*RHO820-shRNA820* eyes was also observed by one month following treatment and sustained for nine months. We conclude that delivery of this vector was effective in preventing retinal degeneration associated with a mutant *human* transgene in a different species than we had previously used to test this gene therapy vector.



Outer Nuclear Layer thickness measured by SD-OCT at 1 month, 3 months and 9 months post-injection with AAV-RHO820-shRNA820 (820) or AAV-GFP (GFP). **** signifies P<0.0001.

726. It Takes Two to Tango: Testing a Dual AAV Gene Therapy Approach for Usher Syndrome

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Usher syndrome is a devastating autosomal recessive disease that causes congenital hearing loss and progressive blindness. While cochlear implants can restore hearing loss, there is no treatment to prevent blindness. Gene replacement therapy using recombinant adeno-associated viruses (AAVs) is a promising approach to restore gene expression in the retina; however, the relatively small capacity of AAVs prevents the delivery of large genes, such as those associated with Usher syndrome. To overcome this, genes can be split and carried in two separate vectors, which, follow transduction, can be reconstituted. In this study, we investigated gene replacement therapy via dual AAV transduction for the prevention of retinal degeneration in Usher type 1F, caused by a mutation in the *PCDH15* gene. Two dual approaches were investigated, the hybrid and trans-splice, in which the *PCDH15* gene was split into two halves with the left construct containing a CMV promoter to drive expression. In vitro validation of our plasmid constructs, showed that the levels of *PCDH15* was dependent on the cell type tested. In HEK293T cells, hybrid plasmids showed a 2-fold increase compared to trans-splice, while in Jurkat cells, trans-splice plasmids showed a 1.79-fold increase compared to hybrid plasmids. Therefore, for our follow up studies using patient iPSC-derived retinal organoids, both hybrid and trans-splice constructs were packaged into AAV2/9 and AAV2/Anc80L65 serotypes. Dual AAV vectors were tested *in vitro* in *i)* patient fibroblasts and *ii)* patient and control retinal organoids (ROs) derived from induced pluripotent stem cells (iPSCs). Compared to untreated, transduction with dual AAVs of both serotypes successfully induced *PCDH15* transcript expression in patient fibroblasts and increased transcript expression in patient-derived ROs. Of the vector strategies tested, hybrid AAV2/Anc80L65 vectors showed higher expression in fibroblasts (3-fold higher compared to AAV2/9), while hybrid AAV2/9 vectors resulted in the overall highest fold increase in *PCDH15* transcript expression in ROs. In ROs treated at day 30, *PCDH15* expression at 15 days post-treatment was higher in AAV2/Anc80L65 treated ROs (2-fold increase compared to baseline) compared to AAV2/9 (1.7-fold increase). However, at 30 days post-treatment (day 60 ROs), AAV2/9 showed a 3.6-fold *PCDH15* expression increase compared to AAV2/Anc80L65 (2.8-fold increase). Similarly, ROs treated at a later time point (day 45) had higher *PCDH15* expression at day 60 (15 days post-transduction) when transduced with hybrid AAV2/9 than hybrid AAV2/Anc80L65. The fold increase in *PCDH15* from untreated day 60 expression was 3.63 for AAV2/9-transduced ROs and 2.84 for AAV2/Anc80L65-transduced ROs. Trans-splice vectors showed overall lower transduction efficiencies and were not evaluated extensively compared to hybrid vectors. Our results demonstrate proof-of-principle expression restoration of gene expression by dual AAV transduction. However, further work is essential for determining the viability of this approach in Usher 1F.

727. RNAi Dependent *Gys1* Knockdown Decreases Glycogen Inclusions in Lafora Disease Mouse Models

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Lafora Disease (LD) is a fatal, autosomal recessive, glycogen storage disorder, and is a rare form of progressive myoclonus epilepsy. LD typically starts in previously healthy adolescents, usually with action and stimulus sensitive myoclonus. Initial symptoms rapidly evolve into progressive dementia, refractory epilepsy, cerebellar ataxia, and respiratory failure which lead to death within about a decade. Currently, there is no treatment available for LD. LD is caused by loss of function mutations in *EPM2A* or *NHLRC1*, which encode the glycogen phosphatase laforin or the E3 ubiquitin ligase malin, respectively. Both laforin and malin are involved in regulating glycogen metabolism by an unclear mechanism. The absence of either protein results in long, poorly branched, hyperphosphorylated, aberrant cytoplasmic glycogen inclusions (Lafora bodies) in almost all tissues. However, the molecular basis of this devastating disease is still poorly understood. Studies indicate that the intracellular inclusions are a principal driver of neurodegeneration and other brain abnormalities seen in LD mouse models. Thus, one approach for the treatment of LD is preventing formation of these inclusions. It was previously shown that partial or full reduction of glycogen synthase in LD mouse models prevents Lafora body formation, neurodegeneration, and seizures. This finding led to the hypothesis that the reduction of brain glycogen synthesis through inhibiting *Gys1*, the glycogen synthase isoform that expressed in the brain, might prevent polyglucosan formation and rescue the neurological phenotype. To test this hypothesis as a proof of principle study, we have pursued an AAV-RNAi dependent gene therapy approach. After screening multiple candidates in vitro, we packaged the artificial miRNA with highest *Gys1* knock down efficiency in a recombinant adeno-associated virus (rAAV2/9). We moved forward to in vivo experiments using LD mouse models. Neonatal mice were injected with the rAAV-RNAi vectors via bilateral intracranial ventricular (ICV) injections. Mice were aged to 3 months and brain tissue was harvested for biochemical and histopathological analysis. Our preliminary results show that RNA interference (RNAi)-mediated silencing of *Gys1* mRNA provides therapeutic benefit in LD mouse models by decreasing abnormal glycogen formation in the brain.

728. Newborn Screening for Menkes Disease Based on Targeted-Next Generation Sequencing

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Purpose: Population-based newborn screening (NBS) allows early detection and treatment of inherited disorders. As the field of viral gene therapy advances, there is increased incentive to detect inherited disorders at birth prior to development of symptoms. This is especially

true for neurodegenerative conditions of brain. For certain medically-actionable conditions, however, newborn screening is limited by the absence of reliable biochemical signatures amenable to detection by current platforms. We sought to assess the feasibility and diagnostic yield of targeted next generation DNA sequencing as a newborn screen for one such disorder, Menkes disease, for which a promising viral gene therapy preclinical profile (CSF-directed AAV9-reduced size *ATP7A* + subcutaneous Copper Histidinate (CuHis)) has been established. **Methods**: After Institutional Research Board (IRB) approval of a specific protocol amendment, Menkes disease subjects enrolled in a phase 3 clinical trial (NCT 00811785) of CuHis (IND 34,166) were recruited to participate in a pilot study on DNA-based newborn screening. Dried blood spots from control or Menkes disease subjects (n=22) were blindly analyzed for pathogenic variants in *ATP7A*. We utilized a tNGS panel consisting of 544 genes (7Mb) relevant to pediatric inherited diseases, a subset of which present in the newborn period. In addition to *ATP7A*, the tNGS panel included the most common genes associated with disorders now listed on the US Recommended Uniform Screening Panel (RUSP) of the Advisory Committee on Heritable Diseases in Newborns and Children (ACHDNC; https://www.hrsa.gov/advisory-committees/heritable-disorders/index.html), and offers a model of broad DNA-based NBS coverage. The entire coding sequences, including first and last exons, adjacent untranslated regions and splice junctions of the 544 genes were sequenced. The clinical test pipeline was optimized for use in neonates, utilizing DNA extracted from small blood volume (DBS punches) to detect single-nucleotide variants (SNVs), small insertions and deletions (INDELs) up to 20bp in length, and large copy number variants (CNVs) with rapid turnaround time (≤120hr). Over 90% of the panel genes were typically sequenced to a depth of 20x or greater. This analytical method was optimized for cost-efficiency and rapid turnaround time. **Results**: The algorithm correctly identified pathogenic *ATP7A* variants, including missense, nonsense, small insertions/deletions, and large copy number variants, in 21/22 (95.5%) of subjects, one of whom had inconclusive diagnostic sequencing previously. For one false negative, that also had not been detected by commercial molecular laboratories, we identified a deep intronic variant that impaired *ATP7A* mRNA splicing. **Conclusions:** Our results provide proof-of-concept for primary DNA-based NBS utilizing tNGS to detect Menkes disease for which potential successful treatments have emerged but for which no reliable neonatal biochemical analyte exists. Routine tNGS would improve early detection of Menkes disease, and improve survival and long-term outcomes. If applied broadly to include other currently unscreened disorders for which gene therapy is being developed, the tNGS-based approach would complement current newborn screening efforts. Consideration of newborn screening and engagement with state and federal policy makers remains critical to optimize the impact of gene therapy advances by identifying affected individuals while still asymptomatic.

729. Dual AAV Gene Therapy for Inherited Childhood Epilepsy, Dravet Syndrome

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Dravet Syndrome (DS) is an inherited childhood epilepsy caused by a mutation in the *SCN1A* gene, which encodes the voltage-gated sodium channel $Na_V1.1$. DS has an incidence rate of 1:12,000 live births. Patients suffer from refractory and generalized seizures, can experience *status epilepticus* and increased mortality with significant cognitive impairments. This disease remains untreatable by either medical or surgical means. Gene therapy for DS is complex; we and others (Feldman and Lossin, 2014) have experienced difficulties propagating wild-type *SCN1A* plasmids in E.coli competent cells. The size of *SCN1A* (6kb) prevents its incorporation into an adeno-associated vector (AAV). To overcome these challenges, we designed a dual AAV8 approach, by splitting *SCN1A* into two complementary halves. We hypothesized that the two constructs would produce polypeptides which would reconstitute post-transcriptionally into a functional $Na_V1.1$ protein (Stühmer et al. 1989). We have established a DS knock-out (KO) mouse model and observed 100% mortality in the KO mice by day P14 due to spontaneous seizures and severe ataxia (Fig. 1A). *Scn1a* expression was significantly lower in the cortex and hippocampus (Fig. 1B) of KO mice compared to WT. Open field test has shown significant differences in KO mice compared to WT.We therefore designed two AAV8 vectors where the two halves of *SCN1A* were driven by a pan neuronal promoter, human Synapsin. For *in vitro* validation, fluorescent genes were linked by a bicistronic linker (T2A) to either half. *In vitro,* co-transfection appeared toxic to cells (data not shown), in a subset of recordings a small amount of sodium current was observed. Western blot showed the expected bands, but they were faint (Fig. 1C). Our data suggest that *in vitro*, these constructs are not able to effectively assemble, that they may be only able to produce small currents, and are generally toxic in isolated cells. Some sodium channels may rely on neuronal processing to assemble properly, so we asked whether *in vivo* expression was more effective. We have applied our AAV8 vectors to KO mice via bilateral intracerebroventricular injection at P0. KO mice received both vectors (n=9) or no treatment (n=12) in a randomised, blinded study; no difference in survival or motor development was found. In summary, we have shown that *SCN1A* can be incorporated into plasmids and vector, but its expression *in vitro* was insufficient and toxic to cells. We have established an animal model of DS and have produced AAV8 vectors to treat KO mice, however these constructs were unable to modify their phenotype. We are developing new strategies to test in the future using our established DS mouse model.

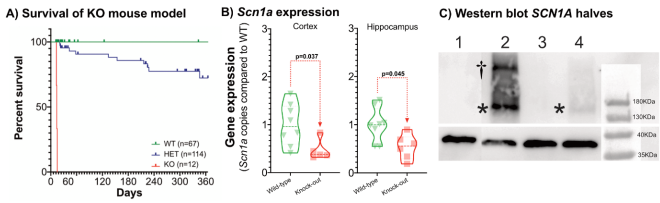

Fig. 1. ***A)*** *KO mice had a significant mortality ($p<0.001$). WT had no dead events, heterozygous had 11 events ($p=0.098$ vs WT).* ***B)*** *There was a significant difference in Scn1a expression in KO mice compared to WT in the cortex and hippocampus.* ***C)*** *Western blot showing detection of the first half (*, lane 2 and 4) and the whole $Na_V1.1$ protein (†, lane 2). Lane 1: control, lane 2: both plasmids, lane 3: second half, lane 4: first half. The antibody only detects the first half of SCN1A, therefore, lane 3 is negative.*

730. Implications of Gene Therapy for Alzheimer's

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Good Problems to Have? Implications of a Nucleic Acid-Based Disease-Modifying Therapy for Presymptomatic Late-Onset Alzheimer's DiseaseRunning title: Implications of Gene Therapy for Alzheimer's Keywords: Alzheimer's; therapy; access; diagnosis; infrastructure; drug pricing Despite decades of frustration in the effort to develop an effective therapy for late-onset Alzheimer's disease (LOAD), we can nevertheless imagine a scenario in which a nucleic-acid-based disease-modifying therapy (DMT) becomes available. Consequently, it is imperative that we begin to contemplate the challenges accompanying the availability of such a therapy. A DMT for the large population of pre-symptomatic LOAD patients will result in a greater burden on diagnostic infrastructure (including expenses spent on brain imaging and cognitive testing), as well as increased demand for geriatricians, neurologists, and memory clinics. An additional challenge unique to Alzheimer's disease is the age of the target population. LOAD patients have fewer productive years of work remaining vis-a-vis the general population, and successfully treated LOAD patients will remain susceptible to a variety of other aging-related diseases. Arguably the most daunting immediate problem posed by an effective "one and done" DMT for LOAD will be cost (and the closely related problem of access). Pharmaceutical companies, in a bid to both recoup research and development and manufacturing expenses and reward shareholders, will ensure that any DMT commands a high price. Thus, to make a DMT for LOAD widely available without bankrupting third-party payers (let alone patients), will likely require innovative payment models. Potential options include the subscription payment plan (or "Netflix Model"), which was used to offset the costs of treating hepatitis C in Louisiana, and the sequential payment model being pursued by gene therapy startup Bluebird Bio for its transfusion-dependent β-thalassemia treatment. Here we examine some general considerations that can inform a rational approach to meeting both the logistical and financial challenges posed by an effective DMT for LOAD.

731. A Dosage Study to Assess the Long-Term Effects of Gene Therapy for AB-Variant GM2 Gangliosidoses in a Mouse Model Using Adeno-Associated Virus Serotype 9

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AB-Variant GM2 Gangliosidoses (ABGM2) is an autosomal recessive lysosomal storage disease that affects the central nervous system (CNS). In a properly functioning cell, GM2 ganglioside is catabolized into GM3 ganglioside by the enzyme β-Hexosaminidase A (HexA) and its essential co-factor, the GM2 activator protein (GM2AP). HexA is composed of two subunits, α and β, which are encoded by *HEXA* and *HEXB*, respectively. Similarly, GM2AP is encoded by *GM2A*. A pathogenic mutation in *GM2A* leads to the formation of a dysfunctional GM2AP, thus resulting in a decrease in its ability to help catabolize GM2 ganglioside. GM2 ganglioside build-up results in neuronal apoptosis and widespread neurodegeneration throughout the entire CNS. Patients suffering with this disease have a reduced quality of life (i.e. cerebellar ataxia, atrophy, dysarthria, etc..) that can begin in early infancy. Despite advances in palliative care, ABGM2 remains one of the most devastating inherited neurological disorders, resulting in death by age 4, in its human infantile form. Thus, there is a clear, unmet need, for an effective revolutionizing therapeutic treatment. The objective of replacement gene therapy is to introduce a functional copy of an otherwise mutated gene into the body. This concept is particularly attractive for monogenic diseases, such as ABGM2, and could provide a one-time curative treatment. In our study, we used a self-complimentary Adeno-associated virus serotype 9 carrying *GM2A* (scAAV9-*GM2A*). As a proof-of-concept study, this therapy was trialed on a *Gm2a*$^{-/-}$ mouse model, through intravenous administration, producing promising data. The results demonstrated that in comparison to mice that received a vehicle injection, the treated mice had: (1) reduced GM2 ganglioside accumulation within the CNS and (2) a long-lasting and widespread vector distribution (unpublished). These results strongly suggest that a one-time administration of this treatment could restore protein activity, thus possibly provide curative effects.In order to further optimize the proposed treatment, the influence of dosage on therapeutic efficacy must be determined. In a previous study, we assessed the short-term dose effects of scAAV9-*GM2A* on the *Gm2a*$^{-/-}$ mouse model, sacrificing mice at 20 weeks of age. We saw that the high dose (1.0E11vg/mouse) was performing better than the low dose (0.5E11vg/mouse) and vehicle treated mice, with a larger vector biodistribution and a larger reduction in GM2 accumulation in the CNS. To assess these benefits long-term, we carried out the same methods until the mouse reached its humane endpoint.

Additionally, we have also added another cohort which received a dose double our previous 'high dose' (2E11vg/mouse). Preliminary results suggest that, long-term, doses are also directly proportional to the therapeutic benefits; the higher the dose, the larger the benefit in GM2 reduction. Our results are a step forward towards clinical Gene therapy for ABGM2. \

732. Allele Specific Knockdown of *APOE* e4 Expression: A Novel Platform for Precision Gene Therapy in Alzheimer's Disease

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The e4 allele of the apolipoprotein E gene (*APOE)* is the strongest genetic risk factor for LOAD, is associated with lower age of onset, and over the ensuing years since the original discovery, it has become the most highly replicated. Furthermore, differential *APOE* expression was described in disease vulnerable brain tissues between LOAD and control. Although the normal function of ApoE protein in health and how it's disrupted in disease is still not completely understood, accumulating evidence lends support to the concept that *APOE*e4 allele inherent hyperfunction that is comparable to the effect of higher e3 expression. Stems from this concept, we proposed a gene therapy approach that combines strategies of overall reduction of *APOE* expression with *APOE*e4 specific knockdown as means to reverse LOAD-pathological phenotypic perturbations. Our strategy is based on CRISPR/dCas9-editing tools for targeted modification of the epigenome landscape of regulatory elements within the *APOE* locus. First step we identified the target regulatory region/s. We demonstrated LOAD-associated differential DNA-methylation in *APOE* promoter/enhancer and exon 4 regions, and LOAD specific changes in chromatin accessibility profiles in the *APOE* locus. In addition, we observed hypomethylated sites at the promoter region of *APOE*. Altogether, these results defined the target region for the development of our novel lentiviral (LV)-based system integrating CRISPR/dCas9-editing tools to enhance targeted and precise DNA-methylation. Applying this LV- CRISPR/ deactivated(d)Cas9 DNA-methylation editing tools into hiPSC-derived neurons showed decreased *APOE*-mRNA and protein levels, validating the target region. Next, we developed a system to specifically downregulate of the APOE e4 expression. We have taken an advantage of a newly engineered VRER-Cas9 protein which specifically and accurately targeting novel protospacer adjacent motif (PAM) created with SNP-rs429358 T-C transition. To enrich DNA methylation and reduce *APOE* e4 expression in the haplotype-specific manner we created a novel all-in-one lentiviral vector carrying VRER-dCas9 fused with the catalytic domain of DNA-methyltransferase 3A (DNMT3A). We evaluated the specificity of the system using *isogeneic* APOE-human induced pluripotent stem cell (hiPSC)-derived excitatory neurons homozygote for the *APOE* e4 compared to the e3 homozygous line. Here, we provide the groundwork and *proof-of-concept* for the development of new gene therapy-based approach for LOAD mediated by targeted DNA-methylation editing for manipulation of overall and e4-specific *APOE* expression. Our epigenome therapy strategy for intervention with *APOE* expression based on dCas9 technology is *translational* toward the development of 'smart drug' for LOAD.

733. Approaches for Gene Therapy of Neurofibromatosis Type 1(NF1) Using Mini-NF1 and Trans Splicing Dual Adeno-Associated Virus (AAV) Vectors

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Development of gene therapy for NF1, a genetic disorder that predisposes affected individuals to highly aggressive malignant peripheral nerve sheath tumors (MPNSTs), have been complicated by the neurofibromin 1(*NF1)* cDNA size that encodes for a GTPase-activating protein (GAP), neurofibromin. The primary known function of neurofibromin is to regulate RAS activity by binding to GTP-bound RAS through the GAP-related domain (GRD) to inhibit RAS signaling through activation of RAS GTPase activity. Loss of neurofibromin results in the dysregulation of multiple signaling pathways downstream of RAS, which leads towards an overlapping set of neurocutaneous manifestations, including benign and malignant tumors, pigmentation changes as well as attention deficits and learning disabilities. Presently recombinant adeno-associated viral (AAV) vectors are regarded as the leading platform of *in vivo* gene therapy of numerous diseases. However the relatively small transgene capacity (~4.7 kb) of recombinant AAV vectors makes development of treatments for disorders caused by genes with cDNAs >3.5kb challenging. Two approaches are being investigated to address this limitation for NF1. One approach consists in designing mini-NF1 proteins that at a minimum regulate the RAS pathway. In the present study, we explored the viability of restoring GTPase activity of RAS through the expression of various constructs which includes the GAP domain alone (GAP-M), GAP domain in combination with cral-trio domain (GAP-MCT) and GAP domain in combination with cral-trio and lipid binding domains (GAP-MLB) of *NF1* driven by a CBA promoter. As the expected product will be a truncated form of neurofibromin, a HA-tag was fused to the C terminus of these constructs for quantitative and qualitative detection of the expressed proteins. The second approach consists in the development of a dual trans-splicing AAV vector (AAV-NF1^{dual}) system to restore expression of full length neurofibromin. This system explores the concatemerization of AAV genomes in the host cell nucleus where the joined ITRs become part of an artificial intron that is removed to generate the full length NF1 mRNA. In this system one AAV vector carries an Mecp2 promoter, 4.17 kb of NF1 cDNA and the splice donor half of an intron. The second AAV vector carries a small intron and splicing acceptor followed by the c-terminal 3.49 kb of NF1 fused to HA-tag and an SV40 polyA. The NF1 cDNA was split between the two vectors using a natural exon-intron boundary. AAV-DJ vectors encoding mini-NF1s and dual AAV-NF1 were used to infect HEK293T cells at MOI 100,000 to assess protein expression by western blot. Our results revealed that all mini-NF1 vectors produced mini neurofibromin isoforms, but the GAP-MLB protein was expressed at the highest levels. Likewise, our data showed that transduction HEK-293T with the trans-splicing AAV-NF1^{dual} system leads to expression of full length neurofibromin. Functional studies in human MPNST

cell lines will compare the efficiency of mini-NF1s and dual AAV-NF1 system to restore RAS pathway activity, which will lead to selection of lead candidate(s) for *in vivo* studies in NF1 mouse models.

734. Efficient Identification of Therapeutic Base Editing Candidates to Correct Human Mutations in the USH2A Gene

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Mutations in USH2A are the most prevalent cause of Usher Syndrome (retinitis pigmentosa and hearing loss), with >1000 pathogenic mutations in USH2A reported in humans. Over 300 of these mutations are transition mutations, which can be directly corrected by base editing, a form of CRISPR-Cas-based genome editing which can convert A:T to G:C or C:G to T:A in the genome. We have recently demonstrated [Nat Biomed Eng. 2020 Jan;4(1):97-110] base editing at potentially therapeutically-relevant efficiencies in murine retina in vivo. Therapeutic applications of base editing are constrained by the requirement for a protospacer-adjacent motif (PAM) recognition sequence, the potential generation of nearby bystander edits, and potential off-target effects. In cases where bioinformatic design criteria are met, empiric testing is then needed to identify gRNA sequence / base editor combinations with high on-target editing activity. In this study, we integrate a tandem array of 35 USH2A mutant sequences into the AAVS1 locus in HEK293 cells, allowing for rapid testing of base editor activity at these USH2A disease-associated mutations using a single cell line. These mutant sequences were selected based on bioinformatic design criteria and the prevalence of the mutations in humans. Using A-to-G base editors (ABEs) and C-to-T base editors (CBEs), we identified multiple mutations of USH2A which showed high efficiency of base editing (>50%) with a low indel frequency (~3%). Off-target effects at predicted cutting sites were evaluated empirically for promising targets. There were specific (but limited) differences detected between various testing methods, including: integrated editing target versus target plasmid transfection; single-plex versus multiplex guide transfection; and Sanger versus NGS editing efficacy evaluation. Testing of intein-split editors which can be delivered by AAV in vivo is ongoing. This work describes a method that improves the rate and throughput with which promising base editor targets can be identified in cultured cells, speeding testing of therapeutic hypotheses in vivo.

Musculo-skeletal Diseases

735. Characterization and Exploration of Treatment in a Novel Mouse Model of Duchenne Muscular Dystrophy Using Adeno-Associated-Virus.U7snRNA Mediated Exon Skipping of a Dystrophin Mutation Hot Spot

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Duchenne Muscular Dystrophy (DMD) is a severe neuromuscular disorder caused by mutations in the *DMD* gene encoding dystrophin, affecting ~1:5,000 males. DMD patients experience progressive muscle weakness that ultimately results in death in the third decade of life due to failure of the heart or diaphragm. DMD is caused by a variety of mutations, and future gene therapies that allow restoration of slightly truncated but functional dystrophin are likely to target only specific subsets of mutations, as the DMD gene is too big to fit in the most common virus used in the field, the Adeno-Associated-Virus (AAV). One approach allowing restoration of a functional truncated protein is called exon skipping. This strategy uses antisense oligonucleotides (AONs) to modulate pre-mRNA splicing by excluding target exons from mRNA. Because AONs require perfect binding to their targets, mouse models with the humanized *DMD (hDMD)* are necessary. Thus, the creation of new hDMD mice lineages harboring disease-causing mutations enables the development of treatments for new subsets of DMD patients. Here our objective is to develop and characterize a new DMD mouse to model patients with mutations in exons 6-8 of the *DMD* gene which encode for the second calponin homology domain (CH2), part of dystrophin's first actin-binding domain. Although CH2 is implicated in a crucial part of dystrophin, evidence in a dog model of DMD supports that a dystrophin lacking CH2 retains its function. Inducing expression of this functional protein by skipping exons 6-8 could benefit 3-4% of DMD patients. To test possible therapies for CH2 mutations, mice containing *hDMD* on an mdx (no murine dystrophin expression) background were modified by CRISPR/Cas9 to introduce a nonsense mutation in exon 7 creating the hDMDex7/mdx mouse. This mouse has been established and is currently being characterized. We are currently testing whether these mice show no protein expression and a decrease in muscle function in line with other *hDMD* mutated mouse models. After characterization, we will test the ability of removing the entire CH2 region to ameliorate the DMD phenotype in hDMDex7/mdx mice. We designed and tested 8 various constructs to skip exons 6-8 *in vitro*. As mentioned earlier, AONs can mediate exon skipping, but they poorly penetrate cardiac tissue and their instability requires frequent reinjection. To avoid these obstacles, we used U7snRNA constructs packaged in AAV (AAV.U7) which allows constant AON expression and efficient skeletal and cardiac muscle transduction. We tested

our constructs *in vitro* in myotubes derived from DMD patients and identified several constructs that enable efficient exon 6-8 skipping. We are in the process of combining these constructs into a single vector (AAV.U7-ΔCH2). After characterization of the hDMDex7/mdx mouse, we will evaluate the result of AAV.U7-ΔCH2 intramuscular injection on exon skipping and dystrophin restoration 1-month post-injection. Altogether, *in vitro* results and previous AAV.U7 programs support that this multi-exon skipping approach could help 3-4% of DMD patients.

736. Precise *COL7A1* Gene Correction in Primary Patient Cells as a Therapeutic Option for Epidermolysis Bullosa

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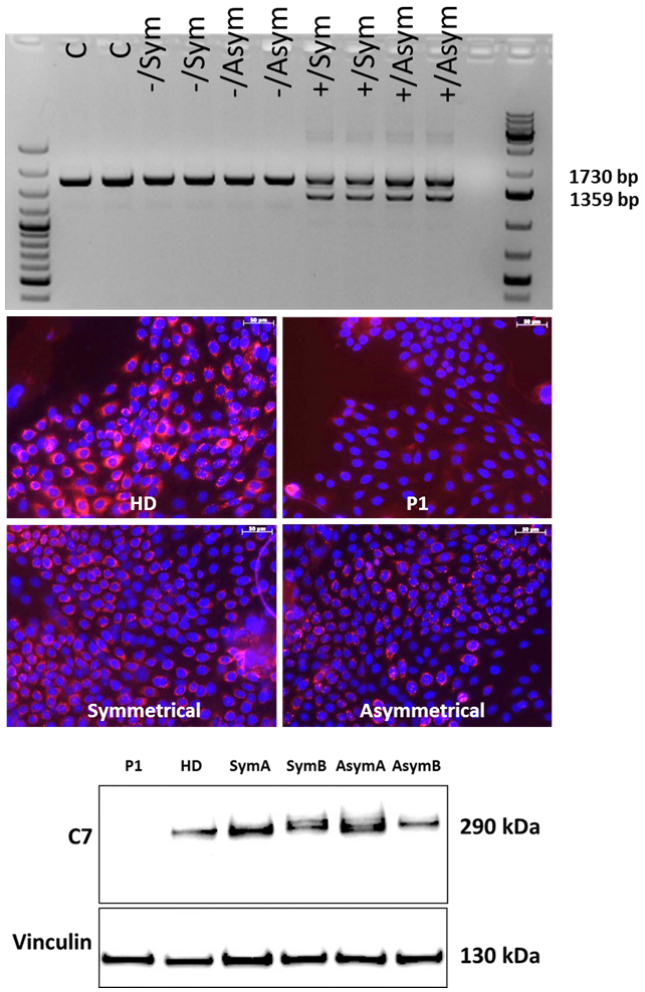
Clinically relevant protocols for correction of genetic disease-causing mutations in patient cells require highly efficient tools to achieve gene modification in a therapeutically useful fraction of cells. Epidermolysis bullosa (EB) is a group of rare genetic skin fragility diseases, characterized by the loss of dermal-epidermal adhesion. The most severe subtype, Recessive Dystrophic Epidermolysis Bullosa (RDEB), is caused by mutations in *COL7A1*. Pharmacological, gene and cell therapies have been proposed but no curative treatment are currently available. Here, we report an efficient and versatile *ex vivo* gene editing approach for homology-directed repair-based (HDR) gene correction using CRISPR/Cas9 RNPs in combination with AAV6-delivered donor template. HDR correction rates close to 40% were achieved in primary patient RDEB keratinocytes, an efficacy sufficient to reach restoration of the skin adhesion defect. Type VII Collagen (C7) expression was observed in skin grafts regenerated from an edited bulk RDEB keratinocytes population, with normal skin structure and differentiation. Gene-corrected skin equivalents can be grafted onto affected skin of RDEB patients. This gene therapy strategy offers traceless gene correction for a wide spectrum of EB-causing mutations, potentially spreading the benefit of precise correction therapies to a large cohort of patients.



737. Characterizing the Histological and Behavioral Phenotypes of a Humanized Knock-In Mouse Modeling a Deep Intronic Mutation in Collagen VI-Related Dystrophy

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Introduction: Collagen VI is an extracellular matrix protein, which forms networks of beaded microfilaments. The *COL6A1, COL6A2,* and *COL6A3* genes encode the three major α-chains, α1(VI), α2(VI), and α3(VI), respectively, that coalesce to form the heterotrimeric collagen VI monomer. A recurrent and common *de novo* C>T dominant-negative mutation in *COL6A1* causes the insertion of an in-frame 72-base-pair-long pseudoexon between exons 11 and 12 for about 50% of transcripts. The mutant α1(VI) chain disrupts the triple-helical domain needed for the proper assembly and function of collagen VI. Patients carrying this mutation present with early-onset muscle weakness, joint contractures, and respiratory insufficiency. To better

study this *COL6A1* mutation and to test exon skipping therapies *in vivo*, the lab has generated a humanized knock-in mouse model that carries the human sequence of *COL6A1* from exon 9 to exon 14. One allele carries the C nucleotide (HumC), and the other allele carries the mutant T nucleotide (HumT) at the mutation locus. The goal of this study was to examine the histological and behavioral differences between *Col6a1*$^{+/+}$, *Col6a1*$^{+/HumC}$, *Col6a1*$^{HumC/HumC}$, *Col6a1*$^{+/HumT}$, and *Col6a1*$^{HumT/HumT}$ mice. **Methods:** Quadriceps muscles from *Col6a1*$^{+/+}$, *Col6a1*$^{+/HumC}$, and *Col6a1*$^{+/HumT}$ mice that were at least 44 weeks old were harvested and stained with hematoxylin and eosin (H&E), Sirius Red stained to assess fibrosis, and immunohistochemically dual stained for collagen VI and laminin. Extensor digitorum longus, gastrocnemius, soleus, tibialis anterior, and triceps muscles were harvested from the *Col6a1*$^{+/HumT}$ mouse and were stained similarly to quadriceps to compare the involvement of different muscles in the disease model. Male and female *Col6a1*$^{+/+}$, *Col6a1*$^{+/HumC}$, *Col6a1*$^{HumC/HumC}$, *Col6a1*$^{+/HumT}$, *Col6a1*$^{HumT/HumT}$ mice were weighed once every 2 days starting at postnatal day (PND) 14 to PND 28, then weighed once every 2 weeks to PND 56, then weighed once per month to PND 84. Grip strength of the same mice was measured once per month starting at PND 28 to PND 84 for males and to PND 56 for females. **Results:** The H&E staining showed a marked increase in the number of central nuclei in *Col6a1*$^{+/HumT}$ mouse quadriceps compared to *Col6a1*$^{+/+}$ and *Col6a1*$^{+/HumC}$ mice. Additionally, the tibialis anterior and quadriceps muscles showed the highest abundance of central nuclei among the *Col6a1*$^{+/HumT}$ muscles. The preliminary Sirius Red and immunohistochemical stains did not show any noticeable difference between the collected muscles. At PND 84, male *Col6a1*$^{+/HumT}$ and *Col6a1*$^{HumT/HumT}$ mice had slightly lower average body weights (27.5 g. and 27.1 g., respectively) compared to *Col6a1*$^{+/+}$ (28.5 g.), *Col6a1*$^{+/HumC}$ (29.1 g.), and *Col6a1*$^{HumC/HumC}$ (28.7 g.) mice. The female mice did not have noticeable body weight differences. The average absolute grip strength and weight-normalized grip strength values of male and female *Col6a1*$^{+/HumT}$ and *Col6a1*$^{HumT/HumT}$ mice were consistently lower than that of all other groups. There was no appreciable difference between the grip strength measurements of the other groups. **Conclusions:** These preliminary histological and behavioral findings suggest that muscle fibers central nuclei quantification and grip strength measurements could be appropriate outcome measures to assess the efficacy of molecular therapies in this humanized knock-in *COL6A1* mouse model. Data collection is still ongoing to assess the behavioral and histological phenotypes of older mice. This model represents a unique opportunity to study a common human intronic mutation, and test human-ready antisense drugs.

738. AAV RNAi Muscle Restricted Expression for Myotonic Dystrophy Gene Therapy

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Dominant disease results from 'gain of function' toxicity. For myotonic dystrophy type 1 (DM1) the mutant mRNA from the *dystrophia myotonica protein kinase gene* (*DMPK*) is responsible for the adult muscular dystrophy marked by muscle hyperexcitability, muscle wasting and other multisytemic effects. The rare genetic DM1 mutation (1:8000 individuals) is a CTG microsatellite repeat expansion in the *DMPK* 3' UTR. This mutation causes an RNA gain of function mainly due to repeat-expanded RNA (RER) sequestration of splicing factors. Targeting the RER with antisense DNA oligonucleotide technologies was able to reverse the disease phenotype in animal studies, but not in clinical trials because of insufficient nuclear accumulation. Our approach for developing a therapy for DM1 combines AAV gene delivery with RNAi gene silencing. Constitutive expression of miR30-based RNAi cassettes was successful in mitigating toxicity in the human skeletal actin-based mouse model of DM1, the *HSA*^{LR} mouse, carrying a CTG long repeat expansion. Application of gene therapy vectors for RNAi-mediated gene silencing for muscle disease has not been tested in clinical trials. For initial safety testing of AAV6 RNAi gene therapy with systemic delivery, we plan to reduce expression in non-myogenic cells to reduce the potential for off-target effects in an effort to balance expression levels needed for efficacy with cell type restricted RNAi expression. In advancing vector development for muscular dystrophy clinical applications we have engineered promoters for RNA polymerase II (Pol 2) tissue restricted expression. Combining our expertise developing strong Pol 2 muscle promoters with experience in RNAi delivery *in vivo* we have designed and are testing combinations of single and multiple RNAi hairpins in the context of muscle restricted gene expression in myoblast cell lines. Our goal it to optimize Pol 2 muscle RNAi gene expression relative to constitutive Pol 3 promoter expression currently in use. In future studies we will test candidate AAV6 Pol 2 HSA RNAi vectors determine their potential for reducing the mutant HSA RER in the *HSA*^{LR} mice in preclinical studies.

739. Development of Administration Protocols for Reduction of the Necessary Dose of rAAV for Treatment

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Background: AAV vectors are highly safe and allow long-term expression of therapeutic genes. Gene therapy using rAAV has been attempted for systemic diseases. It is used to treat genetic diseases such as SMA and DMD. When performing intravenous administration of rAAV, a dose of $3x10^{13}$ to $1x10^{14}$v.g./kg BW is common. Systemic administration of $1x10^{14}$ v.g./kg BW and above produces direct liver injury, immune response to viral vectors, and the risk of germline transmission, thus requiring an attempt to reduce the dosage. Duchenne muscular dystrophy (DMD) is a congenital disease causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. Supplementation of dystrophin using rAAV-microdystrophin is sufficient to reduce pathogenesis of animal models of DMD. Here we investigated strategies using somatic stem cells in combination with rAAV to induce immune tolerance to the rAAV9 vector and to achieve adequate transgene expression at lower doses. MSCs have been employed in various inflammatory diseases including

graft-versus-host disease (GvHD) by their immunosuppressive effects. Furthermore, immune modulating effects of MSCs on rAAV transduction were examined. **Methods:** Bone marrow-derived MSCs and rAAV9-luciferase or rAAV9-microdystrophin were intravenously injected into normal or CXMDJ dogs at eight weeks old. Seven days after injection, MSCs were systemically injected again. At eight days after the first injection, rAAV9-luciferase or rAAV9-microdystrophin were intravenously injected into the same dog. To examine the immune response against rAAV9, IFN-γ expression in the purified canine peripheral leukocytes was analyzed using qRT-PCR. Expressions of the transgene in skeletal muscles of the rAAV9-luciferase or rAAV9-microdystrophin transduced animals were confirmed by immunohistochemistry. Evasion of neutralizing antibodies induction against rAAV was determined at various time points of rAAV-transduced dogs. In addition, MSCs-treated CXMDJ with rAAV9-microdystrophin transduction and non-treated CXMDJ were compared to assess gait function and lameness of the limb. **Results:** Administration of rAAV9 following MSCs treatment resulted in higher expression of the transgene (luciferase or microdystrophin) at the skeletal muscle, compared to the rAAV transduction alone. Expression of IFN-γ in the purified peripheral blood leukocytes after the rAAV9 exposures were not enhanced in the rAAV9 with MSCs, suggesting the immune suppressive effects of the MSCs. The CXMDJ treated with MSCs and rAAV9-microdystrophin showed a better functional improvement than other DMD dogs of the same age. **Conclusion:** Our results demonstrate that rAAV injection with MSCs pre-treatment improved expression of the rAAV-derived transgene in dogs. This strategy would be a practical approach to analyze the expression and function of the transgene *in vivo*. These findings also support the future feasibilities of rAAV-mediated protein supplementation strategies to treat DMD.

740. Extracellular RNA as a Molecular Biomarker of Central Nervous System Manifestations in Myotonic Dystrophy Patients

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Background: Myotonic dystrophy (*dystrophia myotonica* or DM) is the most common muscular dystrophy in adults (prevalence 1/8,000) and an autosomal dominant multisystem disease caused by a CTG trinucleotide repeat expansion in *DMPK* gene (DM type 1) or CCTG tetranucleotide repeat expansion in *CNBP* gene (DM type 2). These DNA expansions are expressed as repeat expansion RNA molecules that are pathogenic in several tissues, including skeletal muscle, heart, and brain. Mis-regulation of alternative splicing is the most well understood molecular disease mechanism in DM. Alternative splice events in extracellular RNAs (exRNAs) of urine have demonstrated a role as DM1-specific biomarkers (Antoury, et al., 2018). Daytime sleepiness and cognitive impairment are frequent central nervous system (CNS) manifestations that significantly interfere with the quality of life of DM patients. Reliable molecular biomarkers of CNS involvement in DM are lacking; their identification would have enormous value in a better understanding of disease pathogenesis and at directing future therapy design. **Objective:** To identify an extracellular RNA transcriptomic pattern in DM patients' biofluids as a molecular biomarker of CNS manifestations of the disease. **Methods:** Urine samples were collected from our DM1 patient cohort (N = 37) and unaffected controls (N = 31). DM1 subjects underwent clinical assessment for cognitive impairment using Montreal Cognitive Assessment (MOCA) and excessive daytime sleepiness using the Fatigue and Daytime sleepiness scale (FDSS) and/or Epworth Sleepiness Scale (ESS). Excessive daytime sleepiness (+EDS) was defined as the need to take daytime naps, use of stimulant medication (such as modafinil), and/or use of nighttime non-invasive ventilation (CPAP or BIPAP).To isolate extracellular RNA, we removed cells by centrifugation at low speed (2450 x g), passed the supernatant through a 0.8 μm filter, followed by ultracentrifugation. We used droplet digital PCR to quantify the percent exon inclusion of *INSR, MBNL1, MBNL2, CLASP1, MAP3K4, and GOLGA4* transcripts. The percent exon inclusion of each transcript in urine sample was correlated +EDS, MOCA, FDSS, and ESS scores for each subject. In a pilot study, we also examined splice events in exRNA and cells collected from cerebrospinal fluid. **Results:** Quantification of*INSR*(containing exon 11) and *MAP3K4* (containing exon 17) transcripts in urine exRNA of DM1 patients demonstrated statistically significant differences in +EDS group in comparison with DM patients without EDS ($P< 0.0001$ *INSR*; < 0.05 *MAP3K4*) and unaffected control subjects ($P< 0.0001$, both *INSR* and *MAP3K4*). Evaluation of cognitive impairment by using the MOCA scale appears to be influenced by the presence of EDS in our cohort. *DMPK* transcripts and splice events of transcripts *INSR*, *MBNL2*, *MAP3K4*, and *GOLGA4* are quantifiable in extracellular RNA and cells from cerebrospinal fluid of non-DM patients. **Conclusions:** The urine exRNA transcriptome may serve as a convenient and renewable molecular biomarker of EDS in DM patients. Exon inclusion of transcripts *INSR* and *MAP3K4* are candidate individual biomarkers of EDS in DM patients. The exRNA transcriptome in cerebrospinal fluid may be an alternative source of molecular biomarkers for cognitive impairment in DM patients. Our results support the further development of exRNA as molecular biomarkers of central nervous system involvement in DM.

741. A Compact Muscle and Heart-Specific Promoter for Efficient rAAV Mediated Expression of Exogenous Genes and Genome Editing

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Systemic delivery of recombinant adeno-associated virus (rAAV) 6, 8, 9, rh74 and rh10 vectors mediate efficient transduction of the entire striated musculature, making this an attractive strategy for muscle gene therapy. When combined with a muscle specific promoter, systemic rAAV delivery can achieve highly muscle-specific expression of transgenes. Owing to the small packaging capacity of rAAV, it is important to minimize the promoter for delivering large transgenes such as Clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9). Here we describe a small regulatory cassettes (MHP1, 483 bp) based on enhancer/promoter regions of murine muscle creatine kinase (CK) and α-myosin heavy-chain genes. MHP1 (483 bp) drove high-level expression of EGFP in C2C12 myotubes, but not in C2C12 myoblasts or HEK293 cells. Following systemic rAAV9 delivery in mice, MHP1 directed high-level expression of EGFP comparable to

cytomegalovirus (CMV) promoter in skeletal and cardiac muscles, with low to undetectable expression in the liver, lung, kidney and spleen. Finally, we showed that MHP1 enabled efficient muscle and heart-specific genome editing in mice with systemic rAAV9 delivery. Taken together, this study provides a novel compact promoter for muscle and heart-specific expression of transgenes and genome editing.

742. Biodistribution of AAVpo1 and AAVpo1A1 in Mice with Myotubular Myopathy

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Recombinant adeno-associated viral (AAV) vectors are powerful vehicles for *in vivo* delivery of therapeutic transgenes. Tissue tropism and consequently vector dosage for gene therapy can vary amongst naturally occurring and engineered AAV serotypes. We previously reported that serotype 8 AAV-mediated MTM1 gene delivery can correct the phenotype of murine and canine models of myotubular myopathy, a severe pediatric disorder leading to generalized muscle weakness and respiratory failure in patients. In order to identify AAV vectors with increased skeletal muscle potency in myotubularin deficient muscle, in the present study we compared the biodistribution and transgene expression of serotype 8, 9 and rh10 vectors with AAVpo1, and its derivative AAVpo1A1, of porcine origin that carry a MTM1 expression cassette. A single dose of $2x10^{13}$vg/kg of each vector was administrated intravenously in mutant mice and tissues were harvested 4 weeks post-injection. We found that administration of AAV9-, AAVrh10- and AAVpo1A1 vectors resulted in higher myotubularin expression levels in skeletal muscles compared to AAV8- and AAVpo1 vectors. In addition, the AAVpo1A1 vector detargeted liver transduction. These results indicate that AAVpo1A1 capsid might be suitable for gene transfer in XLMTM muscles.

743. Abstract Withdrawn

744. Titin Splicing Regulates Cardiotoxicity Associated with Calpain 3 Gene Therapy for Limb-Girdle Muscular Dystrophy Type 2A

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Limb-girdle muscular dystrophy type 2A (LGMD2A or LGMDR1) is a neuromuscular disorder caused by mutations in the calpain 3 gene (CAPN3). Previous experiments using adeno-associated viral (AAV) vector-mediated calpain 3 gene transfer in mice indicated cardiac toxicity associated with the ectopic expression of the calpain 3 transgene. Here, we performed a preliminary dose study in a severe double-knockout mouse model deficient in calpain 3 and dysferlin. We evaluated safety and biodistribution of AAV9-desmin-hCAPN3 vector administration to nonhuman primates (NHPs) with a dose of 3E13 viral genomes/kg. Vector administration did not lead to observable adverse effects or to detectable toxicity in NHP. Of note, the transgene expression did not produce any abnormal changes in cardiac morphology or function of injected animals while reaching therapeutic expression in skeletal muscle. Additional investigation on the underlying causes of cardiac toxicity observed after gene transfer in mice and the role of titin in this phenomenon suggest species-specific titin splicing. Mice have a reduced capacity for buffering calpain 3 activity compared to NHPs and humans. Our studies highlight a complex interplay between calpain 3 and titin binding sites and demonstrate an effective and safe profile for systemic calpain 3 vector delivery in NHP, providing critical support for the clinical potential of calpain 3 gene therapy in humans.

745. Precise Gene Editing of the Most Common LGMD2A Mutation

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Gene editing is a powerful tool to repair disease-causing mutations in patient-derived primary cells. However, precise gene correction in these cells remains inefficient. In our study, we aimed to repair mutations in the *CAPN3* gene. Calpain 3, the protein encoded by *CAPN3*, is a cysteine-protease predominantly expressed in skeletal muscle. Mutations in *CAPN3* cause Limb Girdle Muscular Dystrophy Type 2A (LGMD2A), a progressive skeletal muscle disorder without treatment and the most common form of LGMD worldwide. We isolated and expanded human primary muscle stem cells from 35 patients carrying 37 different *CAPN3* mutations. 20% of the patients carry the well-known founder mutation *CAPN3*c.550delA causing frame shift in exon 4, which creates a pre-mature stop codon. In most cases, patients carry compound heterozygous *CAPN3*c.550delA mutations, two patients with homozygous c.550delA mutations are also part of our cohort. From a homozygous patient we isolated human primary muscle stem cells, expanded and transfected them with a plasmid, which carries mutation-specific sgRNAs and Cas9::Venus. After cell sorting and expansion, a subsequent in-depth sequence analysis of the *CAPN3*c.550 DNA region showed base insertions and deletions (indels) at the targeted *CAPN3* locus with an efficiency of up to 60 %. One of the sgRNAs had a preference of a +1 bp insertion at the position of the mutation demonstrating an indel signature bias of specific sgRNAs and reframing of the ORF. The effects on protein level are analyzed using a new custom made monoclonal anti-CAPN3 antibody suitable for immunostaining. The repair strategy is currently being expanded towards compound heterozygous mutations in primary human muscle stem cells. Additionally, we target the *CAPN3*c.550delA mutation with a new technique, Prime Editing, which might correct allele specific mutations by applying reverse transcriptase cloned to a modified Cas9. Patient-derived iPS cells are also part of our gene correction tool-box developed to target LGMD2A. We demonstrate here for the first time precise gene correction in primary human muscle cells for a *CAPN3* founder mutation. The results will support therapy development for this devastating disease.

746. AAV9 Mediated Delivery of RNA Targeting CRISPR Corrects Molecular and Functional Phenotypes in Myotonic Dystrophy Type 1 Mouse Model

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Myotonic dystrophy type I (DM1) is a multisystemic autosomal-dominant inherited disorder caused by CTG microsatellite repeat expansions (MREs) in the 3' untranslated region (UTR) of the DMPK gene. Previously, we showed that a CRISPR-based RNA-targeting gene therapy has the potential to eliminate toxic RNAs expressed from repetitive tracts in DM1 in primary patient cells. We have now investigated the *in vivo* efficacy of this approach in a series of experiments in a DM1 mouse model. RNA-targeting Cas9 and CUG-targeting guide RNAs were packaged in AAV9 vectors and delivered to adult and neonatal DM1 model mice. Sustained expression of RNA-targeting Cas9 and guide RNAs was observed, accompanied by efficient reversal of both molecular (RNA foci, and mis-splicing) and physiological (myotonia) features of DM1. The reversal of DM1 phenotypes indicates the potential of RNA-targeting CRISPR systems as treatments for DM1 and other MRE diseases.

747. Establishing the Efficacy, Safety, and Biodistribution of FX201, a Helper-Dependent Adenoviral Gene Therapy for the Treatment of Osteoarthritis, in a Rat Model

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INTRODUCTION: FX201 (humantakinogene hadenovec) is a novel helper-dependent adenovirus (HDAd) based on human serotype 5 adenovirus (Ad5) expressing interleukin-1 receptor antagonist (IL-1Ra). FX201 is in development for the treatment of osteoarthritis (OA) and is designed to induce IL-1Ra production in the presence of inflammation (via a nuclear factor-kappa B-responsive promoter). Here, we evaluated efficacy, safety, and biodistribution of HDAd-ratIL-1Ra, the rat surrogate of FX201, and the biodistribution of FX201, when administered as a single intra-articular (IA) injection in the anterior cruciate ligament transection (ACLT) rat model of OA. **METHODS:** Sprague-Dawley rats underwent ACLT surgery in the right knee (except sham animals). To assess efficacy, male rats (n=12 per group) received a single IA injection of HDAd-ratIL-1Ra (3.6×10^7 or 3.1×10^8 genome copies [GC]/dose) or vehicle 1 week postsurgery. Efficacy was assessed via histopathology at Week 12 using a semi-quantitative microscopic grading system (Osteoarthritis Research Society International [OARSI] score) for OA-related cartilage changes. To assess safety and vector biodistribution, 2 Good Laboratory Practice studies were performed in which rats received a single IA injection of HDAd-ratIL-1Ra, FX201, or vehicle in the right knee joint 28 days postsurgery. In the first study, male rats (n=12 per group at each time point) were assigned to 6 study groups: HDAd-ratIL-1Ra (3.2×10^8, 3.1×10^9, or 4.3×10^{10} GC/dose), ACLT/vehicle, ACLT/untreated, or sham/untreated. Safety was assessed at Days 29 and 92 postdose. Vector biodistribution was assessed in a second study of ACLT-operated rats receiving HDAd-ratIL-1Ra (4.3×10^{10} GC/dose) or FX201 (4.1×10^{10} GC/dose) at Days 8, 29, and 92 postdose (n=12 [1:1 sex ratio] per group at each time point). **RESULTS:** In the efficacy study, HDAd-ratIL-1Ra demonstrated a dose-dependent decrease in OARSI composite scores compared with vehicle. In the safety study, HDAd-ratIL-1Ra had no adverse effects on mortality, body weight and food consumption, or clinical or anatomical pathology at any dose studied. Anti-Ad5 titers increased with HDAd-ratIL-1Ra dose and decreased from Day 29 to 92. Similarly, dose-dependent anti-Ad5 T cell responses decreased from Day 29 to 92 as assessed by interferon-γ ELISpot with splenocytes from HDAd-ratIL-1Ra-treated rats. In the biodistribution study, HDAd-ratIL-1Ra and FX201 were detected up to Day 92, with the highest concentrations at the injection site. Both were sporadically detected at low levels in the liver, spleen, lung, and bone marrow at Days 8 and 29; however, neither was detected in plasma or organs such as the brain, heart, and kidney at any time point examined, consistent with the absence of systemic circulation. **CONCLUSIONS:** Following a single local IA injection in rats with ACLT, HDAd-ratIL-1Ra ameliorated OA-related changes in cartilage 12 weeks postsurgery at all doses tested. HDAd-ratIL-1Ra was well tolerated, and the no-observed-adverse effect level was considered to be 4.3×10^{10} GC/dose, the highest dose tested, providing a 1000-fold multiple over the minimal efficacious dose (3.6×10^7 GC/dose). Furthermore, HDAd-ratIL-1Ra and FX201 did not enter systemic circulation. These results support further development of FX201; a Phase 1 study in patients with knee OA is currently underway (NCT04119687).

748. Abstract Withdrawn

749. Abstract Withdrawn

750. Targeting Muscle Satellite Cells for *In Vivo* Gene Editing With Adeno-Associated Viral Vectors for Duchenne Muscular Dystrophy

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Adeno-associated viral (AAV) delivery of gene editing tools for treatment of muscular dystrophies have yielded encouraging results in various animal disease models. Although AAV efficiently targets muscle fibers, there is conflicting evidence on whether AAV targets muscle stem cells, also known as satellite cells (SCs). SCs reside in a specialized niche between the basal lamina and sarcolemma of individual muscle fibers and account for ~5% of all muscle nuclei. Because SCs continuously replenish skeletal muscle in response to tissue damage, the genetic correction of these self-renewing cells could generate a

sustained source of therapeutic gene production. Previous studies have detected some level of gene editing in SCs via AAV9 transduction; here, we sought to expand upon these studies by determining the most efficient AAV serotype for SC targeting, quantifying the level of gene editing in SCs, demonstrating *in vivo* functionality of gene edited SCs, and measuring the activity of muscle specific promoters in SCs. We used a dual reporter mouse to permanently mark SCs transduced by AAV. We started with Pax7-nGFP mice, in which GFP is knocked into the first exon of Pax7 to label SCs. We crossed these to Ai9 mice, which harbor a CAG-LSL-tdTomato cassette at the Rosa26 locus. Cre-mediated recombination removes the stop codon, leading to tdTomato expression in cells transduced by AAV-Cre. We assessed a panel of AAV serotypes for SC tropism following intramuscular (IM) or systemic intravenous (IV) injection. For IM injections, we found that AAV9, AAV6.2, AAV8, and AAV1 marked the SCs cells most efficiently, at ~60% as determined by flow cytometry analysis. We injected these top 4 performing AAV serotypes systemically and found AAV8 and AAV9 were most effective, ranging from 20-30% recombination efficiency in SCs for various muscle types. Next, we sought to assess the level of *in vivo* gene editing in SCs with a dual AAV9-CRISPR strategy consisting of one AAV9 vector encoding SaCas9 and the other AAV9 vector encoding two gRNAs designed to excise exon 23 from the *Dmd* gene in *mdx* mice. 8 weeks after IM injection, SCs were sorted and genomic DNA was isolated immediately without culturing the cells. We readily detected deletion bands in SCs isolated after IM and IV injections of AAV9-CRISPR. To quantify the level of gene editing at the *Dmd* locus in SCs, we adapted Illumina's Nextera-transposon-based library preparation method for unbiased sequencing. Using this method, we quantified gene-editing outcomes including exon deletion, indels, inversions, and AAV integration in SCs. To demonstrate that the CRISPR-edited SCs can differentiate and give rise to dystrophin+ muscle fibers, we performed a transplantation experiment. Donor *mdx* mice were injected with AAV9-CRISPR and SCs were harvested 8 weeks later and engrafted into *mdx* host mice. Immunofluorescence staining after 4 weeks revealed significantly more dystrophin+ muscle fibers (5.95±0.40 fibers/mm^2) compared to host mice that received SCs from donor mice injected with PBS (1.46±0.41 fibers/mm^2). Lastly, we tested the activity of the muscle-specific promoters CK8, SPc5-12, and MHCK7 in SCs. Although these muscle-specific promoters are utilized in clinical trials to express transgenes in muscle fibers, it is unclear if they are capable of driving gene expression in SCs. Compared to the CMV promoter, these muscle-specific promoters induced Cre-recombination about half as efficiently. We also tested these promoters in our AAV9-CRISPR system and detected exon23 deletion events by PCR in sorted SCs, confirming that muscle-specific promoters can target SCs for gene editing. Together, our results confirm that SCs can be targeted by AAV in dystrophic muscle contexts and can undergo successful gene-editing to restore the dystrophin reading frame in the *mdx* mouse.

Cancer - Immunotherapy, Cancer Vaccines

751. In Vivo Expression of Plasmid Encoded Checkpoint Inhibitors by Synthetic Enhanced DNA as a New Tool for Cancer Immunotherapy

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Cancers employ various strategies to escape immune surveillance including the exploitation of immune checkpoints. Immune checkpoints are proteins that regulate the duration or potency of an immune response. Tumor cells often upregulate ligands that activate these checkpoints to protect themselves from the host immune response. Monoclonal antibody (MAb) therapeutics which block checkpoint-ligand interactions restore T cell destruction of cancer cells *in vivo*. MAbs that target the inhibitory T cell signaling mediated by immune checkpoints have gained regulatory approval for the treatment of some cancers based on remarkable clinical outcomes. Here we have focused on a new method to improve MAb delivery through direct engineering of MAb (DMAbs) in the form of synthetic enhanced DNA. This technology would improve many aspects of such a therapy by lowering cost, increasing *in vivo* expression times and allowing for simple combination formulations in the absence of a host anti-vector immune response, possibly extending use of these groundbreaking therapies to disadvantaged patient populations. We report that synthetic enhanced DNA technology can be used to direct *in vivo* production of established monoclonal antibodies which can target the immune checkpoints such as PD1, 4-1BB and OX40. These antibodies are produced at physiologically relevant levels in blood and other tissues of mice using electroporation-enhanced delivery of DNA plasmids encoding genes for each antibody. Importantly, we report that checkpoint inhibitor DMAb can be expressed in the Wistar humanized mice (huMice), retaining the ability to bind to their targets and exhibiting immune stimulatory effects for host T cells. These studies have significant implications for prophylactic and therapeutic strategies for cancer, where a broad application of huMice in cancer immunotherapy warrants further attention.

752. Driving ADP-A2M4 SPEAR Expression from an Endogenous Hematopoietic Lineage Promotor for Off-The-Shelf T-Cell Therapy for MAGE-A4+ Solid Tumors

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Herein, we describe progress with our allogeneic T-cell lines (iT-cell) derived from directed differentiation of human induced pluripotent stem cells (hiPSCs). We demonstrate that by using targeted genetic

engineering we can produce clonal iT-cell populations that express the ADP-A2M4 SPEAR (Specific Peptide Enhanced Affinity Receptor) driven from an endogenous promotor. ADP-A2M4 SPEAR expressing iT-cells are shown to exhibit a range of effector functions upon exposure to tumor lines expressing cognate MAGE-A4 peptide (*GVYDGREHTV*) presented by HLA-A*02. The treatment of cancer with adoptive T-cell therapy is an expanding area of therapeutic intervention. Most current approaches use autologous or patient-derived T-cells that must be harvested from the patient, transferred to a manufacturing facility, and usually genetically modified prior to expansion and transfusion back into the patient. These autologous therapies have performed well in the clinic; however, there are advantages to moving away from patient-derived cells, and developing "off-the-shelf" or allogeneic approaches. An "off-the-shelf" approach can significantly reduce the vein-to-vein times and logistical challenges associated with autologous cell manufacturing. It also provides the opportunity to develop a more consistent T-cell product that does not vary from patient to patient. One source of allogenic T-cell products are those differentiated from hiPSCs. HiPSCs are an attractive starting material as they can proliferate indefinitely, in an undifferentiated state, allowing the production of large cell banks that in turn can be differentiated to produce multiple therapeutic doses. HiPSCs are amenable to genetic engineering and can also be directed to differentiate into various lineages that are clinically useful, such as T-cells or NK cells. The challenge with any allogeneic approach is to ensure that the final product expresses a defined T-cell receptor (TCR) to reduce the risk of graft-versus host disease (GvHD). Here we describe a novel approach via targeted knock-in of the ADP-A2M4 SPEAR in hiPSC, exemplified in two separate clones. This results in the formation of a multi-cistronic expression cassette where the native gene and ADP-A2M4 SPEAR expression are both regulated by the endogenous promoter. The temporal pattern of expression of both genes is linked throughout the differentiation process. Each edited clone was characterized to have one edited and one wild type allele. We demonstrate that edited hiPSC lines can produce iT-cells via a series of defined progenitor populations: $CD34^{+/}CD45^{+}$ hematopoietic progenitor cells (HPCs), common lymphoid precursors (CLP), pro T-cells, double negative (DN) cells, $CD4^{+}/CD8^{+}$ double positive (DP) cells, and $CD3^{+}/CD8^{+}/TCR^{+}$ single positive (SP) T-cells without the use of a feeder or stromal cell line. Flow cytometry shows that iT-cells expressing CD3 and ADP-A2M4 SPEAR upregulate CD69/CD25 and produce cytokine (IFNγ/TNFα) in response to antigen. These differentiated cells express high levels of granzyme and are capable of killing antigen positive tumor lines. Overall, this work represents the development of an allogeneic hiPSC derived platform, with limited genome editing, that permits the production of SPEAR iT-cells with potential therapeutic value. The identification of a suitable locus for targeted integration of a defined TCR/SPEAR enables the future production of multiple iT-cell banks directed against specific tumor associated antigens in a defined and reproducible manner.

753. Transposon Mediated CAR-T Cell Manufacturing Using Scalable Electroporation of Resting PBMCs or Activated T Cells

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Autologous CAR-T cell immunotherapies continue to face a multitude of challenges, including manufacturing costs and safety concerns associated with random integration, which ultimately can limit the treatable patient population and lead to unpredictable and highly variable clinical outcomes. As an alternative to viral-based gene delivery, transposon-based engineering provides for stable CAR expression using resting or activated T cells with lower immunogenicity and potentially improved safety profiles. In this poster, we describe high performance, transposon-mediated manufacturing of CAR-T cells using a clinically validated, scalable electroporation system. Data are presented for high efficiency, low toxicity delivery of Sleeping Beauty and Piggy Bac transposon plasmids or mini-circle DNA to primary T cells. Data will be highlighted for the construction, scalability, and *in vitro* and *in vivo* characterization of CAR-T cells specific for 3 different cancer targets currently under investigation - SLAMF7, EPHB4 and GD2. Additionally, data are shown for the engineering of resting PBMC which allows for the expansion post electroporation of CAR-T cells with beneficial cell phenotypes, such as PD-1 low T cells with a naïve/stem cell memory cell profile. Overall, we demonstrate the use of a clinically scalable electroporation platform to support the development and manufacturing of CAR immunotherapies engineered using transposon-based systems.

754. Regulated Membrane-Bound IL15 Drives Controlled Expansion of Tumor Reactive Lymphocytes

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Cell therapies such as CART cells have improved clinical outcomes for patients with hematologic cancers. Application to indications beyond B cell malignancies will require additional engineering that increases the efficacy and potency of immune cell therapy without compromising patient safety. Unlike small molecules or biologics, cell therapies are "living drugs" that may have unpredictable pharmacologic properties after infusion into the patient, which may cause unintended toxicity or limit durability of response. Durable response correlates to immune cell expansion and persistence *in vivo*, both of which are required for tumor clearance and long-term tumor surveillance, respectively. Interleukin 15 (IL15) drives T and NK cell expansion and persistence in an antigen-independent manner which therefore would enhance the anti-tumor activity and clinical potential of these cells. However, soluble IL15 protein has been toxic in patients, thus limiting its clinical application. To enable the use of cytokines in cell therapy, we developed a pharmacologic control system based on Drug Responsive Domains (DRD) that provides precise control over adoptively transferred immune cells using FDA-approved drugs. DRDs are small human proteins that are unstable in the cell but reversibly stabilized when bound to small molecule drugs. DRD fusion to a therapeutic transgene-derived protein, such as IL15, confers drug-dependent, reversible regulation of functional protein expression, which in turn provides a mechanism to control the activity of the engineered cells. To develop IL15-powered immune cell therapy, we gene-modified immune cells

with a pharmacologically controllable, membrane-bound human IL15 (mbIL15) that supports antigen-independent T and NK cell expansion and has the potential to reduce the safety risks associated with continuous or systemic soluble IL15 exposure by localizing cytokine in proximity to the engineered cells. We fused our novel mbIL15 to a DRD derived from the carbonic anhydrase 2 protein (CA2) and after mbIL15-CA2 gene transfer into immune cells, the mbIL15 is expressed only in the presence of the stabilizing ligand, acetazolamide (ACZ). ACZ treatment of mbIL15-CA2 (engineered) T cells increased IL15 in a drug dose-dependent manner, increased cell survival, and supported 15-fold expansion without supplemental cytokine treatment or antigen stimulation. To evaluate antigen-independent T cell expansion *in vivo*, engineered T cells were infused into ACZ treated NSG mice. Significant engineered T cell expansion was observed in ACZ but not vehicle treated mice ($p<0.0001$, 2-way ANOVA). Co-infusion of engineered T cells with donor-matched unmodified NK cells significantly increased bystander NK cell persistence in ACZ but not vehicle treated mice, demonstrating that regulated mbIL15-CA2 also supports NK cell survival ($p<0.0001$, 2-way ANOVA). To determine whether regulated IL15 increased anti-tumor efficacy, T cells were modified to co-express mbIL15-CA2 and CD19-targeting CAR and transferred into NSG mice bearing $CD19^+$ tumors. Regulated mbIL15 increased anti-tumor activity and persistence of CD19-targeting CART cells, supporting rapid tumor clearance, even at suboptimal CART cell doses. These results show that our novel, regulatable mbIL15 supports antigen-independent T cell expansion, increases bystander NK cell persistence, and enhances anti-tumor activity of CART cells. Our DRD pharmacologic control system thus confers 'drug-like' properties to cell therapy and has the potential to increase efficacy, durability of response, and improved clinical outcome for patients.

755. Identifying Tumor-Reactive TCRs for Adoptive Cell Therapies by Combining CDR3 Repertoire Analysis with Transcriptional Signature Analysis

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Personalised Adoptive Cell Therapies (ACT) have great potential, but identifying tumor-reactive T-cell receptors (TCRs) requires the development of highly personalized, scalable, cost- and time efficient pipelines. While the generation of single cell TCR and RNA sequencing data is now easily performed using commercially available kits - most commonly using 10X Genomics' Chromium controller - identifying the tumor-reactive needles within the TCR repertoire haystack remains challenging. Experimental enrichment of relevant TCRs is possible using the *Mutation-Associated Neoantigen Functional Expansion of Specific T-Cells* or 'MANAFEST' assay, however considerable progress has also been made on the bioinformatics front, including repertoire analysis using deep learning (the DeepTCR package from the Baras lab) and convergent selection analysis (the ALICE and OLGA packages from the Walczak lab). In cases where tumor infiltrating leukocytes (TILs) are available, single cell RNA + TCR sequencing can be performed, and tumor-reactive TILs distinguished from bystander TILs by their transcriptional profile. Here we present our progress in combining experimental enrichment, bioinformatic repertoire analysis, and newly developed transcriptional signatures for identifying candidate tumor-reactive TCRs in primary material from various cancer modalities. We also describe the development of a semi-automated, medium-throughput, high-efficiency, single-step cloning pipeline to transfer selected patient-derived TCRs from sequenced 10x single cell VDJ Illumina libraries into a novel, safe, S/MAR based gene therapy vector.

756. Natural Killer Cells Engineered to Target Adenosingeric Immunometabolic Suppression as an Immunotherapy Against Lung Carcinoma

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Genetically engineered natural killer (NK) cells have emerged as a remarkably promising treatment modality for the immunotherapeutic treatment of various cancers. However, solid tumors, such as lung adenocarcinoma, are resistant to traditional treatments and remain a formidable challenge due to their complexity and heterogeneity. The tumor microenvironment (TME) has a modified cellular metabolism that can result in immune suppression and NK cell metabolic reprogramming, leading to altered immune function and inhibition of NK effector functions. In the hypoxic environment, high levels of ATP are released from the cancer cells, which are subsequently dephosphorylated to adenosine (ADO) by cancer-associated ectoenzymes CD39 and CD73. This accumulation of ADO acts as a powerful immunosuppressant that stunts the NK cell function. Our previously published data has determined that the TME adenosine results the suppression of cytokine-primed NK cells through the reorganization of NK cell metabolism and impairment of anti-tumor functions. Also, the cytokine combination of IL-12/Il-15 showed hyperresponsiveness to adenosine compared to IL-2 and IL-15 alone. To overcome the immunosuppression induced by cancer-producing adenosine, we have engineered the NK cells to directly target CD73 by imparting NK-specific signaling derived from FCγRIIIa (CD16). These engineered NK cells are able to specifically target CD73+ lung adenocarcinoma, block adenosine-producing CD73, and enhance their anti-tumor cytotoxicity. To achieve that, peripheral blood-derived NK cells were isolated from healthy human donors and expanded using feeder cells. The novel genetic construct was electroporated into NK cells or was instead transduced virally. Engineered NK cells expressing the construct were then tested for transfection efficiency and killing ability against CD73+ lung carcinoma A549 cells using the lactate dehydrogenase (LDH) test. The cytokine production of the engineered NK cells was also measured through the production of IFNγ and the release of cytotoxic granules as measured by CD107a. The engineered NK cells were then adoptively transferred into NSG mice using a CD73+ lung cancer xenograft model. Mice were assessed for tumor growth inhibition and tumors were visualized using immunohistochemistry

to determine infiltration. Circulating CD73-CAR NK cells were also analyzed for activating marker expression NKG2D, DNAM, and NKp30. CD73-targeting NK cells were shown to prevent the production of immunosuppressive adenosine through a blockade against CD73 when engineered with the synthetic CD73- FCγRIIIa construct. These retargeted NK cells showed heightened anti-tumor killing ability *in vitro* against CD73-expressing A549 cells. Killing ability of the engineered NK cells was also slightly enhanced compared to NK cells co-incubated with CD73-antibody. Engineered primary NK cells showed promise for up to 3 weeks *in vivo* in stunting CD73+ lung cancer tumor growth. Circulating CD73-CAR NK cell activating marker expression remained unchanged compared to that of adoptively-transferred non-modified NK cells ten days post-infusion, indicating persistence of the cells. We are currently evaluating the off-target effects of such engineered NK cells. The TME is highly immunosuppressive via the activity of CD73 and accumulation of adenosine. Therefore, to redirect NK cell function, a novel NK-specific anti-CD73 targeting construct is being pursued as a promising adoptive immunotherapy to prevent tumor growth of CD73+ lung adenocarcinoma, which is potentially extendable to other CD73-expressing tumors.

757. 1st-In-Human CAR T Targets MUC1 Transmembrane Cleavage Product

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We developed a CAR T that recognizes the growth factor receptor form, MUC1*, does not bind to full-length MUC1, hits a wide range of cancers and shows to little or no binding to normal tissues. No therapeutic has ever been tested in humans that targets the MUC1 transmembrane cleavage product called MUC1*. A 1st-in-human clinical trial of huMNC2-CAR44, NCT04020575, for metastatic breast cancers is underway at a cancer research center in Seattle. We expect to have patient data by conference time. MUC1 biology has historically been poorly understood. Several flawed reports are still widely cited in the literature. We will demonstrate that full-length MUC1 plays no role in tumorigenesis, elimination of full-length MUC1 greatly accelerates tumor growth *in vitro* and *in vivo* and the cleaved tandem repeat domain does not form a heterodimer with the remaining transmembrane portion. We will show that MUC1* is a Class I growth factor receptor that is activated by ligand-induced dimerization of its truncated extra cellular domain, which activates the MAP kinase signaling pathway as well as survival pathways. Onco-embryonic growth factor $NME7_{AB}$ binds to an ectopic site on MUC1* that is only unmasked after MUC1 is cleaved and the tandem repeat domain is shed from the cell surface. $NME7_{AB}$ looks like a single chain dimer of pseudo-identical domains that each can bind to a MUC1* extra cellular domain. Because it can dimerize MUC1* as a monomer, it renders the MUC1* growth factor receptor constitutively active. Adult forms of $NME7_{AB}$ limit self-replication by changing multimerization state from the active dimer to inactive higher order multimers. Antibodies such as 5E5 and SM3 bind to aberrant, trapped glycans on O-linked glycosylation sites that are only in the tandem repeat domain, which is shed from the tumor after MUC1 cleavage. Unlike full-length MUC1, MUC1* has no sites for O-linked glycosylation, so MUC1* is missed by antibodies that target aberrant glycans. Importantly, therapeutics that target full-length MUC1 could increase tumorigenesis by enriching for cells expressing the tumorigenic MUC1* growth factor receptor. Minerva's anti-MUC1* antibody, huMNC2, binds to the conformational epitope that is unmasked when MUC1 is cleaved to MUC1*. MMP9, which has been linked to poor prognosis and metastasis, cleaves MUC1 to a tumor-associated growth factor receptor form of MUC1*. huMNC2 and onco-embryonic growth factor $NME7_{AB}$ compete for binding to the same conformational epitope created when MUC1 is cleaved to MUC1* by MMP9. Neither huMNC2 nor $NME7_{AB}$ binds to full-length MUC1. IHC studies of thousands of human tissues - both normal and cancerous - show that the tumor associated antigen is MUC1* and not full-length MUC1. Patient-match primary and metastases show that as cancer stage progresses the amount of MUC1* increases. huMNC2-scFv bound robustly to 95% of the breast cancers, 83% ovarian, 78% pancreatic and 71% of lung cancer tissues (specimens n>2,800). There was little to no staining of normal tissues. *In vivo*, huMNC2-CAR44 T cells inhibited or completely obliterated a variety of MUC1* positive solid tumors in NSG mice. Minerva has developed next-gen CARs, including logic-gated CARs with increased *in vivo* persistence, and intends to file for additional INDs in 2020. **Conclusions:** MUC1* is the predominant form of MUC1 on cancerous tissues. Antibodies that target a conformational epitope in the membrane-proximal MUC1* extra cellular domain are tumor selective. CAR T cells targeting MUC1* extra cellular domain are highly effective against solid tumors in animals. Robust staining of cancerous tissues and minimal to no staining of normal tissues predicts a large therapeutic window for huMNC2-CAR44 T cell dosing. Clinical trial has begun and patient data is expected before conference time.

758. Dominant Negative PD1 Armored CART Cells Induce Remission in Relapsed or Refractory Non-Hodgkin Lymphoma (NHL) Patients

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The chimeric antigen receptor (CAR) T cell treatment has been demonstrated as an effective therapy to treat relapsed/refractory B cell malignancy. However, tumor microenvironment influences and affects the efficacy of CAR T treatment. For example, programmed death ligand 1/2 (PDL1/2) may inhibit the CAR T cells via interaction with up-regulated programmed cell death protein 1 (PD1) after T cells activation, suppressing the tumor-killing capability of the CAR T cells. Thus, blockade of the PD1-PDL1/2 interaction may enhance the anti-tumor efficacy of CAR T therapy. Here, we generated CAR T expressed an anti-CD19 CAR molecule and a dominant-negative PD1 molecule. Compared with conventional CART cells, these "armored" CART cells showed the enhanced capability of tumor-killing and more

"memory-like" phenotypes after multiple-round tumor challenging. These results suggest dominant-negative PD1 molecules may protect CART cells from exhaustion in the tumor microenvironment. Further, we reported the findings of a clinical trial for six relapsed or refractory B-cell non-Hodgkin lymphoma (NHLs) patients treated using our armored CAR T cells. These six patients failed multiple rounds of chemotherapy and radiotherapy. In the clinical trial, the patients were infused with autologous CAR T cells range from1×106/kg to 8×106/kg. PET/CT showed significant tumor shrinkage and SUV max declines in all six patients, and the ongoing responses were monitored. The best overall response rate (ORR)was 100%. **Conclusion:** The results of these six patients in the clinical trial showed that our armored CAR T cells achieved the significant anti-bulky lymphoma response while causing limited and tolerated cytokine release syndrome and central nervous system toxicity. Thus, dominant-negative PD1 molecules may increase CAR T cells persistence in patients, enhancing the efficacy of CAR T cells for treating blood cancer. Finally, dominant-negative PD1 can be used as a platform technology and may be applied to other adoptive cellular immunotherapies such as TCR-T or TIL in the treatment of solid tumors. We are continuing to monitor current patients and recruit more patients for the clinical trial.

759. Dissecting the Immune Cell Progeny of CAR-Modified Hematopoietic Stem Cells in Humanized Mouse Model

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Introduction: Refractory or recurrent B-lineage hematological malignancies have than 50% of chance of cure. Therapy with FDA-approved autologous T-cells expressing chimeric antigen receptors (CAR) have led to complete remissions in chemotherapy-resistant malignancies, but the effector cells do not persist, limiting clinical efficacy. Our hypothesis is the modification of hematopoietic stem cells (HSC) with anti-CD19 CAR will lead to persistent generation of multilineage target-specific immune cells, enhancing graft-versus-cancer activity and leading to development of anti-tumor immunological memory. ***Design/Methods:*** We generated second-generation CD28- and 4-1BB-costimulated CD19-specific CAR constructs using third-generation lentiviral vectors for modification of human HSC for assessment *in vivo* in humanized NSG. Cells were harvested from bone marrows, spleens, thymus and peripheral blood at different time points for evaluation by flow cytometry and ddPCR for vector copy numbers. Cohorts of mice received tumor challenge with subcutaneous injection of lymphoma cell lines. ***Results:*** Gene modification of HSC with CD19-specific CAR did not impair differentiation or proliferation in humanized mice, and led to CAR-expressing, antigen-specific, cell progeny in myeloid, NK and T-cells. Humanized NSG engrafted with CAR-modified HSC presented similar humanization rates to non-modified HSC, with multilineage CAR-expressing cells present in all tissues with stable levels up to 44 weeks post-transplant, similar in both CAR constructs. Animals engrafted with CAR-modified HSC did not present increased autoimmunity or inflammation. T-cell populations were identified at higher rates in humanized mice with CAR-modified HSC in comparison to mice engrafted with non-modified HSC. CAR-modified HSC led to development of T-cell effector memory and T-cell central memory phenotypes, confirming the development of long-lasting phenotypes due to directed antigen specificity. Mice engrafted with CAR-modified HSC successfully presented tumor growth inhibition and survival advantage at tumor challenge with lymphoma cell lines, with no difference between both constructs (62.5% for CD28-costimulated CAR and 66.6% for 41BB-costimulated CAR, p=0.69). In mice sacrificed due to tumor development, survival post-tumor injection was directly correlated with tumor infiltration by CAR T-cells. ***Conclusions:*** CAR modification of human HSC for cancer immunotherapy is feasible and continuously generates CAR-bearing, antigen-specific, cells in multiple lineages of immune cells. Targeting of different malignancies can be achieved by adjusting target specificity, and this approach can augment the anti-lymphoma activity in autologous HSC recipients. It bears decreased morbidity and mortality and offers alternative therapeutic approach for patients with no available sources for allogeneic transplantation, benefiting ethnic minorities.

760. Abstract Withdrawn

761. Il2 and Il21 Chimeric Signaling in Nk92Cirb21 Enhances Anti-Tumor Activity and Safety of Live Nk92 Cells for Cancer Immunotherapy

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IL2 signaling in NK92 cells increases STAT5 and STAT1 activation, while IL21 induces phosphorylation of STAT3 and STAT1 but not STAT5. However, the combined signaling between soluble IL2 and IL21 in NK92 cells leads to STAT3, STAT1 and STAT5 phosphorylation translating in a dramatic increase of NK92 antitumor activity. We created a chimera combining the signaling of cytokines IL2 and IL21 by fusing the cytoplasmic domains of IL2Rβ and IL21R both tethered to interleukin 2 via a linker. The resulting artificial chimeric signaling from IL2 and IL21 receptors favors strong STAT3 and STAT1 activation with minimal STAT5 phosphorylation. NK92^{CIRB21} cells expressing this chimeric signaling showed substantial activation of CD69, CD107a, higher expression of Perforin-1, Granzyme-B, IFN-γ, and TNF-α and translating in a remarkable anti-cancer activity compared to that induced only by IL2 tethered to IL2Rβ signaling in NK92^{CIRB}. While the continuous IL2 signaling in NK92^{CIRB} leads to a proliferation risk in immunodeficient mice, the combined IL2 and IL21 signaling in NK92^{CIRB21}, however, eliminates this oncogenic risk. Live NK92^{CIRB21} cells intrinsic safety is further enhanced by their slower growth and poor response to soluble IL2 or IL15 and can be further enhanced to completely and efficiently eliminate NK92^{CIRB21} cells at will by a safety switch expressing γ-holin under a tight Tet-ON system. The growth and antitumor activity of live NK92^{CIRB21} cells were further enhanced by synergy with CAR-EGFR. Our findings suggest that artificial chimeric

signaling from IL2 and IL21 receptors platform offers enhanced efficacy and safety allowing the use of long-lived NK92 cells for cancer immunotherapy.

762. One Car to Rule Them all - the Exemplary Fitness of Car-Cd19 T Cells and Their Repurposing for the Eradication of Solid Tumors

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The successful development of CAR-T cell technology for the treatment of solid tumors requires that key hurdles be overcome. 1) CAR T cells must expand and persist in the patient, 2) the CAR T cell population must retain cytotoxic activity, 3) CAR T cell therapy must completely eliminate tumors in order to avoid relapses; in many indications this requires multi-antigen targeting, 4) CAR T cells must be able to attack tumor antigens safely, without causing toxicity due to normal cell or tissue antigen expression. We built a novel platform for repurposing CAR T cells that target CD19 (CAR-CD19 T cells). We create bridging proteins that redirect the CAR-CD19 domain to any antigen of interest: each bridging protein contains the CD19 extracellular domain linked to one or more anti-tumor antigen binders such as scFvs or VHHs. The bridging protein serves as a highly selective and potent "CAR-T-engager". When engineered into a lentiviral vector downstream of a canonical CAR-CD19 sequence, the secreted bridging protein endows a CAR-CD19 T cell with the ability to drive itself to any antigen on any tumor type. We have exemplified this technology using bridging proteins for many antigens and many indications, among them, Her2-positive solid tumors. Because bridging proteins are small and modular we can encode multiple antigen binding domains, such as Her2 and EGFR, or Her2 and B7H3. Because the half-life of bridging proteins is short, systemic exposure is very low and antigen-based toxicity will be minimized. Here we elucidate additional advantages of the CAR-CD19/bridging protein technology, focusing on the inherent exemplary features of CAR-CD19 T cells. As designed, our repurposed CAR-CD19 T cells retain the ability to engage CD19 on normal B cells, and therefore can access an immunologically relevant and self-renewing source of antigen, as B cells are constantly produced by the bone marrow. In addition, CAR-CD19 T cells that encounter B cells have a dramatically different fitness profile than CAR T cells that encounter solid tumor cells. *In vitro* restimulation assays show that CAR T cells that are repeatedly exposed to B cells have a much higher proliferative capacity and a more robust cytotoxicity profile than do CARs that repeatedly encounter solid tumor cells. Further, allowing CAR T cells to 'see' B cells first improves their activity when subsequently challenged with solid tumor cells. Finally, provision of B cells to a population of CAR-CD19 T cells secreting the CD19-anti-Her2 bridging protein improved the fitness of the CAR T cell pool, resulting in improved tumor control *in vivo*. These characteristics, combined with CAR-CD19 T cells' exemplary persistence properties, and the ability to encode multi-antigen targeting within bridging proteins, provide a unique and robust platform to attack solid tumors. The indications for the first-in-human clinical trial to begin in 2021 are Her2-positive breast and lung cancers having CNS metastases. CAR-CD19 T cells, activated by B cells in the periphery, will migrate into the CNS to attack Her2-positive tumors cells - a unique approach to the intractable problem of metastatic CNS tumors.

763. Trop-2-Specific CAR-NK Cells in Cancer Therapy

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Background: In recent years, chimeric antigen receptor (CAR) -modified T cells (CAR-T), which targets CD19, have made significant breakthroughs in the study of recurrent and refractory B-cell malignancies. However, limitations of its application in the field of solid tumors have also been reported. Natural killer cells (NK cells), another form of immune effector cells, have potent cytotoxicity and strong killing effects on tumor cells. Recently, CAR NK-92MI cells have also been studied for different antigens, and some of them have been approved for clinical research. Trophoblast cell-surface antigen (Trop-2), a tumor-associated antigen, is overexpressed in many epithelial tumors, especially in more aggressive ones. Therefore, this study aims to develop a novel CAR NK cell targeting Trop-2 to treat solid tumors. Materials and methods: In this study, CAR-modified NK-92MI cells were transduced with an anti-Trop-2 scFv lentivirus containing a 4-1BB costimulatory and CD3-zeta signaling domain. CAR NK-92MI cells were screened and selected in the presence puromycin, under optimal conditions. Moreover, their proliferation and surface phenotypes were characterized. In addition, the expressions of Trop-2 in different tumor cells were detected using flow cytometry. The cytotoxicity of CAR NK-92MI against tumor cells was analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). Results: The results showed that the Trop-2-CAR NK-92MI cells were modified successfully with no significant toxicity against PBMCs derived from healthy donors. Trop-2-CAR NK-92MI cells screened using different puromycin concentrations showed a positive Trop2-CAR expression rate of approximately 62%. In addition, the proliferation and surface phenotypes of Trop-2-CAR NK-92MI cells were not significantly different from NK-92MI cells. The positive rate of Trop-2 in HCT-15, MCF7, PC-9, and HT-29 was 99.3%, 99.6%, 99.4%, and 50.9%, respectively. Trop-2-CAR NK-92MI cells consistently showed much higher *in vitro* antitumor activity than that of the parental lines against HCT-15(39.88%±1.55% vs. 13.41%±3.65% at a 1.25:1 E: T ratio; 54.88%±2.93% vs. 14.96±0.43% at a 2.5:1 E: T ratio; 72.07%±5.71% vs. 16.88±1.23%at a 5:1 E: T ratio; 89.81%±0.72% vs. 23.93±2.42%at a 10:1 E: T ratio), MCF7(31.6%±7% vs. 2.8±2.5%at a 1.25:1 E: T ratio; 48.4%±7% vs. 3.81±1.7% at a 2.5:1 E: T ratio; 53.1%±8.4% vs. 4.8±2.7% at a 5:1 E: T ratio; 73.5%±14% vs. 13.1±4% at a 10:1 E: T ratio), PC-9(6.4%±1.5% vs. 2.1±0.5% at a 1.25:1 E: T ratio; 11.5%±2% vs. 3.8±1.5% at a 10:1 E: T ratio; 15.7%±2% vs. 5.7±1.5% at a 5:1 E: T ratio; 21.7%±3% vs. 6±2% at a 10:1 E: T ratio), and HT-29(8.8%±2% vs. 3.2±1.5% at a 1.25:1 E: T ratio; 15.1%±3% vs. 3.8±1.4% at a 2.5:1 E: T ratio; 19.2%±3% vs. 6.4±1.5%at a 5:1 E: T ratio; 26.3%±5% vs. 9.8±4% at a 10:1 E: T ratio) cell lines. Conclusions and perspectives: When compared to its parental cell lines, Trop-2-CAR NK-92MI cells have superior killing effect on many Trop-2 positive tumor cell lines *in vitro*. Recently, the primary results from *in vivo* xenograft mouse model

experiments have been encouraging. Therefore, these results indicate that our Trop-2-CAR NK-92MI may have a more efficient antitumor effect on solid tumors and can provide "off-the-shelf" products to treat cancer.Correspondence: Yong Diao, Professor. School of medicine, Huaqiao University, Quanzhou 362021, Fujian, P. R. China. E-mail: diaoyong@hqu.edu.cn. Qingwang Han, PhD. Hebei Zhangda Biological Technology Co., Ltd. Hebei, China. E-mail: qw_han@163.com. Acknowledgements: This work was supported financially by Major project of University and Industry Cooperation in Fujian Province of China (Grant No. 2018Y4009)

764. Targeted Cancer Gene Therapy Using a Superior Bacteriophage-Based Vector Encoding Interleukin-15

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Interleukin-15 (IL-15) is a potent immunostimulatory cytokine with a broad range of biological functions in many cell types linking between innate and adaptive immune systems. The cytokine is highly attractive in the field of cancer immunotherapy due to its ability to increase the proliferation and persistence of natural killer (NK) and T cells without stimulating the population of immune-suppressing regulatory T cells (Tregs) or promoting activation-induced cell death (AICD) of lymphocytes. However, recombinant human IL15 (rhIL-15) used in clinical trials has a short plasma half-life and rapidly undergoes renal clearance which abases its anti-tumour effects. To make the matter worse, systemic administration of IL15 carries the risk of potential toxic side effects, including the induction of autoimmunity. Hence, a targeted gene delivery approach of IL-15 to tumours is needed to improve the anti-tumour efficacy and safety of this cytokine. For this reason, a superior hybrid bacteriophage vector developed by our group is proposed as an efficient platform to deliver IL-15 gene to tumours. The vector is systemically targeted to solid tumours with high specificity via the cyclic RGD4C (CDCRGDCFC) ligand on its pIII capsid protein to bind to the $\alpha_V\beta_3$ and/or $\alpha_V\beta_5$ integrin receptors on the tumour cell surface and tumour blood vessels. To enhance the vector efficacy, endosomal escape peptide, H5WYG, is also displayed on its recombinant pVIII protein capsid. This strategy will mediate a local expression of IL-15 and lengthen the time window of its bioavailability within the tumour microenvironment which consequently should enhance the anti-tumour effects while avoiding systemic toxicity. In this study, colon carcinoma (CT26) was chosen as a preliminary tumour model. The cells showed a good expression of α_v, β_3 and β_5 integrins. Targeted vector encoding secreted luciferase reporter gene mediated gene delivery to these cells efficiently and selectively. After IL-15 gene transfer by the targeted phage vector, substantial secretion of IL-15 was observed from the culture media. The bioactivity of the secreted IL-15 was also assessed from the proliferation of mouse cytotoxic T lymphocytes (CTLL-2) in the presence of culture media from CT26 cells transduced with vector carrying the IL15 gene. The CTLL-2 cell proliferation was enhanced when incubating with the media from cells transduced with the targeted vector. Importantly, no IL-15 expression was detected in CT26 cancer cells and no CTLL-2 proliferation was observed when non-targeted vector encoding IL-15 gene was applied. Furthermore, preliminary *in vivo* investigation was performed on syngeneic immunocompetent mice with subcutaneous CT26 tumours. Biodistribution of IL-15 was selectively detected, at the mRNA level, in the tumours but not in healthy tissues of the mice receiving the targeted vector carrying IL-15. Decreased tumour viability and extended survival of tumour-bearing mice were also achieved after repeated injections of the targeted vector. These results prove the efficacy of the vector encoding IL-15 gene as the powerful platform to specifically and efficiently mediate the therapeutic effect of IL-15 within the tumour microenvironment which is truly useful for targeting cancer gene therapy.

765. NK Cell Expansion and Phenotype Shaping Using CD16-Targeted CSTX002-Fc Feeder Cells

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Cancer immunotherapy utilizing cytotoxic immune cells, such as Natural Killer (NK) and T-cells is gaining more attention. NK cells recognize stress ligands expressed on cancerous cells, which leads to NK cell activation and killing of tumor cells. In current therapeutic protocols, NK cells typically are subjected to some type of activation and/or expansion *ex vivo* prior to infusion. *Ex vivo* expansion allows also for imprinting of NK cells to further arm them with preferred functional attributes without genetic modification. This can be hypothetically achieved through inclusion of appropriate stimuli designed to engage selected NK cell surface receptors. This study tested whether stimulation of NK cells by CD16 engagement will enhance NK cell expansion and improve their phenotype and function, particularly in exerting antibody-dependent cell cytotoxicity (ADCC). To mimic stimulation of antibody bound to target cells, the Fc region fused to an influenza virus neuraminidase domain was expressed on K562 cells engineered with membrane-bound IL-21 (CSTX002 cells), known to stimulate NK cell expansion. An unmodified CSTX002 cell line that does not engage the CD16 receptor served as a control. Primary human NK cells obtained from 2-4 different donors were co-cultured with either CSTX002 or CSTX002-Fc for 10 to 21 days. Expanded NK cells were assessed for expression of CD16 and other activation markers. General cytotoxicity as well as ADCC were compared for NK cells expanded under the two different conditions. Engagement of CD16 *via* the Fc region enhanced proliferation rate past day 14. NK cells proliferated at the same rate upon stimulation with CSTX002 or CSTX002-Fc cells, expressing or not surface bound Fc, until day 14, after which the Fc-stimulated cultures divided at an increased rate as compared to not stimulated with Fc. The NK cells stimulated with Fc had altered phenotype and increased cytotoxicity toward tumor targets.

766. Combination Therapy Using CAR T Cells and Virally Delivered Immune Adjuvants

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Chimeric antigen receptor (CAR) T cells face several barriers to effective therapy when used in solid tumors including unfavorable cytokine gradients, poor or immature vasculature, and immunosuppressive cell types residing within the tumor microenvironment. One way to overcome these barriers is to remodel the tumor microenvironment to be more favorable to T cell infiltration, persistence, and function within the tumor. To achieve this goal, we have tested the hypothesis that delivery of a replication defective adenovirus type 5 encoding an immune-stimulatory adjuvant will co-operate successfully with systemically administered CAR T cell therapy. In the first instance we tested the delivery of CD40L which we, and others; have shown previously is able to stimulate antigen presenting cells within the tumor and tumor draining lymph nodes. Ad5-CD40L has also been shown to stimulate endogenous anti-tumor T cell responses which we hypothesized would potentially combine with CAR T cell therapy. We show here that delivery of CD40L into tumor cells via Ad5-CD40L did not inhibit CAR T cell function or their ability to kill tumor cells *in vitro*. In addition, direct infection with Ad5-CD40L upregulated MHC-II and CD86 expression on bone marrow derived $CD11c^+$ cells. *In vivo* combination therapy of CAR T cells and Ad5-CD40L increased survival over either therapy alone in a murine melanoma model. In addition to CD40L we also tested the ability of highly fusogenic membrane glycoproteins (FMG) to complement CAR T cell therapy. In this respect, we have shown previously that the hyperfusogenic variant of the gibbon ape leukemia virus envelope glycoprotein (Ad-GALV. fus) as well as the vesicular stomatitis virus glycoprotein (Ad-VSV-G), increase DAMPs, heat shock proteins, and TNFα *in vivo.* Here, we show that the combination of Ad-VSV-G and CAR T generated a significantly increased median survival over either therapy alone in an immune competent model of melanoma. Therefore, our data show that delivery of immune stimulating adjuvants to solid tumors represents a potential mechanism by which CAR T cell therapy can be improved through removing some of the immune suppressive barriers inherent in solid tumors.

767. Identification of T cell Targets and Defining Viral Vector Tropism Using a Membrane Proteome Array Functional Genomics Platform

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The Membrane Proteome Array (MPA) is an expression array of 6,000 membrane proteins that enables functional analysis of the membrane proteome. Each full-length membrane protein is individually expressed in live cells, ensuring native conformation and signaling ability. Binding and functional interactions are assayed by high-throughput flow cytometry allowing for rapid analysis of multiple markers of T cell activation state or viral infection. To better understand non-CAR mediated CAR-T cell activation, we developed assays to test CAR-T cells for co-stimulatory interactions across the entire membrane proteome. In addition to identifying many well established co-stimulatory proteins, we discovered dozens of novel proteins capable of enhancing CAR-T cell activation. These novel proteins allow a better understanding of the multiple pathways by which CAR-T cell activation could be augmented within the tumor micro-environment, as well as themselves representing potential therapeutic targets for the modulation of T-cell activity. Furthermore, we developed membrane proteome-wide assays to identify putative receptors of multiple viruses, resulting in the identification of numerous novel receptors and attachment factors. The results of these screens allow better understanding of viral tropism within host tissues and are applicable to the future refinement of viral gene therapy vectors.

768. Centrifuge-Less, Automation-Ready Red Blood Cell Lysis and Immunostaining of Human Whole Blood for Flow Cytometry Using Laminar Wash Washer and Plate

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Blood cells are prime indicators of immuno-surveillance, and the ease of blood sampling makes blood analysis a key interest for clinical and research applications. While current flow cytometry methods are high-throughput and provide fine resolution in the segregation of white blood cell (WBC) populations, WBC enrichment involving red blood cell (RBC) lysis are laborious and typically performed manually, contributing to experimental variability especially as blood cells are sensitive to physical and chemical stress. We describe RBC removal and leukocyte immunostaining on a centrifuge-less platform Laminar Wash™ (LW), using a novel wall-less plate and laminar flow washer. The LW96 plate consists of an array of 96 hydrophilic spots surrounded by hydrophobic surface, which functions as a virtual wall that separates each spot. Erythrocytes from whole blood are first depleted with EasySep™ RBC Depletion Reagent and EasyPlate™ magnet from StemCell™ Technologies. Supernatant from 100µL whole blood is transferred to LW96 for wash and antibody staining by a gentle laminar-flow washing process in the LW HT1000 washer, with staining protocol similar to conventional whole blood staining. We demonstrate similar or higher retention of CD45+ leukocytes with centrifuge-less whole blood processing and staining compared to conventional tube centrifuge, by flow cytometry. Centrifuge-less whole blood protocol resulted in better erythrocyte removal and less debris, allowing for cleaner preparation and clear staining resolution. In assays comparing population frequencies of major blood populations (TBNK, monocytes and regulatory T-cells), conventional RBC lysis in tube and our centrifuge-less whole blood staining method did not present differences in frequencies, suggesting that magnet depletion did not alter leukocyte composition after erythrocyte removal. In summary, we introduced a whole blood processing and staining method that eliminates centrifugation. By avoiding centrifugation, cells undergo significantly less mechanical stress that can impact viability and antibody-antigen binding. Importantly, the replacement of centrifuge with Laminar Wash HT1000 washer simplifies automation of whole blood protocol. This protocol can be fully automated by Laminar Wash™ AUTO, a robotic, trackable system that integrates LW HT1000 in a footprint less than half of conventional automation, allowing easy adoption by any laboratory. Whole blood diagnostics can now be economically automated while reducing operator exposure and variation with centrifuge-less whole blood protocol on LW AUTO.

769. Development of Off-The-Shelf Cellular Therapy Engineered with Novel MICA/B-Reactive Chimeric Antigen Receptor for Pan-Targeting of Solid Tumors

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MHC class I related proteins A and B (MICA/B) are cell surface proteins strongly induced by cellular stress including malignant transformation in many types of cancers. MICA/B is recognized by lymphocytes containing the NKG2D receptor, which binds the distal α1 and α2 domains of MICA/B to activate a potent cytotoxic response. However, advanced cancer cells can evade this immune surveillance by proteolytic shedding of the α1 and α2 recognition domains. In order to restore lymphocyte recognition of malignant cells and develop an effective pan-tumor targeting strategy, we created novel chimeric antigen receptors (CARs) that are unaffected by MICA/B proteolysis by specifically targeting the α3 domain and inhibiting MICA/B shedding. Several CAR designs were evaluated containing the single-chain variable fragment from a novel α3 domain specific antibody, attached to various spacers, transmembrane domains, and receptor signaling domains for optimal function in natural killer (NK) or T cells. To this end, preliminary studies showed that the designed α3 targeting CARs (CAR-MICA/B) elicit MICA/B-dependent activation and cytokine production, specific cytotoxicity against MICA/B positive tumor cells regardless of MICA/B shedding, and *in vivo* clearance of B16-F10 melanoma cells expressing MICA. We next aimed to complement CAR-MICA/B with a series of functional modalities to create an ideal product candidate for a universal cancer targeting strategy. We have previously shown that NK cells engineered with IL-15 receptor alpha fusion (IL-15RF) for cytokine independent persistence, high affinity non-cleavable (hn)CD16 for enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), and CD38 knockout for improved function, are potent effector cells that synergize well with monoclonal antibodies to elicit a multifaceted and durable anti-tumor response *in vivo*. However, creating an engineered primary NK cell with complex genetic edits is challenging. In order to create a homogenous product that contains all four modalities, we utilized our clonal master induced pluripotent stem cell (iPSC) platform where at the single cell level, an engineered iPSC is derived to contain all engineering attributes in a defined manner. The derived clonal iPSC is then banked to serve as a renewable starting material for the mass production of precision-engineered CAR NK and T cells. iPSCs were engineered by CRISPR-mediated knock-in of an IL-15RF and hnCD16 cassette into the CD38 locus. Single cell clones were established by flow cytometry and screened for targeted insertion and biallelic disruption of the CD38 gene. This was followed by a second knock-in of the MICA/B CAR cassette and subsequent single cell sorting on CAR expression that yielded clones with precise targeting without random insertions at efficiencies of 22% for CD38 targeting. *In vitro* differentiation of clonal iPSCs produced homogeneous NK cells with uniform expression of the CAR-MICA/B, hnCD16, and IL15RF without any CD38 immunoreactivity. The derived CAR-MICA/B engineered iPSC-derived NK (iNK) cells displayed enhanced cytokine production and cytotoxicity against MICA/B positive tumor cells compared to unedited iNK cells. Further *in vitro* and *in vivo* studies are ongoing. In summary, the advances presented here demonstrate tumor recognition and specificity through our novel α3-specific CAR-MICA/B which is to be combined with additional unique anti-tumor modalities to form the basis of our next generation off-the-shelf iPSC-derived NK and T cell therapeutics for a pan-targeting approach in solid tumors.

770. piggyBac Transposon-Based Engineering Enables Rapid Development of Multiple Immune-Competent Tumor Models for the Assessment of Targeted T Cell-Based Peptide Delivery Platforms

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Immune competent tumor models in rodents are powerful tools for assessing the role of the immune system in tumor progression and oncolytic therapeutics. However, many syngeneic tumor models are inherently immunogenic and fail to efficiently form tumors due to the co-expression of reporters or antibiotic resistance genes which are used to generate stable cell lines. We used piggyBac transposons to engineer multiple clonal tumor cell lines expressing different levels of model antigens without antibiotic selection or use of reporters. Lewis lung carcinoma (LLC) and murine colorectal carcinoma (MC38) cell lines were engineered with a piggyBac transposon driving expression of full-length chicken ovalbumin or human membrane bound CD19 allowing for tumor targeting using OT1-2 TCRs or using hCD19 targeted CARs respectively. In each candidate tumor line, we measured relative antigen expression, interferon-gamma mediated MHC I upregulation, tumor engraftment, and recruitment of Ag- specific T cells. We then evaluated the utility of a T cell-based platform engineered to deliver the chemokine CCL5 to tumors, testing the hypothesis that CCL5 expression by tumor homing cells could drive oncolytic remodeling of the tumor immune microenvironment. We found that T cells engineered to express CCL5 efficiently home to tumors based on in vivo imaging. Furthermore, analysis of tumors by flow cytometry revealed a two-fold increase in the tumor infiltration of CD103+ dendritic cells within 48 hours of CCL5 delivery. These results indicate that transposon systems can be leveraged to rapidly generate syngeneic tumor models expressing model antigens or other genes of interest without use of antibiotic selection or reporter transgenes. Furthermore, these studies support a role for CCL5 in the recruitment of CD103+ dendritic cells into tumors. Ongoing studies leveraging these tumor models are evaluating the effects of delivery of additional chemokines and cytokines aimed at augmenting solid tumor rejection.

771. Combining a Constitutively Active IL-7 Receptor with CLL-1 CAR T-Cells for Treatment of Acute Myeloid Leukemia

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Introduction: C-type lectin like molecule 1 (CLL-1) is expressed by >80% of acute myeloid leukemia (AML) but is absent on normal hematopoietic stem cells (HSCs). We have previously shown that T-cells retrovirally transduced with a CLL-1.CAR encoding 41BB and CD3-ζ endodomains can recognize and eliminate CLL-1 expressing AML blasts using *in vitro* and *in vivo* models[1]. However, although CAR molecules can provide adequate signals for initial T-cell activation upon antigen ligation (signal 1 and 2), they generally do not induce sufficient cytokine secretion (signal 3) to sustain T-cell expansion after repeat exposure to antigen-positive tumor cells. To address this deficiency, we used an engineered IL-7 receptor (C7R) that constitutively activates the STAT5 pathway[2]. We have shown that the effector function of GD2-CAR T cells can be significantly improved by expressing C7R. The goal of this project was now to explore whether C7R can also enhance the effector function of CLL-1.CAR T-cells in leukemia models. **Material and Methods:** Activated T-cells were retrovirally transduced with either C7R, CLL-1.CAR, or C7R and CLL-1.CAR simultaneously (C7R/CLL-1.CAR), and cultured with IL-7 and IL-15. To assess the activity of C7R, we cultured the cells in medium without cytokines and counted weekly to assess persistence. To see if C7R enhanced the anti-tumor activity of CLL-1.CAR T-cells, we serially cocultured T-cells with HL60, an AML cell line expressing CLL-1, at a 1:1 effector to target ratio. Every 3-4 days, each one coculture well was harvested and analyzed by flow cytometry and the remaining cells were split 1:1 and rechallenged with the same number of HL60 cells. **Results:** T-cells expressing C7R and C7R/CLL-1.CAR but not CLL-1.CAR alone had phosphorylated STAT5 after 48 hours of culture without exogenous IL-15 and IL-7 (mean MFI: 202 vs. 200 vs. 49.4, $p<0.005$). In the persistence assay, CLL-1.CAR T-cells failed to expand without cytokines and began contracting after 7 days. In contrast, T-cells transduced with C7R or C7R/CLL-1.CAR expanded until day 14 then gradually contracted during the next 21 days. In our serial co-culture assay, during the first challenge with HL60, C7R/CLL-1.CAR T cells performed similarly to CLL-1.CAR T cells in both T-cell expansion and in elimination of leukemia cells. However, C7R/CLL-1.CAR T cells expanded significantly more upon repeat tumor cell challenge, maintaining their cytotoxic activity even unto the fourth serial co-culture. **Conclusion:** Expression of C7R in CLL-1.CAR T-cells enhances their persistence, expansion and anti-leukemia activity after repeat antigen exposure, warranting further active exploration of our modified CAR T-cell therapy approach for AML. 1. Tashiro H, et al. Mol Ther. 2017 2. Shum T, et al. Cancer Discov. 2017

772. Recombinant T Cell Receptor Immunity Against Hepatitis B Surface Antigen Following Disruptive Base Editing of Endogenous T Cell Receptor

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T cells redirected through expression of recombinant T cell receptors (rTCR) can target viral and tumour antigens, and are being investigated in early phase clinical trials. Targeting of hepatitis B surface antigen epitopes holds promise for therapeutic strategies against HBV associated hepatocellular carcinoma. Limitations of current approaches include competition from endogenous TCR (eTCR) and aberrant cross pairing of TCR alpha and beta chains. The application of CRISPR guided base conversion by cytidine deamination to disrupt endogenous TCR (eTCR) offers the possibility of improved expression of rTCR, reduced risk of cross pairing and mitigation against toxicity in both autologous and allogeneic settings. We generated a lentiviral vector to couple expression of a rTCR specific for Hepatitis B envelope antigen with a CRISPR guide RNA specific for T cell receptor beta chain constant genes. Upon electroporation, delivery of codon optimised base editor mRNA mediated high level disruption of eTCR in human T cells and was associated with enhanced levels of rTCR expression. Discriminatory magnetic bead mediated depletion of T cells expressing residual eTCR, but not rTCR, resulted in selective enrichment of rTCR+ specific populations, increasing in proportion from 56% (SD ±4.9%) to 95% (SD ±1.2%). Modified T cells exhibited cytokine production in response to peptide pulsed target cells, and migration and specific killing in a 3-D microfluidic model. Molecular signatures of base editing confirmed seamless conversion of C>T (G>A) to create stop codons, with only low level (<1.2%) background conversion activity detected at sites of predicted off-target DNA editing, comparable to control cultures without exposure to BE. Sequencing of RNA samples collected at serial time points from 24 hours onwards found no perturbation of cytidine residues within the TCR CDR3alpha and CDR3beta hypervariable regions, and this further alleviated concerns about possible promiscuous BE activity which may otherwise have influenced T cell specificity. In conclusion, targeted disruption of eTCR by cytidine deamination and discriminatory enrichment of HBV specific T cells offers the prospect of enhanced, better targeted immunotherapy. In addition, base editing is ideally suited for multiplexed effects and combinational modification of additional genes to influence checkpoint or cell survival pathways.

Cancer - Targeted Gene and Cell Therapy

773. Evaluating Tumor-Homing Neural Stem Cell Therapy Using Bio-Inspired 3D Models of Brain Cancer

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Engineered neural stem cells (NSCs) have recently emerged as a promising therapy for the highly aggressive and lethal brain cancer Glioblastoma (GBM). Acting as a tumor-homing drug-delivery system, NSCs are known to migrate through brain tissue and seek out primary and invasive tumor foci. NSCs can deliver therapeutic agents, such as TNFα-related apoptosis-inducing ligand (TRAIL), directly to the tumor and have been shown to suppress both solid and post-surgical GBM in mouse models of cancer. Despite this promise, mouse models have been the mainstay for evaluating NSC migration and efficacy where the low-throughput, small scale, and challenges with implanting and tracking cells in the murine brain have left key translational questions unexplored. To circumvent these challenges, we developed a 3D, bio-inspired culture system composed of PLA microfibers suspended in an agarose hydrogel and a custom bioreactor apparatus. We then sought to determine the potential of this model to assess tumor growth, stem cell migration, and stem cell-based treatment efficacy at a mid- and human-scale. First, we investigated tumor growth in the models by implanting a panel of 5×10^5 human GBM and metastatic cancer cells into small scale microfiber/agar models approximately 6 cm in diameter. Real-time serial imaging showed over one week GBM tumor cells proliferated nearly 50-fold, while metastatic-breast cancer cells proliferated more than 3-fold. To evaluate NSC migration in the models, we created another set of microfiber/agar models in which NSCs were implanted 3mm laterally from the GBM foci. Fluorescence imaging showed directional migration of the NSCs towards the tumor over the course of one week, whereas NSCs implanted laterally from non-cancerous fibroblasts showed no directional migration. To investigate the efficacy of NSC therapy in this model, we first explored the impact of NSC distance from the tumo. NSCs were implanted 0, 2, 5, or 10 mm laterally from a GBM foci and imaging was to track GBM growth. We found that NSCs at all distances were efficacious, with NSCs co-injected with tumor inducing 99% reduction in less than 10 days, while NSCs implanted 10 mm laterally from the tumor reached a similar level but not until 30 days post-treatment. At distances of 2 and 5 mm, a similar response was observed at 20 and 30 days post-treatment, respectively. Next, we studied NSC migration and efficacy in a multifocal tumor model. Tumor cells were injected into each hemisphere of the brain model, and NSCs were implanted directly adjacent to the tumor in the right hemisphere. Interestingly, imaging showed 54% of NSCs migrated to the tumor in the left hemisphere while only 29% of NSCs migrated directionally to the tumor in the right hemisphere. In conclusion, our results suggest the microfiber/agar system is a useful tool for investigating multiple parameters around cell therapy for cancer, including migration and tumor response. The precise control over cell implant, compatibility with kinetic imaging, and high-throughput nature of the system could allow this system to be a useful surrogate for investigating key questions that are beyond the reach of traditional animal or cell cultures as new cell therapies are advanced towards human patients.

774. A Blockade of an N-CoR2 Epigenetic Checkpoint Overcomes Chemo- and Immuno-Resistance in Malignant Tumors

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Background: The majority of human malignant tumors are resistant or only partially respond to conventional chemotherapy (C/T) or immunotherapy (I/T) such as immune checkpoint inhibitors (ICIs). Irrespective of the treatments, efficient tumor-cell killing requires amplification of inflammatory signaling, which however is tightly regulated by various "checkpoint" mechanisms evolved by epithelial cells to prevent excessive tissue damage induced by virus and immune attack. Novel approaches to disable theses conserved and cell-intrinsic inflammation checkpoints may provide breakthrough and "tumor-agnostic" strategies to circumvent the innate treatment-resistance to unleash the full potential of C/T and I/T in treatment-refractory and highly lethal malignant tumors. Material and methods: We designed and conducted integrated genomic and proteomic screening combined with molecular and functional studies to identify conserved anti-inflammatory pathways that mediate innate and cell-intrinsic resistance to C/T and I/T agents. Preclinical studies were used to validate a gene therapy strategy to disable the inflammation-checkpoint identified from this process. Results: We uncovered that the cytotoxic and immunogenic death induced by C/T and I/T agents is constrained by repression of a toll-like receptor-2 (TLR-2)/TLR-3- and NF-kB-induced interferon regulatory factor-1 (IRF-1) and interferon (IFN)-gamma anti-viral response program in cancer cells. Loss and gain of function studies implicated that co-repressor-2 (N-CoR2) co-translocated with NF-kB p50 into cell nuclei in response to therapy, wherein it serves as an epigenetic checkpoint of this inflammation program by mediating a histone deacetylase-dependent chromatin remodeling and repression of a specific panel of pro-inflammatory and pro-apoptotic genes. Thus, high N-CoR2 expression predicts treatment refractoriness and poor prognosis in neoadjuvant or adjuvant treated breast cancer patients. Blockade of the epigenetic checkpoint function of N-CoR2 by a small decoy of N-CoR2 (De-CoR2) hyper-sensitized malignant cells to assorted C/T agents, death ligands, and IFN-gamma. Consistently, intratumoral delivery of De-CoR2 dramatically potentiated systemic C/T and ICI therapies, including anti-PD-1 and anti-CTLA-4 antibodies, and completely halted tumor growth or induced remissions in orthotopic and patient-derived xenograft models of triple-negative breast cancer. Conclusion: Our findings suggest that malignant tumors can access intrinsically conserved anti-inflammatory mechanisms that enable them to escape from C/T and I/T. As such, strategies that can override this defense program constitute novel anti-tumor gene therapies that may be applied to overcome resistance in treatment-refractory tumors and improve patient prognosis.

775. Abstract Withdrawn

776. Secondary Structure Analysis by SHAPE-MaP of the EGFR pre-mRNA Transcript to Identify Target Regions for RNA Anti-Sense Therapies

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This project is to develop therapies to bypass challenges to effective and continuous drug delivery to the brain, for the treatment of glioblastoma multiforme (GBM). Currently, individuals diagnosed with GBM have a short life expectancy of 12-14 months. Today, the standard of care and treatment of GBM, the most common malignant primary brain tumor in adults, are often limited by the blood-brain barrier. Our approach has the potential to deliver one single dose of gene therapy directly to the GBM tumor environment and block the production of cancer-driving genes. Epidermal growth factor receptor (EGFR) is dysregulated in 57% of all GBM. Our approach uses an adeno-associated virus gene transfer vector encoding antisense RNA therapeutics targeting critical splicing elements surrounding Intron 10 of the EGFR pre-mRNA transcript. Cryptic poly-A signals within intron 10 have the potential to activate the expression of a stable therapeutic isoform of EGFR. Alternative splicing and polyadenylation is determined by the secondary structure of the pre-mRNA nascent transcript. To advance our therapeutic strategy, we have analyzed the EGFR secondary structure using selective 2' hydroxyl acylation and primer extension followed by mutational profiling (SHAPE-MaP). The SHAPE reagent 1M7 reacts with the 2' hydroxyl of RNA molecules when the RNA molecule is in a conformationally flexible position creating a 2' O-adduct. The modified RNA is reverse transcribed, incorporating mismatches at the acylated positions; a comparison of unmodified to modified RNA allows us to determine RNA nucleotide positions that are bound in secondary structure, complexed with RNA-binding-proteins, or single stranded. The genetic region of Exon 10, Intron 10, Exon 11 of EGFR was transcribed and purified by RNA gel electrophoresis. RNA was treated with 1M7 (+), DMSO (-), or 50% formamide with 1M7, reverse transcribed under SHAPE conditions with Superscript II then converted to cDNA. Reverse transcription conditions for SHAPE experiments incorporate manganese as the divalent cation, which allows the enzyme to incorporate nucleotide mismatches. 3.5 million reads corresponded to (+), (-) and denaturing conditions with the expected mean read length of 936 nucleotides and mean quality score of 9.7. Sequencing output was run through the ShapeMapper2 pipeline. The median read depth at each nucleotide was at least 92K for all conditions after elimination of reads under quality control score of 15. SHAPE-MaP reactivity profiles were generated using RNAfold. Splicing elements within the targeted region were identified and compared to reactivity profiles. SRp40 binding motifs show high accessibility upstream of the 5' splice site. The exon-intron junction of the 5' splice site is structurally available while the 3' intron-exon junction shows moderate accessibility. We identified a putative poly-A signal that exists within intron 10 and the literature shows expression of this shortened EGFR isoform in various cell types. Importantly, this cryptic poly-A signal shows no reactivity indicating high secondary structures blocking recognition by the poly-A machinery. Based on these structural profiles, we generated RNA antisense molecules to unravel the hidden poly-A signal to activate expression of the short isoform. In conjunction, we used eCLIP to identify target specific RNA-binding proteins.

777. A Materials-Based Approach to Enhance Induced Neural Stem Cell Therapy for Post-Surgical Cancer

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Glioblastoma multiforme (GBM) is an aggressive stage four brain cancer. The tumor's migratory nature allows it to evade current standard of care (surgical resection followed by chemoradiotherapy) and results in a median patient survival of approximately 15 months. The invasive tumor foci can invade both hemispheres of the brain, making gross total resection nearly impossible. There is an urgent need to develop a therapy that can target and treat invasive GBM foci more effectively. Recent studies have explored the use of neural stem cells (NSCs) for GBM therapy. NSCs have innate tumor-tropism, and when engineered with cytotoxic proteins, can actively kill cancer cells. This technology has proven promising, and allogeneic NSC therapy has recently entered human clinical trials. Despite the promise of NSC therapy, clearance from the post-surgical tumor resection cavity remains an issue. In this study, we took a two-pronged approach to address these limitations. First, we employed autologous neural stem cells transdifferentiated from a patient's skin, termed induced neural stem cells (iNSCs), to limit immunologic responses. These cells are engineered to secrete the cytotoxic protein, TNFα-related apoptosis-inducing ligand (TRAIL). Second, we encapsulate the iNSCs in a composite gelatin-fibrin matrix to stabilize the iNSCs within the cavity and potentially shield the transplanted cells from immune cells that could induce clearance. In vitro, electron microscopy showed robust encapsulation of the iNSCs in the fibrin component of the matrix as well as homogeneous distribution of the cells throughout the material. When iNSCs were implanted into a surgical cavity in mice, serially bioluminescence imaging showed that encapsulating iNSCs in the gelatin-fibrin matrix increased cell persistence over 4-fold, reaching 90 days post-implant, compared to only 20 days when the cells are injected in suspension directly into the surgical cavity. Additionally, we found that the increased persistence of iNSCs lead to improved suppression of post-surgical cancer and extended survival of tumor-bearing mice using human GBM cell lines. In the highly diffuse GBM-8 tumor model, mice treated with therapeutic iNSCs encapsulated in the gelatin-fibrin matrix survived 70+ days post-resection while mice treated with a suspension of therapeutic iNSCs only survived a maximum of 40 days post-resection. Future studies will explore the impact of the matrix's mechanical properties on the migration, proliferation, differentiation, and tumor-tropism of iNSCs via qRT-PCR. Overall, the gelatin-fibrin

matrix provides significantly increased iNSC persistence and is a promising step towards developing a longer-lasting, more efficacious therapy for GBM.

778. Evaluating the Safety and Toxicity of Autologous Induced Neural Stem Cells Expressing TRAIL and Thymidine Kinase in a Canine Model

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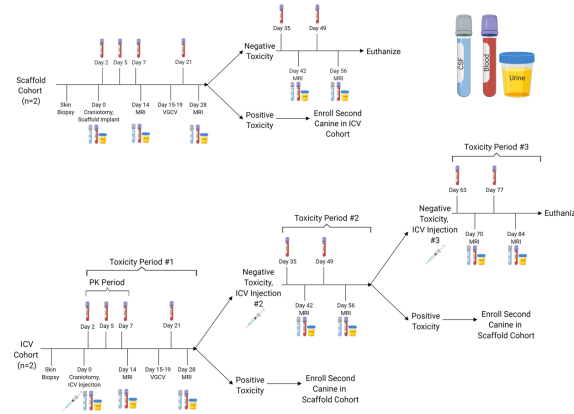
Glioblastoma (GBM) is a deadly brain cancer with a median patient survival of only 15 months. The clinical standard of care for GBM patients is tumor resection, chemotherapy, and radiation treatment; however, the infiltrative nature of GBM allows tumor cells to evade therapy. Neural stem cells transdifferentiated from a skin biopsy, termed induced neural stem cells (iNSCs), have the innate ability to home to tumors and actively kill cancer cells when engineered with cytotoxic proteins. iNSCs have shown significant therapeutic potential in murine models, but safety and delivery of iNSCs in models that more closely reflect human patients remains unexplored. In this study, we explored multiple aspects of personalized iNSCs in a canine model using two delivery methods: intracerebroventricular (ICV) infusion and encapsulation in a biocompatible scaffold. First, autologous iNSCs were made from each canine and engineered to express TNFα-related apoptosis-inducing ligand (TRAIL) and thymidine kinase (TK) and pre-labelled with supraparamagnetic iron oxide nanoparticles for imaging. In the cohort where cells were administered via ICV infusion, each animal received three doses of autologous iNSCs infused every four weeks. Canines who received iNSCs encapsulated a scaffold received a single dose of autologous iNSCs co-administered into a mock resection cavity in a gelatin/fibrin matrix. Doses tested were 1×10^6 iNSCs/kg or 3×10^6 iNSCs/kg. To track the cells after infusion, canines underwent bi-weekly magnetic resonance imaging sessions until euthanasia. The ICV cohort was observed for 12 weeks, and the scaffold cohort was observed for 8 weeks post-iNSC infusion. Imaging revealed hypodense areas present within the brain at all time points for both ICV and scaffold study arms, thus indicating the presence of blood and/or iNSCs. Blood, urine, and cerebrospinal fluid were drawn serially and tested for markers of toxicity; no unanticipated changes in blood chemistry, urine, or cerebrospinal fluid were observed across all time points. Neutropenia as determined by complete blood count was attributed to animal stress prior to sample collection. Samples of major organs were collected at necropsy for histological analysis. Analysis of the brain and spinal cord revealed procedure-related changes; spleen, liver, lung, and bone marrow revealed no lesions. However, testicular degeneration and atrophy progressing to aspermia was detected, likely attributed to the TK pro-drug, valganciclovir, rather than the iNSCs themselves. To our knowledge, this is the first study to assess the safety and toxicity of autologous iNSCs expressing TRAIL and TK in a large animal model. We conclude that, at the tested doses, iNSCs expressing TRAIL and TK exhibited few toxicity signals, and this study provides foundational knowledge for future clinical trials.



779. Combination of Gene Therapy Using rAd-p53 and Immunotherapy Using Anti-PD-1 Antibody for Urogenital Cancers

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Introduction: Urogenital cancers including kidney, bladder, and prostate are common cancers worldwide. Surgical intervention, chemotherapy, and/or hormonal therapy, that are standard of care for urogenital cancers, have some obstacles in terms of efficacy and safety. Thus, the development of a novel therapeutic strategy is desired. The combination of cancer gene therapy and immunotherapy would be a promising approach. Recombinant adenovirus vector carrying *p53* gene was well studied in many previous studies, but the clinical anti-tumor effect was controversial. Programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1), which are key mediators of host immune tolerance, are associated with various cancer progression. Recently immune-checkpoint inhibitors such as anti-PD-1 antibody showed the anti-tumor effect and the prolongation of survival beyond chemotherapy, and it is becoming one of the standard treatments for advanced cancer. This study explored the feasibility the combination of rAd-p53 and anti-PD-1 antibody using animal models of urogenital cancers. **Experimental Summary:** In this study, we employed three human urogenital cancer cell lines, TRAMP-C2 (prostate cancer), MBT-2 (bladder cancer) and Renca (renal cancer) cell lines. For *in vitro* study, these cell lines were infected with rAd-p53 or rAd-LacZ (as a control vector) at multiple concentrations of Multiplicity of infection (MOI) and cultured for 6 days. RAd-p53 showed significantly higher cell cytotoxicity in TRAMP-C2 and Renca cells at 160 MOI compared with rAd-LacZ but not in MBT-2 cells. Also, we examined

the mRNA expressions of CAR (coxsackievirus and adenovirus receptor) in the three cell lines, and confirmed that MBT-2 cells expressed the significantly lower level of CAR mRNA compared to the other two cell lines. In addition, we determined the expression of PD-L1 on those cell lines. The three cell lines were cultured in the presence of mouse IFN-γ at the concentration of 10 ng/mL with or without infection of rAd-p53 or rAd-LacZ at 40 MOI. After 3 days of culture, the cells were stained with phycoerythrin-labeled anti-mouse PD-L1 antibody, and then PD-L1 expression was determined by flow cytometry. As the results, PD-L1 expression was increased in all cell lines by rAd-p53 infection. These results suggested that rAd-p53 might stimulate the PD-L1/PD-1 pathway and lead the T cell exhaustion. The combination of rAd-p53 and anti-PD-1 antibody could inhibit the T cell exhaustion and achieve the strong anti-tumor activity. Based on these in vitro results, we also conducted *in vivo* studies using prostate (TRAMP-C2) and kidney (Renca) murine tumor models. 2×10^6 of TRAMP-C2 and Renca cells were respectively subcutaneously inoculated into C57BL/6 mice and BALB/c mice. The TRAMP-C2 and Renca bearing mice were randomly assigned into 5 treatment groups (n=5) as following; rAd-p53+anti-PD-1 antibody, rAd-LacZ+anti-PD-1 antibody, PBS+anti-PD-1 antibody, rAd-p53 alone, rAd-LacZ alone, and PBS. As the results, in either case, the highest anti-tumor effect and the longest survival are observed in the combination group of rAd-p53+anti-PD-1 antibody. **Conclusion:** The combination of rAd-p53 and anti-PD-1 antibody demonstrated the strong anti-tumor activity *in vivo*. These results suggested that the combination of rAd-p53 and anti-PD-1 antibody could induce the synergistic effects in urogenital cancers. Therefore, gene therapy using rAd-p53 can be a promising candidate as an adjuvant therapy for immunotherapy using immune checkpoint inhibitors including anti-PD-1 antibody for kidney cancer and prostate cancer.

780. Abstract Withdrawn

781. RNA Replacement Based on RNA Reprogramming for Cancer Theranostics

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Group I intron-based trans-splicing ribozyme enables to sense and reprogram target RNA into gene of interest through RNA replacement. Previously, we proposed hTERT-targeting trans-splicing ribozyme downstream therapeutic suicide gene for cancer therapy. Here, we optimized the specific ribozyme for highly efficient antitumor activity with less off-target effect for theranostics application. We enhanced the intracellular expression of the ribozyme at transcriptional/post-transcriptional level and improved tumor selectivity via introduction of microRNA target site at the 3' end of the ribozyme. Then, systemic administration of adenovirus encoding our refined ribozyme achieved great anti-tumor efficacy and improved ability to specifically target tumor without hepatotoxicity in vivo. In addition, noninvasive imaging modalities were successfully employed to monitor both how well a therapeutic gene was expressed inside tumor and how effectively a ribozyme therapy took an action against tumor. Collectively, the advanced therapeutic ribozyme and its image-aided evaluation system may lead to the powerful strategy for successful clinical translation and the development of clinical protocols for cancer therapy.

782. Cytotoxic Human Induced Neural Stem Cells for the Intravenous Treatment of Primary Tumors

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Introduction: Human induced neural stem cells (hiNSCs) transdifferentiated from human fibroblasts exhibit innate tumor tropism. The potential of hiNSCs as personalized anti-cancer drug delivery vehicles has been demonstrated in the treatment of intracranial tumors, but the potential to treat extracranial, primary tumors remains relatively unexplored. Herein, we investigate the tumor-homing potential and therapeutic efficacy of intravenously infused hiNSC against two of the deadliest primary tumor types, triple negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC). **Methods:** In order to study the migration of hiNSCs *in vitro*, we performed time-lapse motion analysis on hiNSCs co-cultured with TNBC and NSCLC. Migration was tested *in vivo* by establishing orthotopic human TNBC or NSCLC tumors and intravenously infusing hiNSCs. Post-mortem tissue analysis was then used to determine the location and persistence of hiNSCs. The therapeutic potential of these engineered cells was assessed by creating a line of hiNSCs that secreted the cytotoxic protein TRAIL and co-culturing these hiNSC-TRAIL cells with NSCLC and TNBC. Therapeutic efficacy was tested *in vivo* by intravenously infusing hiNSC-TRAIL following establishment of orthotopic TNBC or NSCLC tumors. The safety and toxicity hiNSC with and without TRAIL was investigated by following hematology, blood chemistry, and histology for 28 days after mice had received a maximum tolerated dose of either hiNSC or hiNSC-TRAIL. **Results/Conclusion:** Time-lapse motion analysis showed hiNSCs migrated directionally to both human NSCLC and human TNBC in co-culture assays. Kinetic tracking with post-mortem tissue analysis confirmed that intravenously infused hiNSCs co-localized with established orthotopic NSCLC or TNBC tumors within 3 days and persisted through 14 days. Real-time imaging showed hiNSCs that secreted the cytotoxic agent TRAIL induced rapid killing of NSCLC and TNBC in co-cultures in time- and dose-dependent manners. Using orthotopic tumor models, intravenous hiNSC-TRAIL therapy reduced human NSCLC and TNBC tumor volumes 42% and 70%, respectively. Neither hiNSCs nor hiNSC-TRAIL cells induced significant changes in multiple safety parameters through 28 days post-infusion. These results provide the first evidence that intravenous hiNSC therapy safely and effectively seeks out and kills peripheral tumors, suggesting a potential new strategy for treating aggressive peripheral cancers through personalized cell therapy.

783. Phenotypic and Functional Characterization of Gene-Circuit Modified Allogeneic Mesenchymal Stromal Cells (MSCs) for Solid Tumor Immunotherapy

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BackgroundMesenchymal stromal cells represent an attractive therapeutic cell therapy modality, owing to their capacity to express and deliver therapeutic payloads, the clinical feasibility and safety of allogeneic administration, and the potential for large-scale manufacturing to reduce cost. In the context of cancer, where MSCs have been relatively underexplored, the ability of MSCs to naturally resist tumor-mediated immune suppression is an advantage, and it has been shown that MSCs can selectively migrate to tumor sites following locoregional administration in preclinical models of cancer. However, it is not known whether MSCs can retain these advantages following gene modification, cellular expansion, and multiple freeze/thaw cycles. SENTI-101 comprises allogeneic bone marrow-derived MSCs genetically modified to express and locally secrete IL12 and IL21. In this study, we characterized the ability of SENTI-101 to maintain its immunomodulatory function through the manufacturing process. **Methods**The manufacturing process for SENTI-101 involves encoding the genetic circuit into a DNA plasmid, packaging it into a lentiviral vector, followed by stable transduction, expansion and cryopreservation of the MSCs. These cells are then expanded in cell factories, formulated and cryopreserved for shipment to clinical sites. In this study, we tested various transduction conditions including multiplicity of infection (MOI) range, transduction enhancers and optimal cell concentration, to achieve high transduction efficiency while keeping vector copy number below a target threshold. We compared the growth kinetics between unmodified and gene-modified MSCs derived from multiple donors. In addition, we evaluated the stability in gene expression of transduced cells between pre-cryopreservation and post-thaw cells subjected to multiple freeze/thaw cycles and long-term culture. We characterized the functionality of these cells by measuring IL12 and IL21 protein secretion, transduction efficiencies and tumor-homing capacity in an in vivo xenograft mouse model of human ovarian carcinoma (OVCAR8). **Results**We observed consistent cell expansion (12-17 population doublings from initial thaw to final harvest), doubling time (18-25 hrs) and viable cell yield (2E4-4E4 cells/cm2) across multiple donor MSCs (unmodified and gene-modified). Stable and efficient transduction of MSCs was observed at MOIs between 8-15. Depending on process conditions, transduction efficiency ranged between 25-65% with IL12 secretion between 5E5-2E6 pg/24hr/E6 cells and IL21 secretion between 1E5-5E5 pg/24hr/E6 cells. We observed a strong positive correlation between IL12 (R2=0.9) and IL21 (R2=0.9) protein expression levels and the percentage of cells transduced. We observed <1% difference in transduction efficiency and <10% difference in IL12 and IL21 expression levels between pre-freeze and post-thaw. In the OVCAR8 xenograft mouse model, SENTI-101, upon infusion, preferentially localized to tumor sites compared to other organs in the peritoneal cavity (10-fold higher vs normal tissues). Consequently, IL12 and IL21 protein concentration was more than 100-fold higher in the peritoneal fluid compared to systemic circulation (p=0.001), further confirming the potential of SENTI-101 to locally deliver potent cytokines **Conclusion**These results demonstrate that SENTI-101 retains its engineered function through the manufacturing process and supports further development of this novel cell therapy product for the treatment of solid tumors.

784. MicroRNA Expression in Concert With Stable Expression of a Chimeric Virus Capsid Protein for Transient Virus Modification without Genome Alteration

Jian Gao[1], Wenli Zhang[1], Kemal Meese[1], Oskar Bunz[1], Fengmin Lu[2], Anja Ehrhardt[1]

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There are many types of cancer treatments. Nowadays immune cells are emerging as carriers for the delivery of oncolytic viruses (OVs) and improvement of cancer treatment. The natural killer cell line NK-92 and the tumor-specific oncolytic adenovirus type 5 (Ad5) have been widely used in cancer treatment. However, the delivery of oncolytic Ad5 into tumor sites with NK-92 carrier is challenging. The efficacy of treatment depends on delivering sufficient amounts of Ad5 into tumor sites through carrier cells and therefore, the improvement of Ad5 infection efficiency into NK-92 carrier cells is essential. Using an engineered human adenovirus library consisting of a broad range of replicating human Ads with measurable marker genes in our laboratory, we transduced hepatocellular carcinoma (HCC) cell lines Huh7, Hep3B and HepG2 as potential target tumor cells and the natural killer cell line NK-92. Subsequently we measured virus-derived luciferase expression levels to determine transduction efficiencies. We found that Ad37 most efficiently infected NK-92 cells, and therefore we constructed a producer cell line stably expressing a fused Ad5/37 chimeric fiber comprising the Ad5 shaft and the Ad37 knob and a miRNA inhibiting Ad5 knob expression (HEK293-Ad5/37-miRNA). These modified HEK293 cells were transduced with the oncolytic Ad5-hTERT vector, and the cell lysate was harvested 48 hours post-transduction. After purification of the chimeric Ad5-hTERT vector, we transduced NK-92 cells and incubated them with HCC cell lines. Among all the adenovirus candidates, Ad5 demonstrated the highest infection efficiencies in HCC cell lines. However, Ad5 showed poor infection for NK-92 cells while the highest infection efficiency was observed for Ad37. NK-92 cells transduced with chimeric Ad5/37 showed higher luciferase expression compared to the cells solely transduced with Ad5. Particularly, encapsidation of the oncolytic Ad5-hTERT vector genome into the chimeric Ad5/37 capsid showed efficient transduction of NK-92 carrier cells. These infected carrier cells then delivered the oncolytic adenoviral vector to tumor cells, which resulted in enhanced Ad5-hTERT-mediated tumor cell killing. We conclude that engineering of oncolytic Ad5 vectors with Ad37-derived knob for improved delivery into tumor sites with NK-92 carrier cells holds great promise for cancer treatment.

785. Abstract Withdrawn

786. Potential Utility of Retroviral Replicating Vector-Mediated Prodrug Activator Gene Therapy for Human Ovarian Cancer

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Retroviral replicating vectors (RRVs) have been shown to achieve efficient tumor transduction and enhanced therapeutic benefits in a wide variety of cancer models. Here, we evaluated two different RRVs derived from amphotropic murine leukemia virus (AMLV) and gibbon ape leukemia virus (GALV), which utilize different cellular receptors (PiT-2 and PiT-1, respectively) for viral entry, in human ovarian cancer cells. Quantitative RT-PCR analysis for both receptors showed that high levels of expression of were observed in RMG-1 and SKOV3 ovarian cancer cell lines compared to normal and non-malignant cells. Efficient RRV replication and spread was observed in vitro in RMG-1 and SKOV3 cells with >90% transduction achieved by Day 10-13. Furthermore, after transduction of these cells with RRVs expressing the cytosine deaminase RRV-CD, significant reduction of cell viability was observed in a 5-FC prodrug dose-dependent manner. These data indicate the potential utility of RRV vector-mediated prodrug activator gene therapy in the treatment of human ovarian cancer.

787. Targeted Gene Therapy Against Chondrosarcoma Using Superior Bacteriophage-Based Vector

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Chondrosarcoma is a malignant cartilage-forming bone tumour ranked as the second most common bone tumor. It is well known for intrinsic resistant to chemotherapy and radiotherapy. Surgical removal is current treatment, but impossible to completely eradicate the tumour. Therefore, novel approaches for chondrosarcoma therapy are urgently required. We have used filamentous bacteriophage (phage) as a cargo to deliver therapeutic genes. This phage-based vector shows several superiorities over other eukaryotic viral vectors. Propagation of phage-based vector is cost and time efficient, the phage viral particle is stable for years at 4ºC, phage capsids can be modified to accommodate drugs or therapeutic elements. Phage is a virus for prokaryotes, it has no tropism to mammalian cells, allowing engineered phage to be targeted. Here, a superior hybrid phage vector was developed as an efficient targeted gene delivery platform for chondrosarcoma treatment. The vector displays a cyclic RGD4C ligands on pIII capsids to serve as targeting ligand, to specifically bind to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin receptors overexpressed on chondrosarcoma but not chondrocytes. The endosomal escape peptide, H5WYG, was also displayed on recombinant pVIII major coat proteins to enhance gene delivery. Importantly, human TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) therapeutic transgene cassette driven by a CMV promoter was incorporated into the phage genome. After gene transfer, the targeting chondrosarcoma cells should express TRAIL and subsequently resulting in cancer cell death. Human chondrosarcoma cells, SW1353, was chosen as a model. The cells showed high expression of $\alpha_v\beta_3$, $\alpha_v\beta_5$ integrin receptors and TRAIL receptor 2 (Death receptor 5). Targeted vector encoding secreted luciferase reporter gene (RGD-Phage-*Lucia*) efficiently and selectively mediated gene delivery to these cells. The targeted vector failed to mediated gene delivery to primary human articular chondrocytes (HACs), used as control. The expressions of the integrin receptors and TRAIL receptor 2 were relatively low in HACs cells. When SW1353 cells were transduced with the targeted vector encoding human TRAIL transgene (RGD-Phage-*TRAIL*), higher expression of TRAIL and apoptosis-related genes including caspase 3, 7, 8, BAX, AKT, MDM2 were detected. Cell killing was determined on day 7 post transduction, 42 percent cell death was observed in the targeted phage vector treatment group. These data show our Phage-based vector is a promising selective and efficient tool for chondrosarcoma gene therapy. In the future, we will apply this platform in animal models of chondrosarcoma and clinical trials to investigate the efficiency and safety.

788. Novel CAR-T Cell Therapy that Can be Activated, Silenced, and Reprogrammed *In Vivo* with Soluble Protein Adapters in a Dose Dependent Manner

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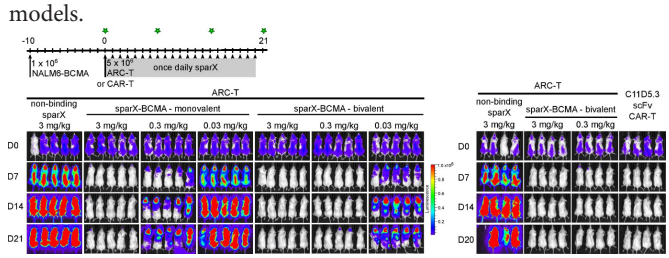
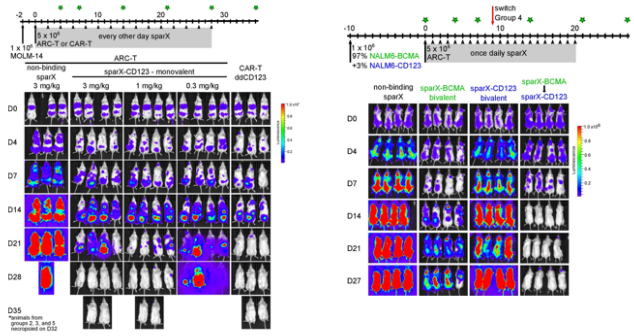
Arcellx, Gaithersburg, MD

Current engineered immune cell therapies often target tumors through a mono-specific receptor that is constitutively expressed and active. Toxicity and disease relapse in this setting are problematic. We have developed **A**ntigen **R**eceptor **C**omplex **T** (ARC-T) cells that are readily activated, silenced, and reprogrammed *in vivo* by administration of a novel non-scFv tumor-targeting **s**oluble **p**rotein **a**ntigen-**r**eceptor **X**-linker (sparX) that has been deimmunized. ARC-T cells bind exclusively to sparX. The formation of the ARC-T, sparX, and tumor complex is required for the ARC-T to kill the tumor. We have created a library of sparX that bind different cell surface targets including BCMA and CD123. In vitro studies demonstrate that co-culture of ARC-T cells, sparX-BCMA and BCMA-expressing target cells drives T-cell activation, expansion, cytokine secretion and cytotoxicity of target cells in a dose-dependent manner. T-cell activation and expansion does not occur with $BCMA^{-/-}$ cells, or with a negative control sparX. In vivo proof-of-principal studies with ARC-T/sparX-BCMA demonstrate complete BCMA-expressing tumor cell eradication in a sparX-BCMA

dose-dependent manner. Moreover, sparX-BCMA in a bivalent format conferred greater potency with both in vitro assays and in vivo tumor models.



In vivo proof-of-principal studies with ARC-T/sparX-CD123 similarly demonstrate complete eradication of CD123-expressing AML tumor cells. Using a model of tumor antigen heterogeneity, we demonstrated that ARC-T cells can be reprogrammed *in vivo* to target a second antigen, after failure of the sparX-BCMA to fully clear a tumor admixed with BCMA-negative cells.



Conclusion: This body of work shows that ARC-T cells can be activated and reprogrammed in mouse models by administration of tumor-targeting sparX adapters, and ARC-T activity is dependent on the sparX dose that can be managed to control the rate of tumor kill. SparX are functional as monovalent or bivalent constructs, and could be administered simultaneously or sequentially to address the inherent phenotypic heterogeneity of tumors.

789. The Utility of Claims Data Analysis for Gene Therapy Trials and Commercial Planning

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The Utility of Claims Data Analysis for Gene Therapy Trials and Commercial Planning Abstract: Commercialization of gene therapy can be accelerated significantly by integrating insights from longitudinal claims data. Gene therapy trials face challenges pertaining to complex preparation and implementation phases, as well as identifying qualified patients who would ultimately succeed in the trial. Specific targeting of patient populations who might respond better to trials can be achieved by examining deep patient journeys. Patient journeys help in identifying trends (when, where and why patients access care) in related disease factors as well as historic diagnoses and procedures that might affect the results of gene therapy trials. Filtering methods can be applied to design effective marketing strategies to target the right patient population based on demographics and geographical information gleaned from a claims-based analysis. Application of machine learning approaches, combined with claims data science expertise, and deep understanding of rare diseases can accelerate the identification of eligible patients for clinical trial recruitment and the launch of new drugs or therapy regimens, bringing faster relief to patients as well as being able to connect healthcare providers (HCPs), clinicians and patient advocacy groups faster and more efficiently to the people who are in desperate need for a medical breakthrough. When claims data is combined with EMR in mapping protocols, we can apply machine learning and information theoretic techniques to provide a level of analysis that can accelerate genetic trials, capturing significant quantities of medical information in the process. This leads to valuable insights into the effectiveness of a therapy as well as enabling comparison of outcomes of different treatment regimens, thus improving the commercialization outlook and market success of the therapy in question.

790. A Molecular Foundation of CRISPR-Directed Gene Editing as an Augmentative Therapy for NSCLC. I. Nuclear Uptake Kinetics and Site-Specific Cleavage of Tumor DNA

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Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-directed gene editing is approaching clinical implementation in cancer. Thus, it is imperative to define the molecular framework upon which safe and efficacious therapeutic strategies can be built. Two important reaction parameters include the biological time frame within which the CRISPR/Cas complex enters the nucleus and executes gene editing, and the method of discrimination that the CRISPR/Cas complex utilizes to target tumor cell, but not normal cell, genomes. We are developing CRISPR-directed gene editing for the treatment of non-small cell lung carcinoma (NSCLC) focusing on disabling Nuclear Factor Erythroid 2-Related Factor-Like (NRF2), a transcription factor that regulates chemoresistance and whose genetic disruption would enhance chemosensitivity. In this report, we define the time frame of cellular events that surround the initialization of CRISPR-directed gene editing as a function of the nuclear penetration and the execution of NRF2 gene disruption. We also identify a unique protospacer adjacent motif (PAM) that facilitates site-specific cleavage of the NRF2 gene present only in tumor genomes. We will discuss that our results set a scientifically meritorious foundation for the exploitation of CRISPR-directed gene editing as an augmentative therapy for lung cancer and other solid tumors.

791. Development and Biological Optimization of Star-Shaped Polymeric Nanoparticles for the Delivery of Therapeutic siRNA to Lung Cancer Cells *In Vitro* and *In Vivo*

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Introduction: Non-small cell lung cancer (NSCLC) is a major cause of cancer mortality worldwide. NSCLC is highly chemoresistant and requires novel therapeutic strategies. We have previously identified the microtubule protein, βIII-tubulin (TUBB3) as a *bona fide* therapeutic target for NSCLC[1-3]. Silencing βIII-tubulin in NSCLC cells using RNA interference agents (shRNA) increases chemosensitivity and reduces lung cancer growth and metastases in mice[1,2]. Unfortunately, TUBB3 is considered undruggable using chemical inhibitors due to its high amino acid sequence homology with related proteins. siRNA therapeutics offers a solution but requires a delivery vehicle. **Aim:** To identify the biological properties required for polymeric star-shaped nanoparticles (Star) to act as highly efficient delivery vehicles for siRNA to silence TUBB3 in NSCLC cells *in vitro* and *in vivo*. **Methods:** Star nanoparticles were synthesized as described by our team[4]. Physicochemical properties of Star-siRNA were determined using TEM and dynamic light scattering. Star-siRNA internalization, intracellular trafficking and exocytosis were examined using florescence-based assays and confocal microscopy. Star-TUBB3 siRNA gene silencing activity was measured in NSCLC cells (H1299, Calu-6, H1975, A549, H460) by western blotting and qPCR. An orthotopic syngeneic NSCLC mouse model was used to examine Star-siRNA tumour biodistribution. **Results:** Star-siRNA formed monodisperse nanoparticles (size 14.2 nm ± 0.8, zeta potential 8.7 mV ± 2.7, n = 3 experiments). At different Nitrogen / Phosphate (N/P) ratios (7:1, 14:1, 28:1) Star-siRNA were internalised into NSCLC cells. At 28:1 (N/P) ratio, Star-siRNA was able to rapidly enter NSCLC cells and escape from early endosomes. This led to potent inhibition of TUBB3 protein levels in NSCLC cells [H1229: 79 ± 8.7%*; Calu-6: 62 ± 7.8%*; H1975: 61 ± 7.2%*; A549: 47 ± 4.2%; H460: 58 ± 5.6% decrease compared to Star-non-functional (control) siRNA, n = 3, *p<0.001] 48h post-treatment. Star-siRNA when delivered systemically or locally to the lungs of mice using a nebulizer was not toxic. Notably, fluorescently labelled Star-siRNA was present in growing orthotopic mouse lung tumours up to 24h post-treatment. **Conclusions:** This study demonstrates for the first time the intracellular uptake and trafficking profile of Star-siRNA in NSCLC cells. Star-siRNA can silence TUBB3 expression in NSCLC cells *in vitro* and accumulate in mouse lung tumours *in vivo.* Taken together, Star-siRNA has the potential to be used as a novel therapeutic tool to silence TUBB3 and other undruggable genes in NSCLC cells.

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792. AAV3-JAZ Vectors for the Potential Gene Therapy of Human Liver Cancer

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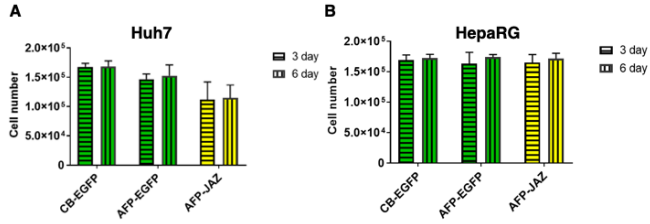
Liver cancer is the fourth leading cause of cancer death worldwide, with about 841,000 new cases and 782,000 deaths annually. During the past two decades, the incidence of liver cancer in the US has tripled while the 5-year survival rate has remained below 12%. Thus, novel therapeutic strategies are needed to target this disease. We have identified AAV3 to be the most efficient serotype for targeting human liver cancer cells *in vitro* (*Mol. Genet. Metabol*., 98: 289-299, 2009; *Hum Gene Ther*., 21: 1741-1747, 2010) and in a murine xenograft model *in vivo* (*Gene Ther*., 19: 375-384, 2010; *Hum. Gene Ther*., 25: 1023-1034, 2014). We have also described the isolation and cloning of a novel zinc finger gene, termed JAZ (*J. Biol. Chem*., 274: 27399-273406, 1999) that is evolutionarily conserved and negatively regulates the cell cycle in a p53-independent mechanism regulating cell proliferation and tumor suppression (*Blood*, 108: 4136-4145, 2006). Since forced expression of JAZ has been shown to potently induce apoptosis in murine fibroblast cells (*Cell Cycle*, 10: 2390-2399, 2011), we reasoned that the use of AAV3 vectors expressing JAZ might be a useful strategy to target human liver cancer. In the present studies, we observed that normal human hepatocytes express high levels of the JAZ, whereas JAZ expression is significantly reduced in human hepatocellular carcinoma (HCC) cells. We transduced two HCC cell lines (Huh7 and HepG2), and a non-transformed hepatic stem cell line (HepaRG), with scAAV3 vectors in which the EGFP reporter gene was under the control of either a chicken β-actin promoter (scAAV3-CB-EGFP) or the human alpha-fetoprotein promoter (scAAV3-AFP-EGFP) vectors, which were used as approriate controls. scAAV3-AFP-JAZ vectors expressed the JAZ gene under the control of the AFP promoter. Cells were transduced at a multiplicity of infection (MOI) of $1x10^5$ vgs/cell, and total cell counts were determined at day3 and day 6. JAZ protein expression was detected by Western blots using anti-HA-tag antibody. As can be seen in **Figure 1**, a modest level of growth inhibition of Huh7 cells (~25%) was observed, but only with scAAV3-AFP-JAZ vectors, suggesting that the observed inhibition was mediated by JAZ (Panel A). No growth inhibition was observed in HepaRG cells (Panel B). In a preliminary experiement, Huh7 cells transduced with scAAV3-AFP-JAZ vectors, but not mock-transduced Huh7 cells, failed to form tumors in a xenograft mouse model (n=2), suggesting that JAZ may also induce apoptosis in human HCC cells. Further studies are warranted to corroborate whether scAAV3-AFP-JAZ vector-mediated suppression of growth is an effective gene therapy strategy to target p53-negative human liver cancer. **Figure 1:** scAAV3 vector-mediated expression of JAZ leads to suppression of growth of human HCC cells, Huh7 (**A**), but not non-tumorigenic HepaRG cells (**B**).



793. A Novel Model of High-Grade Glioma in Göttingen Minipigs

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Background: Prior studies have applied driver mutations targeting the RTK/RAS/PI3K and p53 pathways to induce the formation of high-grade gliomas in rodent models. In the present study, we report the first immunocompetent, reproducible, high-grade spinal cord glioma model in pigs using lentiviral gene transfer. **Methods:** Six Gottingen Minipigs received thoracolumbar (T14-L1) lateral white matter injections of a combination of lentiviral vectors, expressing platelet-derived growth factor beta (PDGF-B), constitutive HRAS, and shRNA-p53 respectively. All animals received injection of control vectors into the contralateral cord. Animals underwent baseline and endpoint magnetic resonance imaging (MRI) and were evaluated daily for clinical deficits. Hematoxylin and eosin (H&E) and immunohistochemical analysis was conducted. Data is presented using descriptive statistics including relative frequencies, mean, standard deviation, and range. **Results:** 100% of animals (n = 6/6) developed clinical motor deficits ipsilateral to the oncogenic lentiviral injections by a three-week endpoint. MRI scans at endpoint demonstrated contrast enhancing mass lesions at the site of oncogenic lentiviral injection and not at the site of control injections. Immunohistochemistry demonstrated positive staining for GFAP, Olig2, and a high Ki-67 proliferative index. Histopathologic features demonstrate consistent and reproducible growth of a high-grade glioma in all animals. **Conclusions:** Lentiviral gene transfer represents a feasible pathway to glioma modeling in higher order species. The present model is the first immunocompetent pig model of high-grade spinal cord glioma and may be used in preclinical therapeutic development programs.

794. CDH6/YAP1/OCT4 Interactions Mediating Differentiation Resistance of Mesenchymal Stem Cells Participate in Solid Tumor Development

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Background Despite the promising clinical benefits, some detrimental effects of mesenchymal stem cells (MSCs) have been reported, in some cases including tumorigenicity. The reason for this remains unclear. YAP1 (Yes-associated protein 1) and its associated pathway play a key role in organ development, but their potential involvement in MSC tumorigenicity remains inconclusive. Therefore, we conducted this study to investigate YAP1-related signaling networks in both MSCs and solid tumors. **Methods** MSCs were cultured in ostegenic differentiation medium according to manufacturer's instructions, and harvested on days 12, 19 and 25. Cells were stained for cell differentiation analysis using Alizarin Red. Discrete stages of osteogenesis, including differentiation/osteogenesis-resistant MSCs (DR), differentiated osteoblasts (DO) and precursor osteoblasts (PO), were recorded based on a single-cell transcriptome analysis of individual MSCs during osteocyte differentiation with single-cell RNAseq. Gene Specific Analysis was performed for differential expression analysis. Verteporfin was administered to test YAP1 inhibition in HeLa and HepG2 cell lines. Cell viability was assessed by trypan blue staining, and RT-PCR was performed to test for expression of YAP1 and associated proteins octamer-binding transcription factor 4 (OCT4) and cadherin6 (CDH6) after YAP1 inhibition. Clinical data was analyzed for correlation of YAP1, OCT4 and CDH6 in solid tumors. **Results** We found 1780 genes were significantly differentially expressed in both PO and DO compared to the DR MSCs. Importantly, CDH6, OCT4 and YAP1 expression were significantly up-regulated within the DR MSCs in comparison to PO or DO. A survey of 240 clinical cohorts revealed a high correlation among the expression of CDH6, YAP1 and OCT4 in a variety of solid tumors. Additionally, YAP1 was expressed in only a fraction of cancer cells as well as DR MSCs. YAP1 inhibition down-regulated solid tumor cell lines viability and gradually inhibited YAP1 nuclear localization with doses of 2, 5 and 10μM verteporfin, while reducing the transcriptions of CDH6 and OCT4 as well. **Conclusion** CDH6/YAP1/OCT4 interactions mediating DR MSCs participate in the development of solid tumors.

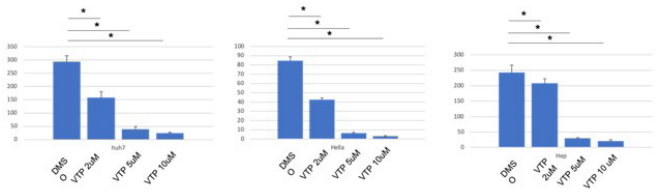

Fig5. Cell viability was assessed using trypan blue staining in HeLa, Hepa2 and Huh7 solid tumor cell lines.

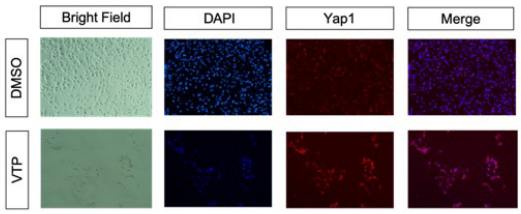

Fig6. Representative Images of the effects of Verteporfin 2uM yap1 inhibition in HeLa cells (X20). Cells were stained with anti YAP1 antibodies (red) and live cell nucleus was stained using DAPI (blue).

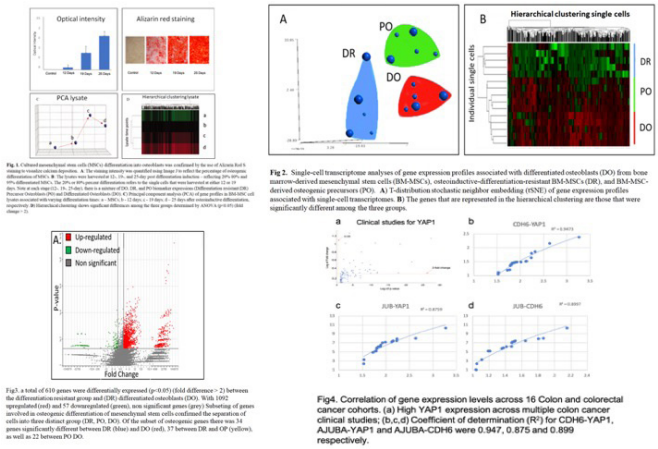

Fig3. a total of 610 genes were differentially expressed ($p<0.05$) (fold difference > 2) between the differentiation-resistant group and (DR) differentiated osteoblasts (DO). With 1092 upregulated (red) and 57 downregulated (green), non-significant genes (grey) Subsetting of genes involved in osteogenic differentiation of mesenchymal stem cells confirmed the separation of cells into three distinct group (DR, PO, DO). Of the subset of osteogenic genes there were 34 genes significantly different between DR (blue) and DO (red), 37 between DR and OP (yellow), as well as 22 between PO DO.

Fig 2. Single-cell transcriptome analyses of gene expression profiles associated with differentiated osteoblasts (DO) from bone marrow-derived mesenchymal stem cells (BM-MSCs), osteoinductive-differentiation-resistant BM-MSCs (DR), and BM-MSC-derived osteogenic precursors (PO). **A)** T-distribution stochastic neighbor embedding (tSNE) of gene expression profiles associated with single-cell transcriptomes. **B)** The genes that are represented in the hierarchical clustering are those that were significantly different among the three groups.

Fig4. Correlation of gene expression levels across 16 Colon and colorectal cancer cohorts. (a) High YAP1 expression across multiple colon cancer clinical studies; (b,c,d) Coefficient of determination (R^2) for CDH6-YAP1, AJUBA-YAP1 and AJUBA-CDH6 were 0.947, 0.875 and 0.899 respectively.

795. Hypoxia Lung Cancer Establish Liver Pre-Metastatic Niche by Exsomal CD151 Delivery

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Introduction: Hypoxia plays a critical role during the evolution of malignant cells and tumor microenvironment (TME). Cell-cell interactions via exosome between primary cancer cells and the microenvironment of distant organs are crucial for pre-metastatic niche (PMN) formation and metastasis. This study aims to clarify how hypoxic condition remodeling liver PMN through exosome shed from lung cancer cells. **Methods:** Lung cell CL1-5 cells were seeded at a100 mm dish and cultured for 24 hours under hypoxic (1% O_2) or normoxic (20% O_2) conditions in cell culture medium containing 1% exosome-free serum (Life Technologies, Grand Island, NY). The exosomes derived from lung cancer cells were purified by total exosome isolation reagents (from cells) (Life Technologies, Grand Island, NY). The exosomal protein was determined by LC-MS/MS. The tube formation of human hepatic sinusoidal endothelial cells (HHSEC) was used evaluated the effect of lung cancer-derived exosome in angiogenesis. **Results:** Previous our study has revealed that hypoxia increased lung cancer cells to secrets exosome, which are involved the Shaping of TME by microRNAs. We investigate the biologic role of exosomal proteins in lung cancer after hypoxic stimulation. We found that there are 14 proteins only in the exosome produced hypoxic lung cancer, but not the exosome isolated from normaxic condition. HHSCS could uptake the exosome of lung cancer, and the levels of CD151 was higher after hypoxic lung cancer-derived exosome treatment. In addition, hypoxic lung cancer-derived exosome increased the angiogenesis of HHSEC, whereas the effects of exosome were inhibited when CD151 was silenced in lung cancer. Moreover, the recruitment and transendothelial migration of neutrophil in HHSEC was increased when HHSEC uptake hypoxic lung cancer-derived exosome. Our research reveals that lung cancer cells-hepatic sinusoidal endothelial cells communicated by exosomal CD151 released from hypoxic cancer cells, which may facilitate the formation of liver PMN. **Conclusions:** Taken together, our study reveals the therapeutic value of cancer-derived exosomal CD151 under hypoxic conditions, and investigates a unique intercellular interaction, mediated by cancer-derived exosomes, which modulates liver vasculature prior lung cancer metastasis.

The proteins only in hypoxic lung cancer exosome	
TBA1A_HUMAN	Tubulin alpha-1A chain
GBG12_HUMAN	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12
TENX_HUMAN	Tenascin-X
KKCC2_HUMAN	Calcium/calmodulin-dependent protein kinase kinase 2
ASPH_HUMAN	Aspartyl/asparaginyl beta-hydroxylase
RED2_HUMAN	Double-stranded RNA-specific editase B2
AT12A_HUMAN	Potassium-transporting ATPase alpha chain 2
RL32_HUMAN	60S ribosomal protein L32
APOM_HUMAN	Apolipoprotein M
CD151_HUMAN	CD151 antigen
TTBK1_HUMAN	Tau-tubulin kinase 1
H4_HUMAN	Histone H4

796. In Vivo Targeted Delivery of Functional Anti-CD19 CARs in Human Lymphocytes with Chemically Encapsulated Lentiviral Vectors as an Alternative to Ex Vivo CAR T-Cell Therapies

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Aratinga.Bio TNP, Villejuif, France

With Yescarta® and Kymriah® marketed therapies for B cell hematological malignancies and more than 300 ongoing clinical trials, genetic modification of T cells with chimeric antigen receptors

(CARs) recognizing surface antigens on tumor cells has emerged as a revolutionary therapeutic strategy in oncology. Despite impressive clinical benefits, the complex logistics required to manufacture *ex vivo* CAR-T cells from individual patients and the associated costs represent major hurdles to the widespread use of these therapies. aratinga.bio TNP is a preclinical-stage biotechnology company developing *in vivo* CARs based on lentiviral vectors encoding anti-CD19 CARs encapsulated in oligopeptide-modified poly(beta-amino ester)s (OM-PBAEs) biodegradable polymers. Here we report on a method to reprogram *in vitro* primary mouse and human T cells to express a functional CAR directed against the CD19 antigen with a cytotoxic activity demonstrated in peripheral blood mononuclear cell preparations obtained from 10 different healthy donors. These LV/OM-PBAE nanoparticles have been engineered to specifically target CD3-positive cells and to selectively trigger the expression of the CAR in these target cells. The repeat intravenous administration of these nanoparticles in Balb/C mice was safe and well tolerated as no obvious sign of distress, body weight loss, change in blood cell count or cytokine levels were reported. The pronounced tropism for blood cells observed *in vivo* with these nanoparticles provides an obvious advantage for CAR T-cell therapy of blood malignancies. Preliminary *in vivo* efficacy data will also be presented. These encouraging results indicate that OM-PBAE encapsulated LVs have highly promising properties with a great potential for further preclinical and clinical development of *in vivo* universal CAR T-cell therapy.

Hematologic and Immunologic Diseases

797. Generation of Multi Modal Lentiviral Vectors for High-Level Fetal Hemoglobin Expression

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Induction of fetal haemoglobin (HbF) in sickle cell disease (SCD) is a proven approach to ameliorate disease phenotype. Several drugs effectively increase expression of the γ-globin gene (HBG1/HBG2). Alternatively, expression can be increased by gene addition or knockdown of BCL11a. BCL11a is an attractive candidate to induce production of native HbF in patients with hemoglobinopathies, and gene transfer technologies using lentiviral vectors (LV) have been used to introduce γ-globin sequences and are not limited by the γ promoter. Both approaches have advanced to clinical testing. We are investigating the potential of combining these strategies to express an introduced γ-globin gene expressed specifically in erythrocytes while simultaneously inducing expression of the native gene. We developed and optimized a series of vectors encoding the individual components. To downregulate BCL11a we used a LV vector with an shRNA embedded into an mirE scaffold, which has been shown to have efficient knockdown potency and used the β-globin promoter for erythroid-specific expression. CD34+HSPCs were transduced and differentiated into erythroid cells and analysed for the expression of *BCL11A*, HBG1 and HbF. Compared to control cells transduced with scrambled shRNA, there was a >70% reduction of BCL11A resulting in 5-fold upregulation of HBG1 transcripts and 20% increase in cells expressing HbF. This was achieved without selecting the transduced erythroid cells. This data shows that this vector provides significant erythroid-specific knockdown of *BCL11A*. For gene addition, we developed several self-inactivating (SIN) LV vectors with the human ^{A}γ-globin sequence. Each contains identical HBG1 sequences, various lengths of the β-globin (HBB) promoter region and a human locus control region (LCR) containing DNA hypersensitive sites (HS) 2-4. These vectors were evaluated for HbF expression and amelioration of disease phenotype in an in vivo model of SCD. In brief, Sca-1^{+} cells from Townes SCD mice were isolated and transduced with the HBG vectors and were transplanted into lethally irradiated C57BL/6 mice. These mice were monitored for 20 weeks to evaluate HBG1 induction and reversal of SCD phenotype. In the primary transplants, our CMCEU3 vector had greatest expression of HBG1 with an average vector copy number (VCN) of ~1 as measured by cellulose acetate electrophoresis and qPCR, respectively. These mice also had improved elongation index (EI) measurements under hypoxic conditions as measured by LORRCA oxygen scan. Importantly, upon secondary transplantation of bone marrow from CMCEU3 mice, we achieved a 60/40 ratio of γ to β-globin as measured by HPLC with an average VCN of ~4. The secondary transplanted mice had improved haemoglobin levels of 13 g/dl as compared to 8 g/dl in mock transplanted controls. These mice also had decreased sickling in response to hypoxic conditions as measured by a combination of a hemox analyzer and imaging flow cytometry. Therefore, CMCEU3 can achieve therapeutic levels of HbF with correction of the SCD phenotype at low to moderate gene copy numbers. To further elevate fetal haemoglobin, we generated bicistronic vectors with the BCL11a shRNA contained in intron 1 and/or 2 of our γ-globin gene. The expression cassette was used for production of VSV-G pseudotyped LV vector and high titer vector (>10^7) was produced. The vector was used to efficiently transduce human CD34+ haematopoietic stem and progenitor cells (HSPCs) in vitro, and is being tested in SCD mice, which are currently evaluated for levels of γ-globin expression.

798. Targeted CRISPR/Cas9-Mediated Gene Addition to a Safe Harbor in Placental Cells for the Treatment of Hemophilia A

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Hemophilia A (HA) is an X-linked inheritable disorder caused by a defect in the gene for coagulation factor 8 (FVIII), which leads to decreased or absent clotting activity. Since HA is a monogenic disease,

gene therapy represents a promising therapeutic approach for achieving lifelong phenotypic/clinical correction. Considering the heterogeneity of FVIII mutations and the need for a universal treatment that could benefit all HA patients, a "gene addition" approach to knock-in a functional fVIII transgene would be preferable compared to attempting to correct the mutation via gene-editing, which would require gRNA and homology arms specific to each patient's mutation. Although highly efficient gene addition can be achieved by lentiviral gene delivery, using CRISPR/Cas9 to achieve location-specific insertion of a fVIII transgene into a genomic safe harbor, such as the AAVS1 site, should allow efficient gene addition, while avoiding the potential for insertional mutagenesis that is potentially inherent to all integrating viral vectors. In order to start investigating the efficiency and specificity of our CRISPR/Cas9 delivery system, we co-transfected human placental stem cells (PLCs) and liver-derived endothelial cells (HLECs) with the plasmids pCas-Guide-AAVS1 and pAAVS1-puro-EF1a-liver-optimized bioengineered FVIII (lcoET3). Genomic insertion of the EF1a-lcoET3 cassette was assessed via PCR, and expression of the lcoET3 transgene was confirmed by measuring FVIII mRNA by RT-qPCR. Clotting activity was measured in the supernatants of the modified PLCs by aPTT assay, using unmodified PLCs and PLCs transduced with a lentiviral vector encoding the same FVIII transgene (lcoET3) as negative and positive controls, respectively. RT-qPCR with lcoET3-specific primers confirmed successful insertion and expression of this bioengineered fVIII transgene in both HLECs and PLCs following CRISPR/Cas9-mediated gene knock-in. aPTT assays demonstrated that the CRISPR/Cas9-engineered PLCs (trial 1: 11.47 ± 1.15 and trial 2: 6.39 ± 0.28 IU/10^6 cells) secreted significantly more FVIII:C than engineered HLECs (<1 IU/10^6 cells) and control PLCs (2.82 ± 0.07). Importantly, the differences in the levels of FVIII:C measured by aPTT correlated with the fold-change seen at the mRNA level by RT-qPCR. Next-generation sequencing to confirm successful insertion at the AAVS1 site and define the rate and location of any off-target genomic insertion is currently underway. In conclusion, the levels of FVIII:C produced by PLCs following CRISPR/Cas9-mediated knock-in of the bioengineered lcoET3 fVIII transgene were sufficient to be in the therapeutic range, and this cell-gene delivery platform could thus be used to treat HA. The ability to successfully edit HLECs *in vitro* represents an important first step to establishing the feasibility of using CRISPR/Cas9 to mediate gene-editing in HLECs *in vivo*, thereby achieving FVIII expression within the cells that serve as the natural site of synthesis of this protein and thereby correcting HA.

799. Target Optimized Variant of CRISPR Associated Nuclease Enables Allele-Specific Knock Out of *ELANE*-Related Neutropenia

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Emendo Biotherapeutics, Ness Ziona, Israel

Heterozygous mutation in ELANE causes the majority of Severe Congenital Neutropenia (SCN). Over 100 heterozygous mutations in ELANE associate with the disease. We identified three SNPs that can be linked with the majority of mutated alleles and designed CRISPR based strategies to knock out these alleles. We used our directed-evolution platform to optimize a CAS nuclease to effectively and specifically cleave at the SNPs. We demonstrated that the editing of normal human CD34+ cells with the optimized CRISPR composition specifically knocked out single ELANE allele and the edited cells mature to active neutrophils. We develop a CRISPR composition for the ex-vivo editing of SCN patients' CD34+ cells. The approach we present here is novel and broad; it opens up unlimited opportunities for gene editing therapy in cases of dominant-negative mutations, compound heterozygous mutations, and haploinsufficiency.

800. Lentiviral-Mediated Preclinical Studies for the Gene Therapy of *RPS19*-Deficient Diamond-Blackfan Anemia Patients

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Allogenic hematopoietic stem cell transplantation (HSCT) currently represents the only curative treatment for the bone marrow failure (BMF) of patients with Diamond Blackfan Anemia (DBA). Mutations in *RPS19* are the most frequent, and account for approximately 25% of all DBA patients. Aiming at developing a gene therapy approach for *RPS19*-deficient DBA patients, we have first investigated the HSC compartment in the BM of these patients. Compared to other bone marrow failure syndromes, such as Fanconi anemia (FA) patients, numbers of BM $CD34^+$ cells and Colony Forming Cells were 10 and 3.3 fold increased respectively in age-matched DBA patients, suggesting that collection of HSCs should not constitute a significant limitation for the gene therapy of DBA patients. Additionally, functional HSC assays based on the analysis of the repopulation potential of DBA CD34+ cells in NSG mice did not show significant defects compared to BM cells from HDs. With the aim of correcting the phenotype of *RPS19*-deficient HSCs, clinically applicable lentiviral vectors (LV) were generated using a codon-optimized version of *RPS19* driven by the *PGK* or the *EF1α* promoters. As a first proof of concept, studies in K562 cells in which *RPS19* was down-regulated with sh-*RPS19* LVs showed that transduction with either of the therapeutic LVs restored the expression of *RPS19* and corrected defects in the ribosomal biogenesis of sh-*RPS19* transduced cells. The therapeutic efficacy of these LVs was confirmed in primary $CD34^+$ cells from *RPS19*-deficient patients. Transduction of these $CD34^+$ cells with either of the therapeutic LVs increased 1.5 and 3.9 times the number of hematopoietic granulo-macrophage and erythroyd colonies, respectively, as compared to the control group transduced with a non-therapeutic EGFP-LV. Moreover, therapeutic

LVs reverted the red blood cell differentiation defect characteristic of DBA $CD34^+$ cells, increasing between 2.5-3.5 the output of $CD71^+$/$CD235^+$ mature erythroid cells. Transduced $CD34^+$ DBA cells were also capable of repopulating the hematopoiesis of NSG mice. In addition, no toxicity was observed due to the transduction of HD $CD34^+$ driven with any of the therapeutic vectors. In conclusion, our preclinical studies support that the lentiviral-mediated gene therapy of RPS19-deficient DBA patients should constitute an efficient and safe approach for the treatment of the hematopoietic defects characteristics of DBA patients.

801. Human Lentiviral Gene Therapy Restores the Cellular Phenotype of IFNγR1 Deficient MSMD

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Complete recessive interferon γ receptor 1 (IFNγR1) deficiency, known as one genetic etiology of Mendelian Susceptibility to Mycobacterial Disease (MSMD), is a life-threatening congenital disease leading to premature death in early childhood. Affected patients present a pathognomonic predisposition to recurrent and severe infections with environmental mycobacteria or the *Mycobacterium bovis* Bacillus Calmette Guerin (BCG) vaccine. Current therapeutic options are limited to antibiotic administration, which is only symptomatic. Given the clinical success of gene therapy, our research group previously established a lentiviral gene therapy approach, feasible to restore antimycobacterial activity in an IFNγR1-deficient mouse model. In this study, we used a human lentiviral mediated gene therapy approach for IFNγR1-defiency and pave the way for the first causative treatment of IFNγR1-deficiency in human MSMD patients. To correct the cellular phenotype of INFγR1 deficient cells, we developed third generation SIN lentiviral vectors expressing the human IFNγR1 cDNA constitutively from a viral SFFV or physiological EFS promotor. In our study, we demonstrate stable transgene expression for more than four weeks in healthy human K562 cells without interference with cell viability and proliferation of transduced hematopoietic cells. Using an IFNγR1-deficient HeLa cell model, we further demonstrate stable receptor reconstitution with a 4- to 5-fold increase of CD119 (IFNγR1) compared to uncorrected IFNγR1-deficient HeLa cells. Overexpression of IFNγR1 was associated with a restored IFNγ-IFNγR1 signaling pathway without interfering type I interferon response. Detailed evaluation of the IFNγ downstream signaling pathway revealed a 20-fold induction of STAT1 phosphorylation upon IFNγ-stimulation and normalized activation of the IFNγ downstream targets SOCS3, MHC-II and LMP7, which was comparable to wildtype HeLa cells. Similarly, corrected HeLa cells sorted for various levels IFNγR1 surface expression showed an IFNγ dose dependent phosphorylation profile of STAT1, highlighting low IFNγR1 expression to be sufficient for functional correction. In addition, transduction of SV40-immortalized and primary fibroblasts from IFNγR1-deficient MSMD patients was able to recover IFNγR1 expression in both cell sources. Similar to the HeLa cell model, also transduction of primary patient cells was able to restore type II interferon signaling upon stimulation with IFNγ. In summary, we here present lentiviral vectors to correct IFNγ signaling and present the first gene therapy approach to treat patients suffering from IFNγR1-deficient MSMD.

802. Differential Engraftment of Hematopoietic Stem and Progenitor Cells with High Numbers of Lentiviral Vector Integrations in the NBSGW vs NSG Xenograft Models

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Ex vivo gene therapies rely on efficient genetic modification of hematopoietic stem and progenitor cells (HSPCs) and their subsequent ability to engraft and repopulate the hematopoietic system. The degree of transgene integration in a product can be determined by vector copy number (VCN). While high VCNs generally ensure sufficient transgene expression to achieve a therapeutic effect, it is also hypothesized that the theoretical risk of insertional oncogenesis increases with increasing integrations per cell. This hypothesis assumes that clones within the cell product containing very high VCNs are equally capable of supporting long-term engraftment as those with lower or no vector integrations. The relative maintenance of engraftment capability of genetically modified human cells compared to unmanipulated cells is commonly assessed via xenotransplantation in the immunodeficient NSG mouse model. We previously demonstrated that cell populations sorted by discrete VCN ranges demonstrate different levels of engraftment in NSG mice. As VCN increases, engraftment efficiency decreases, suggesting high copy clones within a lentiviral vector (LVV) transduced cell population, which may otherwise contribute to a higher risk of insertional oncogenesis, do not persist amongst the engrafted population in this model. We have also previously shown data suggesting marked reduction in high-VCN progenitors from peripheral blood mononuclear cells and bone marrow (BM) post-infusion in comparison to the manufactured drug products in a gene therapy clinical trial. The NBSGW mouse model is increasingly utilized for investigating the effects of genetic manipulation in human HSPCs, particularly for hemoglobinopathies. Unlike the NSG mouse, the NBSGW model allows for high and sustained levels of human cell engraftment without conditioning, as well as the production of human erythroid progenitor cells in the BM. These critical differences led us to question if high VCN cells would also engraft and be maintained in the NBSGW model. Briefly, we transduced mobilized peripheral blood CD34+ HSPCs with an LVV encoding a GFP reporter and subsequently sorted the transduced cells based on GFP expression (untransduced [GFP-Neg], GFP-Low, GFP-Mid, and GFP-High). We performed methylcellulose cultures to quantify the colony-forming potential of cells with different VCNs and to determine VCNs of individual

hematopoietic colony-forming cells. The sorted cells were transplanted into NBSGW or busulfan-conditioned NSG mice at equivalent doses to evaluate the engraftment potential of the different cell populations across the two models. As shown previously, there were statistically significant differences between the engraftment of human cells in NSG mice across the GFP-Neg group (38% hCD45), GFP-Mid (12% hCD45, $p<0.05$) and GFP-High (5% hCD45, $p<0.05$) groups. The same cells in the NBSGW model demonstrated a modest though statistically significant decrease in engraftment between the GFP-Neg group (77% hCD45) and GFP-High group (64% hCD45, $p<0.05$); the GFP-Low and GFP-Mid group engraftment efficiencies were equivalent to the GFP-Neg group. These results highlight the differences in engraftment efficiency and maintenance of human cell grafts between the NSG and NBSGW models. These data suggest that cell products containing a population with an engraftment deficit might not be detected in the NBSGW model, which may result in different interpretations of the risk profile of the given cell product. These results should be carefully considered in the selection of an appropriate mouse model for the assessment of the engraftment potential of any gene-modified stem cell product.

803. Direct Comparison of DNA, RNA, AAV Nucleic Acid Delivery Technologies for *In Vivo* Mouse Expression of a Human IgG

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Delivery of nucleic acids into a patient's cells enables expression of protein drugs to prevent or treat disease. There are three well established nucleic acid delivery platforms: adeno-associated virus (AAV) viral vector delivery, mRNA encapsulation in lipid nanoparticles (LNPs), and DNA delivered via electroporation. While each platform has been shown to express proteins or antibodies at µg/mL levels *in vivo*, to date, they have not been directly compared. AstraZeneca, in partnership with Defense Advanced Research Projects Agency (DARPA), is the first to directly compare multiple *in vivo* protein expression technologies which promise to revolutionize biologics-based medicines. These platforms are being evaluated for expression using a single broadly-neutralizing, anti-influenza mAb, MEDI8852, to establish their potential as an effective response to a pandemic outbreak. Here we show that our lead AAV, mRNA, and DNA candidates induce peak MEDI8852 serum levels in mice of 100-150, 200-300, and 20-30 µg/mL, respectively. The time required to reach these peak titers varied between platforms from a day to several weeks. Expression duration ranged from days to months as mRNA has robust early onset mAb expression but is transient in nature, AAV delivery takes weeks to provide peak mAb expression but expression is persistent, and DNA delivered via electroporation is between the two. Functional expression of MEDI8852 was also confirmed, as each platform protected mice from a 30xLD_{50} lethal influenza challenge. This protection was within 7 days of administration, indicating that these platforms hold promise in case of an actual pandemic situation. Our work demonstrates that these platforms can be efficient delivery vehicles for pandemic pathogen prevention if results translate to larger animal models and sets the stage for broader adoption as delivery platforms for other biological targets. Funding source: This research was developed with funding from the Defense Advanced Research Projects Agency under HR011-18-3-001. The views, opinions and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government. Approved for Public Release, Distribution Unlimited

804. Genomic Characterization of the Berkeley Sickle Cell Mouse Model

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Preclinical studies to re-activate fetal hemoglobin (HbF) in patients with sickle cell disease (SCD) by genome editing have shown promising results. We have shown CRISPR/Cas9-mediated editing of the gamma-globin (*HBG1* and *HBG2*) promoters in primary human hematopoietic stem and progenitor cells (HSPCs) results in robust HbF ($\alpha2\gamma2$) induction both in tissue culture and following xenotransplantation. While these results are promising, editing of the *HBG* promoters to induce gamma-globin to a therapeutic level and improve SCD-related organ pathology is not well understood. To address this, we performed genome editing of Berkeley SCD mouse HSPCs. The transgenic Berkeley model has not been well characterized and transgene sequencing information is limited. Therefore, we investigated the human sequences comprising the transgene by target locus amplification sequencing and identified that the Berkeley mouse has greater than ten copies of the human transgene integrated into the mouse chromosome (mChr1). Based on the known toxicity of DNA double-strand breaks (DSBs) in mammalian cells, we hypothesized that classical genome editing using CRISPR/Cas9 at several *HBG* promoter sites within the transgene would result in multiple DSBs. This, in turn, would result in a heightened DNA damage response and negatively affect the survival and engraftment of mouse HSPCs in a transplant model. To test this, we edited Berkeley HSPCs with CRISPR/Cas9 + gRNA ribonucleoprotein (RNP) complex targeting the *HBG* promoters. Proliferation, cell cycle analysis, and apoptosis were monitored for 5 days to evaluate DNA damage responses. Fluorescence in situ hybridization (FISH) was performed to observe large-scale chromosomal changes resulting from multiple DSBs. Targeting *HBG* promoter sites (77.6±1.6% indels at 72 hrs) with gRNA had a statistically significant negative impact on total cell number (3.56E5±1.22E5 vs 1.05E6± 1.65E5 in controls, $p<0.05$). The decrease in cell number was associated with increased apoptosis and necrosis (56.4%±2.2% vs. 27.4%±2.4% in controls, $p<0.01$) and with a lack of proliferation (16.3±2.8% vs. 32.8%±3.4% in controls, $p<0.001$) by annexin V and BrdU assays, respectively, at 48 hours. In addition, a heightened p21 response (six-fold increased over controls) was observed in *HBG* edited cells, indicating p53-mediated activity. We measured, by FISH, that 50.4% of *HBG* promoter edited cells (n=1000 cells) had chromosomal abnormalities over unedited

controls (0.04% abnormal). Preliminary results in transplantations of edited cells showed >60% of mice that received edited HSPCs (n=6) died after 8 weeks compared to no effect on survival in the Cas9-only electroporated HSPC recipients (n=6), suggesting a lack of successful engraftment. The remaining mice are monitored for engraftment of donor cells, editing efficiency, HbF induction, and hematological parameters for up to 18 weeks to determine long-term effects of editing the human transgene within HSPCs in the Berkeley model. Our results showed, (1) a characterization of the human beta-globin locus in the Berkeley transgenic model and (2) that classical genome editing within the human beta-globin locus transgene has a negative effect on Berkeley HSPCs. The extent of DNA damage impairs engraftment of edited HSPCs, limiting the capacity of transplantation studies, HbF quantification, and assessment of organ pathology. These results support previous findings of heightened DNA damage and p53 response after multiple DSBs and highlight limitations of the Berkeley SCD mouse as a preclinical model. Alternatively, it opens the door for the Berkeley SCD mouse to test the efficacy and cytotoxicity of new base-editor strategies, which create DNA nicks rather than DSBs.

805. Inhibition of p38 MAPK Improves the Engraftment of Hematopoietic Stem and Progenitor Cells Derived From a Pro-Inflammatory Environment

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Phagocytes from patients with chronic granulomatous disease (CGD) are unable to kill pathogens due to lack of NADPH oxidase activity, which renders patients susceptible to severe, life-threatening bacterial and fungal infections. The only curative treatment options for CGD patients are allogenic hematopoietic stem cell (HSC) transplantation or retroviral gene therapy. However, previous gene therapy trials for CGD were hampered by insertional mutagenesis of the applied LTR-driven gamma-retroviral vectors and lacked long-term engraftment of corrected HSCs. Hyperinflammation and expression of the pro-inflammatory cytokine interleukin-1 beta (IL1B) in the bone marrow of CGD patients might induce aberrant cell cycling of HSCs accompanied by loss of their long-term repopulation potential. To address this problem, we recently showed that the sgRNA-mediated inhibition of p38 MAPK, a downstream signal transducer of the IL1 signaling pathway, could improve the repopulating capacity of hematopoietic stem and progenitor cells (HSPCs) during transplantation. To mimic CGD pathophysiology and the engraftment defect, we challenged donor mice with IL1B prior to HSPC isolation. Using a competitive transplantation assay, we explored the possibility to rescue the IL1B-induced engraftment deficit via p38 MAPK inhibition during *ex vivo* cultivation of HSPCs. For potential clinical translation we applied a small molecule inhibitor instead of a genetic knockout to target p38 MAPK. HSPCs cultured in the presence of a p38 MAPK inhibitor exhibited reduced proliferation, lower apoptosis rates and increased expression of the HSC homing factor CXCR4. We demonstrated that inhibition of p38 MAPK significantly improved the engraftment of IL1B-challenged cells by over 20% compared to untreated controls upon analyses of peripheral blood and spleens of transplanted animals. Moreover, the HSPC engraftment in the bone marrow of transplanted mice was almost restored to wild type levels in p38 MAPK inhibitor-treated IL1B-challenged cells compared to untreated cells. Similarly, via p38 MAPK inhibition we were able to improve the repopulating capacity of HSPCs derived from X-linked CGD mice by up to 20%. In summary, our findings demonstrate that p38 MAPK may serve as a potential druggable target to improve engraftment of HSPCs derived from a pro-inflammatory environment.

806. *barcodetrackR*: An R Package for the Visualization of Cellular Barcoding Data

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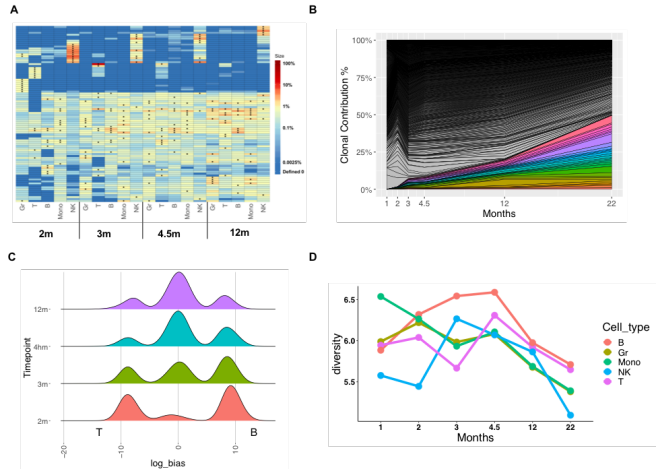
Cellular barcoding is a technique by which individual cells are labeled with unique genetic "barcodes" allowing for quantitative analysis of clonal progeny in fate-mapping and lineage-tracing studies as well as high-throughput screens. Cellular barcoding has been used to study lineage differentiation during hematopoiesis both *in vitro* and *in vivo* using murine and primate models. Due to the large number of research laboratories engaging in cellular barcoding experiments, there is an increasing need for transparency and reproducibility in the experimental methods, data processing, and secondary analysis. Here, we set out to make secondary analysis of cellular barcoding data more accessible and reproducible for researchers across the spectrum of computational expertise. We do so through the open-source R package *barcodetrackR* which is freely available on Github. > The *barcodetrackR* package includes easy-to-use functions which store results from barcoding experiments into the SummarizedExperiment object class in R which is compatible with other types of data such as single cell RNA sequencing data. The package includes functions for normalizing barcoding data and visualizing the results. Users can conduct analysis using command-line function calls in R or through a Shiny app which allows for point-and-click data visualization. > The *barcodetrackR* package can help users gain insight into clonal patterns over time, correlation between samples, clonal bias between different samples, and clonal diversity of samples. Figure 1 illustrates visualizations from the *barcodetrackR* package applied to cellular barcoding data from a long-term non-human primate study of hematopoiesis. The package compiles visualizations that are used frequently in published papers such as heat maps and diversity plots; it also includes new visualization techniques created by the authors. We demonstrate the use of *barcodetrackR* on our own published datasets as well as other cellular barcoding datasets from the literature. Importantly, the package's use is not constrained to cellular barcoding data. The analysis methods and visualization tools can be used to analyze any data that counts features across samples. We illustrate this by using *barcodetrackR* to visualize T-cell receptor sequencing data, viral integration site retrievals, and

microbiome abundance data. > The *barcodetrackR* package allows users to easily and reproducibly analyze cellular barcoding data. Through simple command-line functions or point-and-click within a Shiny app, users can create information-rich visualizations. This open source R package will enhance communication and transparency within the cellular barcoding community. >



> **Figure 1:** Use of *barcodetrackR* package to visualize cellular barcoding data from a long-term *in vivo* study of hematopoiesis. **A)** Barcode heat map shows the abundance of the top ten clones from each sample across five cell types and four timepoints. **B)** Clonal contribution of individual clones within NK cell samples over six timepoints, with top 30 clones from the final timepoint colored. **C)** Ridge plot quantifying the relative bias of clones from B cell and T cell samples across four timepoints. **D)** Shannon diversity of samples from five cell types across six timepoints of cellular barcoding data. *Gr*, granulocytes; *Mono*, Monocytes.

807. Lentivector versus CRISPR/Cas9/AAV6 Gene Editing in X-Linked Severe Combined Immunodeficiency CD34+ Hematopoietic Cells

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Gene therapy using integrating lentivector (LV) with busulfan conditioning has achieved clinical benefit in patients with X-linked severe combined immunodeficiency (X-SCID). However, in the previously-transplanted older X-SCID cohort, the improvement of immunity and of clinical status is gradual over several years and the immune restoration may be incomplete. This may be attributable to factors such as the vector design using promoters that lack physiological regulation and potency, and the relatively low gene correction levels in the quiescent hematopoietic stem/progenitor cells (HSPC)s. Transduction enhancers can increase vector copy numbers (VCN) but whether higher VCN will improve the clinical outcome is not known, nor are the risks of increased VCN yet understood. Genome editing of X-SCID patient hematopoietic cells by the insertion of a corrective *IL2RG* at the endogenous locus has previously been shown to restore function and *in vivo* differentiation of multi-lineage immune cells following transplant into immunodeficient mice. However, there remain concerns regarding the overall feasibility of an editing approach using AAV-delivered donors with respect to the problem of maintaining gene correction levels observed *ex vivo* when edited human HSPCs are engrafted as human xenografts in immune deficient mice. We sought to compare LV versus CRISPR/Cas9/AAV6 gene therapy approaches with respect to subsequent lineage specific correction of X-SCID patient CD34+ HSPCs. The lentivector-*IL2RG* gene is driven by the non-specific promoter EF1a-short, versus genome editing using CRISPR/Cas9/AAV6-IL2RG into the *IL2RG* gene to use the natural promoter to express from a codon divergent cDNA. Peripheral blood CD34+ HSPCs from X-SCID patients collected after G-CSF and plerixafor mobilization (IRB-approved) underwent enhanced transduction with our clinical LV with LentiBoost and dimethyl-prostaglandin E2 or GE with CRISPR/Cas9 targeted insertion (TI) of *IL2RG* cDNA. Treated cells were cultured *in vitro* to evaluate (i) T-cells using the Artificial Thymic Organoid (ATO) system, (ii) Natural Killer (NK) cell differentiation, (iii) TI rates by digital droplet PCR, or (iv) transplanted into immunodeficient mice. The ATO system demonstrated similar correction of T-cell differentiation blockade in X-SCID patient cells with both approaches. However, there was a striking contrast in the NK cell development following GE (mean 37.9%) or following LV-gene (2.1%) ($p<0.0001$) correction. The poor NK correction following LV is consistent with clinical outcomes following LV gene therapy in both infants and older children, suggesting that suboptimal IL2RG expression may be the limiting factor to NK cell development. Immunodeficient mice transplanted with CD34+ HSPCs from patients (treated with LV or gene editing) and healthy donors analyzed at 16 weeks showed comparable engraftment rates of human CD45+ cells in bone marrow. Tumorigenicity studies did not reveal any malignancies in either groups of mice, and the rates of off-target insertions or deletions were 100% in LV compared to <1% with the genome edited cells. In summary, we show data demonstrating similar T-cell correction and engraftment rates following lentivector or targeted genome editing approaches, but significantly improved NK restoration following restored physiological expression and regulation of IL2RG.

808. A Novel Base Editing Approach to Directly Edit the Causative Mutation in Sickle Cell Disease

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Gene therapy represents an exciting new frontier in the treatment of sickle cell disease (SCD). So far in a clinical setting, these approaches have focused on either expression of artificial anti-sickling globin or upregulation of fetal hemoglobin. However, direct editing of the sickle causing mutation (Glu6Val) has not been possible at high efficiency and without causing double strand DNA breaks. Adenine base editors (ABEs) have been shown to site-specifically mediate A-T to G-C basepair conversions with low rates of insertions and deletions and without causing double strand DNA breaks. Using SCD patient-derived fibroblasts, we identified ABE variants that efficiently recognized and edited the sickle mutation, converting the sickle-causing valine to an alanine. This conversion generates a naturally occurring form of β-globin, HbG-Makassar. This variant was previously identified in asymptomatic homozygous individuals that have normal hematologic parameters and no evidence of hemoglobin polymerization or sickling of red blood cells. Editing of SCD patient fibroblasts with our variant ABE editors was able to consistently achieve 40%-70% conversion of the target adenine. Several ABE variants were chosen to edit mobilized peripheral blood $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) isolated from donors heterozygous for the sickle cell trait. These HSPCs were electroporated with guide RNA and mRNA encoding these ABE variants, yielding editing rates ranging from 40%-60% at 48 hours post-electroporation with high cell recovery and viability. Additionally, HSPCs subjected to base editing did not display any phenotypic changes 48 hours post-electroporation. Subsequent *in vitro* erythroid differentiation (IVED) of edited $CD34^+$ cells confirmed that this level of editing (>70%) could be retained throughout erythropoiesis. In addition, we were able to confirm editing in non-mobilized whole blood $CD34^+$ cells isolated from homozygous SCD patients. Similar to the editing observed in sickle trait $CD34^+$ cells, we achieved >30% editing in SCD $CD34^+$ cells 48 hours post electroporation, with >65% editing following IVED (Fig 1A). As successful resolution of HbG-Makassar from sickle β-globin proteins has not been previously achieved, we sought to develop a high sensitivity ultra-high-performance liquid chromatography (UHPLC) assay to detect and distinguish the HbG-Makassar β-globin protein from sickle β-globin (HbS) in IVED cells. Using these newly developed methods, we assessed the conversion of sickle HbS to HbG-Makassar in our edited IVED cells derived from a homozygous SCD patient. We found that we could achieve conversion at levels near 70% (Fig 1B) with a concomitant reduction of HbS globin to 30% of control levels (Fig 1C). Generation of the HbG-Makassar variant was further confirmed by liquid chromatography mass spectrometry (LC-MS). Coupled with autologous stem cell transplantation, the direct editing of the causative sickle cell mutation to the naturally occurring and asymptomatic HbG-Makassar represents a promising new treatment paradigm for patients with SCD.

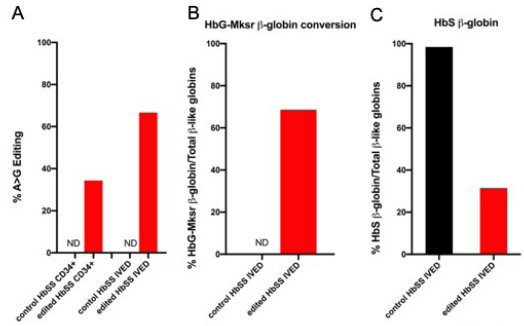

Figure 1

809. Human Dose Prediction of a Novel Factor IX Variant Gene Therapy Candidate (AMT-180) Mediating Clotting Independently of Factor VIII

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The Netherlands Recent data suggest that expression of FVIII constructs after AAV delivery in patients with severe hemophilia A may wane over time, perhaps due to liver stress or other factors. In addition, up to 30% of patients are currently ineligible for FVIII-based gene therapy due to a current or past inhibitors. Management of patients with inhibitors is particularly challenging and relies on the use of bypass agents, such as recombinant FVIIa, prothrombin complex or bispecific antibodies that bridge FIX(a) and FX(a), which have been reported to be associated with an increased risk of thrombosis. Hence, there is a need for a non-FVIII, non-thrombogenic and durable gene therapy in hemophilia A. The novel AAV5 gene therapy clinical candidate (AMT-180) encodes a transgene for a variant of FIX, FIX-FIAV, which possesses 4 amino acid substitutions compared to wild-type FIX under the control of a propriety liver specific promoter. Upon activation of FIX-FIAV, FIX-FIAVa is able to induce hemostasis through FVIII-independent activation of FX (Quade-Lyssy et al., 2014), and therefore is a strong candidate to treat inhibitor and non-inhibitor hemophilia A patient populations. Administration of AMT-180 to mice and non-human primates was well-tolerated and displayed no evidence of an increase in coagulation activation markers. Toxicological studies in non-human primates also showed no increase in coagulation activation markers. We have used the in vivo data from rodents and NHP to predict suitable clinical doses. This was achieved by first assessing FVIII-independent clotting activity in hemophilia A mice and correlating this to FIX-FIAV levels. These data were then used to predict theoretical FVIII-independent clotting activity in the NHP. Based on the current data set, our model predicts a dose-dependent increase in FVIII-independent activity that can readily achieve meaningful hemostasis in hemophilia A patients with and without inhibitors.

810. MGTA-145/Plerixafor-Mediated HSC Mobilization and Intravenous Gene Therapy in Mice Allows for Efficient In Vivo HSC Transduction and Stable Gene Marking in Peripheral Blood Cells

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In vivo HSC gene therapy via systemic injection represents a simpler approach to treating hemoglobinopathies without requiring myelosuppressive preconditioning or autologous HSC transplantation. We have developed a helper dependent adenovirus (HDAd5/35++)-based platform that enables efficient *in vivo* transduction of mobilized HSCs upon i.v. injection via CD46. Transduced HSCs can be positively selected by low-dose O^6BG/BCNU-treatment to achieve ~90% marking rates in peripheral blood cells. Initial proof-of-concept in mouse models as well as in rhesus macaques demonstrates high level of γ-globin expression after gene addition by a *Sleeping Beauty* transposase. While the current mobilization regimen—4 days of G-CSF injection followed by an injection of plerixafor on day 5—robustly mobilizes HSCs from the bone marrow to the periphery, several issues do exist. Despite widespread use as a mobilization agent, G-CSF is contra-indicated in patients with sickle cell disease. Additionally, G-CSF results in unselective bone marrow cell mobilization, which leads to leukocytosis and higher numbers of cytokine-producing cells in the periphery. This increases the number of cytokine-producing cells in the periphery that come in contact with intravenously injected HDAd particles, which in turn, contributes to higher cytokine levels in mobilized vs non-mobilized animals. Mobilized (committed) bone marrow cells in the periphery also sequester HDAd thus reducing the effective dose for primitive HSCs. Further, the five-day treatment regimen and high costs associated with G-CSF/plerixafor justify the development of an alternative mobilization regimen. Here we tested HSC mobilization by MGTA-145, a truncated GRO-beta, a CXCR2 agonist, and plerixafor in the context of in vivo HSC transduction. CD46-transgenic animals were mobilized with GCSF + plerixafor (5 days) or with MGTA-145 + plerixafor (given subcutaneously at the same time) and then injected one hour later with an integrating HDAd5/35++ mgmt/GFP vector. MGTA-145 + plerixafor triggered less leukocytosis. With both mobilization regimens, after in vivo selection with O^6BG/BCNU, >90% of PBMCs expressed GFP and marking rates were stable long-term. Mice were sacrificed 12 weeks after in vivo transduction and bone marrow lineage-negative cells were harvested for transplantation into secondary recipients. GFP marking in secondary recipients (>90% at week 16 after transplantation) was also not significantly different for the two mobilization regimens. Studies in a mouse model for thalassemia using MGTA-145/plerixafor mobilization are ongoing. These data suggest that MGTA-145 + plerixafor could serve as an efficient one-day mobilization regimen for *in vivo* gene therapy in patients with Sickle Cell Anemia, and potentially other diseases.

811. In Vivo Transduction of Mobilized HSCs with an HDAd5/35++ Vector Expressing Gamma-Globin from a 26kb Beta-Globin LCR Cures Thalassemia in a Mouse Model

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We developed a minimally invasive in vivo hematopoietic stem/progenitor cell (HSPC) gene therapy strategy. It involves the mobilization of HSPCs from the bone marrow, and while they circulate at high numbers in the periphery, an intravenously injection of an HSPC-tropic helper-dependent HDAd5/35++ adenovirus gene transfer vector system. Transduced HSPCs return to the bone marrow where they persist long-term. Random integration is mediated by an activity-enhanced Sleeping Beauty transposase (SB100x). We have recently shown that our approach resulted in an amelioration of murine thalassemia intermedia. However, the level of the therapeutic human gamma-globin gene expression would not be sufficient for a cure of severe hemoglobinopathies, partly, due to a suboptimal 5.5kb mini-beta-globin locus-control region driving gamma-globin expression. We therefore constructed a HDAd5/35++ vector that contained a 29kb gamma-globin expression cassette including 26kb of the beta-globin locus-control region (LCR). Our ex vivo and in vivo HSPC transduction studies with CD46-transgenic mice as well as our in vitro studies with human HSPCs demonstrated that, in addition to conferring higher gamma-globin expression levels (~20% of adult mouse alpha-globin), the long LCR also provided more stringent erythroid-specific expression. We then performed a therapy study in thalassemic CD46/Hbb-th3 mice. GCSF/AMD3100-mobilized mice were injected with HDAd-SB100x plus an HDAd5/35++ vector carrying either a 5.5kb mini-LCR or the 26kb LCR. With both vector systems nearly 100% of peripheral red blood cells expressed gamma-globin as measured by flow cytometry. However, the gamma globin levels (measured by HPLC) were ~4-fold higher for the 26kb LCR vector. This resulted in a correction of the disease phenotype including normalized red blood cell values, normal reticulocyte counts and absence of extramedullary hematopoiesis and iron deposition. Overall, our study indicates that the use of endogenous long LCRs increases transgene expression levels, cell lineage specificity, and, theoretically, reduces trans-activating effects on genes surrounding the vector's integration site.

812. In Vivo Characterization of an AAV Vector Expressing Complement Component C2

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The complement system is a network of proteins and receptors that are part of the innate immune response to pathogens. Inherited deficiencies of nearly all complement components and regulators

have been identified and found to be associated with severe diseases including primary immune deficiency, autoimmunity, chronic renal disease as well as severe recurrent infections with gram-negative and gram-positive microbes. Most complement deficiencies are treated symptomatically with immunosuppressors as in the case of overactive immune response or with precautionary prophylactic antibiotics for underactive immune response. However, there is very little literature on protein replacement of complement proteins in the setting of an inherited or acquired complement deficiency. Recently, liver directed gene therapy using recombinant adeno-associated virus (rAAV) has shown efficacy in the clinic for expression of plasma proteins. As part of our preclinical studies, we explored the feasibility of using an AAV gene therapy to deliver complement component C2. Vectors were constructed expressing human (hC2) from a ubiquitous promoter. Pilot studies were first undertaken to establish assays to detect human C2 (hC2) and evaluate expression. Following transient transfection, robust hC2 expression was detected from both Huh7 hepatocytes as well as HEK293 cell supernatants by ELISA and western blot. The secreted hC2 protein from both cell lines was functional as assessed by a complement hemolysis assay (CH50). The protein levels determined by ELISA (3.5-10.3 ug/ml) correlated well with the CH50 activity, suggesting efficient protein folding and secretion of functional protein. The hC2 construct was vectorized and efficient transduction and protein secretion from HEK293 cells was confirmed with the AAV. hC2 vector. Subsequently, male C57Bl/6 mice (n=3/group) were dosed with AAV.hC2 intravenously (tail vein) at 1E10, 3E10 or 1E11 genome copy (GC)/mouse. Following systemic administration, circulating C2 levels of ~40ug/ml were achieved at 1E10 GC dose, staying consistent through the 30 day observation period. Vector expressed human C2 levels were within the normal reference human range (20-40ug/ml) even at this low dose level. At higher doses, increased expression of hC2 was detectable at day 3, but subsequently declined at day 10. The levels rebounded in two weeks and were stable to day 30. This early transient decline may indicate complement consumption due to classical pathway activation in the first week following vector transduction. Transduced mice did not display overt signs of toxicity or other adverse effects either during the initial phase or for up to 3 months following vector administration. Complement activation was not observed in animals dosed with an irrelevant transgene. We discuss possible reasons for complement activation in animals dosed with AAV.hC2 vectors and its potential relevance to AAV clinical trials. This exploratory work supports the rationale for a novel AAV-expressed complement therapy to correct a component deficiency. We unexpectedly observed increased complement activation when mice were administered AAV expressed complement C2 component. Our data underscores the importance of additional preclinical studies to evaluate the safety of expressing complement components delivered from an AAV vector.

813. The Impact of Curative Therapy on Socioeconomic Disparities Among Children with Sickle Cell Disease

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Background Sickle cell disease (SCD) predominantly affects Non-Hispanic black and Hispanic Americans, creates significant disease burden and materially affects the financial future of the affected children and their families. While one in 500 Non-Hispanic black and one in 1,400 Hispanic Americans are estimated to be currently living with SCD in the United States (U.S. National Library of Medicine, 2019), the overall societal impact of the disease is not well understood. Research is warranted to quantify the impact of SCD on socioeconomic attainment and on the potential benefits of emerging transformative, potentially curative, treatment options for these patients. **Objective** Using real world data, we will perform a microsimulation of the socioeconomic trajectories of children with SCD compared to a matched control of children without SCD. Based on these inputs, we will estimate the potential reduction in socioeconomic disparities generated by a future potentially-curative therapy. Here, the objective is to present a novel modeling framework that accounts for broader societal impact that is relevant, yet not typically considered, in the context of health economic evaluation. **Methods** This study will identify a cohort of children for whom a parent self-reported the diagnosis of SCD, using data from the 2006 to 2015 waves of the National Health Interview Survey (NHIS). In an initial study step, we will describe the demographic characteristics of this cohort compared to a matched cohort for which the parents self-reported that their children do not have SCD. Propensity score matching will be implemented through a combination of demographic and socio-economic characteristics that have been previously identified as associated with SCD status in the NHIS. (Boulet et al., 2010) Next, we will apply predictors of cohort assignment in the NHIS to identify a comparable cohort of children with a high likelihood of SCD in the 2006-2015 Panel Survey of Income Dynamics (PSID), a longitudinal survey of health, education, and income. To estimate the effect of a potentially-curative or transformative treatment on socioeconomic disparities, we will compare lifetime earnings trajectories for patients with SCD and a matched cohort of individuals without SCD. Given that educational attainment, a major driver of future earnings, has been shown to be lower for patients with SCD compared to individuals without SCD (Schatz, 2004; Harris et al., 2019), our analyses will further evaluate various scenarios around the age when a patient receives a potentially-curative therapy, and their ability to close the earnings gap, relative to a non-SCD trajectory. Additionally, we will provide perspective on the broader economic impact to caregivers or families. **Policy Implications** As has been well documented previously, families with a child with sickle cell disease face significant health challenges with lower economic resources and education compared to other families in the United States. This research will continue to evaluate how these disparities may be alleviated if a potentially-curative therapy for sickle cell disease were to become available. We believe that our model, based on empirical data from the PSID, will provide a first estimate of the potential economic value of curing a disease such as SCD that disproportionally affects populations of lower socioeconomic status. By varying parameters such as age when patients receive a potentially curative therapy, which impacts lifetime earnings and education pathways, this study will improve our understanding of the dynamics underlying the relationship between SCD and economic wellbeing.

814. Correction of β-Thalassemia Through the Generation of Genomic Rearrangements in the β-Globin Locus

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β-hemoglobinopathies are caused by mutations affecting the production of the adult hemoglobin (Hb) β-chain. In β-thalassemia, the reduced production of β-chains causes α-globin precipitation and insufficient red blood cell (RBC) hemoglobinization. In sickle cell disease (SCD), the $\beta6^{Glu\rightarrow Val}$ substitution leads to Hb polymerization and RBC sickling. The clinical course of β-hemoglobinopathies is ameliorated in the presence of large deletions in the β-globin locus, resulting in elevated fetal Hb (HbF) levels, a condition termed hereditary persistence of fetal Hb (HPFH). The molecular mechanisms underlying HbF reactivation in deletional HPFH are poorly understood. HPFH deletions could remove γ-globin inhibitory regions. Moreover, these deletions encompass the β-globin promoter, thus its absence could induce a preferential binding of the β-globin locus control region to the γ-globin promoters that results in HbF reactivation. We have recently shown that CRISPR/Cas9 disruption of a 13.6-kb HPFH-like region induces a significant γ-globin reactivation in an adult erythroid cell line (HUDEP2) and in RBCs derived from SCD hematopoietic stem/progenitor cells (HSPCs). The 13.6-kb region includes the β-globin promoter and the 7.4-kb Corfu region, whose deletion is associated with elevated HbF in β-thalassemia patients. The Corfu region encompasses a putative 3.4-kb HbF silencer. Here, we used a therapeutic approach based on the disruption of the 13.6-kb region to correct the β-thalassemia phenotype. In addition, we designed a CRISPR/Cas9 strategy to identify factors contributing to γ-globin reactivation upon disruption of the 13.6-kb region, and regulatory elements involved in HbF silencing. In particular, we dissected the 13.6-kb region by disrupting: (i) the 1-kb β-globin promoter; (ii) the 5.2-kb region located between the β-globin promoter and the Corfu region; (iii) the 7.4-kb Corfu region; (iv) the 3.4-kb putative HbF silencer. We tested this strategy in a HUDEP2 β-thalassemic cell line that showed increased γ-globin levels compared to wild-type cells. However, this increase was not sufficient to compensate for the lack of β-globin expression, leading to globin chain imbalance and to the generation of α-globin precipitates. Importantly, this cell line was useful to (i) exclude the contribution of β-globin down-regulation to HbF expression when targeting the β-globin promoter; (ii) evaluate if β-globin down-regulation is necessary to obtain γ-globin reactivation when targeting the Corfu region. Efficient generation of genomic rearrangements was obtained after transfection of Cas9- and gRNA-expressing plasmids. Disruption of the 13.6-kb region led to increased γ-globin levels, amelioration of the α/non α ratio and reduction of α-globin precipitates, as evaluated by RT-qPCR, flow cytometry and HPLC. Targeting the β-globin promoter or the 5.2-kb region failed to induce γ-globin expression, demonstrating that disruption of these regions does not contribute to the increase in γ-globin production observed upon targeting of the 13.6-kb region. Interestingly, disruption of the full 7.4-kb Corfu region but not the putative 3.4-kb silencer led to γ-globin reactivation. In contrast, targeting the Corfu region failed to reactivate γ-globin expression in wild-type HUDEP2. These experiments show that disruption of the Corfu region is able to induce γ-globin expression only when associated with a reduction in β-globin expression. To validate these results, we performed experiments in clinically-relevant primary HSPCs from β-thalassemia patients. Overall, this study showed that complex regulatory mechanisms are involved in γ-globin de-repression in deletional HPFH and identified the Corfu region as a new potential target for genome editing approaches to treat β-thalassemia.

815. CRISPR-Cas Genome Editing of Gamma-Globin Gene Promoters in Human Hematopoietic Stem and Progenitor Cells Towards Sickle Cell Disease Therapies

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Sickle cell disease (SCD) is an inherited blood disorder that affects approximately 100,000 Americans and millions of individuals worldwide. Patients with a single nucleotide change in the adult beta globin gene produce an abnormal sickle hemoglobin (HbS) and are affected by severe pain crises, chronic anemia, multi-organ dysfunction, and early mortality. Re-activation of fetal hemoglobin (HbF) to replace defective sickle hemoglobin in adults by editing *BCL11A*-binding site in the gamma-globin gene (*HBG1/HBG2*) promoters in human hematopoietic stem and progenitor cells (HSPCs) (Traxler et al. Nat. Med 2016) is a promising therapeutic strategy to treat SCD. Notably, individuals that co-inherit homozygous sickle mutation and natural genetic variants that cause persistence of fetal hemoglobin expression into adulthood are typically asymptomatic. We optimized editing of human primary CD34+ hematopoietic stem and progenitor cells (HSPCs) and compared activity of Cas9-2xNLS, a wild-type S.pyogenes Cas9 containing a two C-terminal SV40 nuclear localization with an engineered Cas9-3xNLS variant with 3 distinct nuclear localization signals in N- and C-terminal positions (Wu et al. Nat. Medicine 2019). We delivered Cas9-NLS variants as ribonucleoprotein (RNP) complexes by electroporation to primary CD34+ HSPCs and transplanted them into immunodeficient NBSGW mice. After 17 weeks, we observed higher editing activity of Cas9-3xNLS in repopulating hematopoietic stem cells (HSCs) than Cas9-2xNLS, which resulted in up to 29.6% fetal hemoglobin (HbF) compared to Cas9-2xNLS (<10%) in erythroid progeny. To model the potential efficacy of our approach we edited plerixafor-mobilized CD34+ HSPCs from one normal donor and three adult individuals with SCD using Cas9-3xNLS RNPs and transplanted them into NBSGW mice. We achieved high and consistent editing rates ranging from 80.6% to 94.5% in both CD34+/CD90- progenitor and CD34+/CD90+ stem-cell enriched populations. Our xenotransplant studies showed that indels were maintained at an average of 74.5% in repopulating HSCs, resulting in HbF induction of up to 34.3%

in human erythroid progeny compared to <5% in erythroid cells from unedited controls. Single-cell western blot analysis showed 49-58% of edited erythroblasts expressed gamma-globin compared to <6% in controls. Experiments are in progress to assess unintended genome-wide off-target activity mediated by Cas9-3xNLS. Overall, our pre-clinical experiments suggest that Cas9- mediated disruption of gamma-globin gene to reactivate therapeutic levels of HbF represents a promising approach for genome-edited cell therapies to treat SCD.

816. Adeno-Associated Virus Mediated Delivery of TFPI Neutralizing Antibody as a Novel Gene-Therapy Approach for Hemophilia

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Hemophilia is an X-linked recessive bleeding disorder due to a deficiency in clotting factors, factor VIII (FVIII) in hemophilia A and Factor IX (FIX) in hemophilia B. Both factor VIII and factor IX are components of the intrinsic pathway of coagulation, and their deficiency leads to insufficient thrombin generation for conversion of fibrinogen to fibrin and development of a stable clot. Though replacement factor therapy has been a standard of care for prophylaxis or for on-demand treatment of bleeds in patients with hemophilia, some patients develop inhibitory antibodies to factors making them refractory to protein replacement therapy. Tissue factor pathway inhibitor (TFPI) is a Kunitz domain type inhibitor that negatively regulates thrombin generation in the extrinsic pathway. We have previously shown that augmenting the extrinsic pathway by a neutralizing monoclonal antibody (mAb) to TFPI can facilitate hemostasis initiated by tissue factor/Factor VIIa compensating for loss of factor VIII (FVIII) or factor IX in hemophilia A or B mouse models (Jasuja et al, 2016). Adeno associated virus (AAV) mediated delivery of genes encoding an anti-TFPI mAb (TFPI-mAB) has the potential to restore hemostasis in multiple mouse models of hemophilia . To test this hypothesis, anAAV8.TFPI-mAB vector was designed to express the heavy and light chains of anti-TFPI mAB 118 from a ubiquitous (CAG) promoter. mAB-118 binds to K2 domain of human TFPI, neutralizes TFPI activity in vitro and in vivo in hemostatic assays, and cross reacts to mouse TFPI. Vectors were made from adherent cells, concentrated and purified by gradient centrifugation. In vitro evaluation using a functional dilute Prothrombin Time (dPT) assay with plasmid transfected and vector transduced cells demonstrated expression and secretion of functional TFPI-mAB 118 antibody with appropriate processing of heavy and light chain. Subsequently, male hemophilia A mice (n=3/group) were dosed intravenously with $1X10^{10}$ or $1X10^{11}$ vg/mouse and blood was collected to evaluate expression and activity. TFPI-mAB 118 was detectable in circulation within 2 weeks in all animals following both low and high dose AAV8.TFPI-mAB administrations. Analysis for clot formation time (R time) using whole blood thromboelastography (TEG) at 2, 4 or 8 weeks post dosing showed an 8 to 9 fold reduction in clot formation time at 2 and 4 weeks. No reduction in clot time was observed in control animals treated with an irrelevant vector (AAV.GFP). In hemophilia A mice administered AAV8.TFPI-mAB, the clot formation time, even in the low dose treated group did not differ significantly from untreated wild-type mice (ANOVA $p < 0.005$). More importantly, clotting activity measured by TEG and TFPI-mAB 118 levels in the circulation were persistent up to 8 weeks post- vector administration. Long term follow up studies are needed to determine durability of antibody expression and development of ADA.

817. Gene Transfer Strategy for the Correction of CD34+ Cells from Artemis-SCID Patients: Towards a Prospective Open Phase I/II Clinical Trial in Europe

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Genetic deficiency of the endonuclease DCLRE1C/Artemis -a key factor for the nonhomologous end-joining (NHEJ) mechanism- causes radiosensitive severe combined immunodeficiency (RS-SCID) characterized by a complete lack of T and B cells associated with hypersensitivity to ionizing radiations (RS-SCID). The only curative treatment is allogeneic hematopoietic stem and progenitor cells (HSPCs) transplantation that is associated with a poor outcome in the absence of a compatible donor. In this context, gene correction of autologous HSPCs could represent an alternative therapeutic strategy. Proof-of-concept studies were obtained with a lentiviral vector (LV) which contains a short intron-less EF1α promoter regulating expression of human DCLRE1C cDNA. A pre-GMP grade LV batch was used for preclinical and biosafety analyses in mice, confirming the ability of Artemis corrected cells to restore a functional T and B lymphoid compartment and to confer long-term engraftment without any toxic effects (Charrier et al. MTMCD, 2019). Lack of toxicity of the vector on human CD34+ cells growth and myeloid differentiation was also demonstrated. Altogether, these results demonstrate that Artemis gene therapy could be an efficient approach for this RS-SCID. A GMP clinical-batch of vector was manufactured for a phase I/II trial. This batch was used to optimize the gene transfer protocol on human bone-marrow HSPCs isolated from healthy donor and from Artemis-deficient patients. In the best condition, the vector copy number was stable at different time points and compatible with a clinical use (VCN=1 to 1.5). The T cell differentiation was restored in patient's cells after in vitro culture on OP9-DL1 culture. Altogether, the results support the submission of a phase I/II multicentric clinical trial called ARTEGENE that is expected to start in mid-year 2020.

818. DREPAGLOBE Clinical Trial for Sickle Cell Disease Patients: Preclinical and Clinical Results

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In sickle cell disease (SCD), the $\beta 6^{Glu \rightarrow Val}$ substitution leads to sickle hemoglobin (HbS) polymerization and red blood cell (RBC) sickling. Transplantation of autologous, genetically modified hematopoietic stem cells (HSCs) represents a promising therapeutic option for patient lacking a compatible donor. We previously designed a new lentiviral vector, (βAS3 LV) expressing a potent anti-sickling βAS3 globin and demonstrated its safety and efficacy in SCD patient cells (Weber et al., 2018). We tested the efficacy of βAS3 LV in transducing SCD patient's cells under *Good Manufacturing Practice* (GMP) conditions. We used plerixafor-mobilized hematopoietic/stem progenitor cells (HSPCs) from SCD patients included in our previous clinical trial demonstrating the efficacy and safety of HSC mobilization by plerixafor in SCD patients (Lagresle et al., Haematologica, 2018). Three pre-clinical runs in GMP conditions were performed. Plerixafor-mobilized SCD HSPCs were pre-activated for 48 hours; then βAS3 LV was added together with transduction enhancers. Sterility and endotoxin tests were negative. After transduction, cells were grown either in liquid culture or in a semi-solid medium allowing the growth of erythroid (BFU-E) and granulomonocytic (CFU-GM) progenitors. The vector copy number (VCN) was between 0.88 and 1.19 in liquid culture, 1.12 to 1.91 in BFU-E, and 0.66 to 1.60 in CFU-GM. The expression of the Hb containing the βAS3 globin chain in BFU-E represented ~30% of the total Hb as determined by HPLC. We assessed the efficiency of this transduction protocol *in vivo* in *bona fide* SCD HSCs. Transduced HSPCs engrafted in busulfan-conditioned NSG Immunodeficient mice gave rise to the different blood lineages, as evaluated by flow cytometry in the bone marrow, spleen, thymus and blood 18-20 weeks post-transplantation. VCN in repopulating human HSCs ranged between 1.77 and 3.80. Overall, these preclinical studies demonstrate the safety and efficacy of a gene therapy protocol based on a novel and efficient LV. Based on these results a Phase 1/2 clinical trial (NCT03964792) has started in January 2020. Three SCD patients have been recruited. Deformability, density, viscosity, hemolysis markers and Hb expression were evaluated before the treatment in total RBCs. We also sorted patient' RBC populations by magnetic separation based on blood group antigens to evaluate adherence, O_2 association and dissociation curves and senescence markers. The first patient was mobilized with plerixafor and HSPCs were transduced using our optimized protocol achieving a VCN of 0.75 in liquid culture, 1.80 in BFU-E and 0.60 in CFU-GM. The patient was hypertransfused to reach HbS levels <30%. After busulphan-based myeloablative conditioning, he received a drug product $8.1x\ 10^6$ per kg. The results obtained in the two SCD first patients will be presented.

819. Abstract Withdrawn

820. Synovial Gene Therapy for Hemarthrosis in Patients with Hemophilia A

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Spontaneous hemarthrosis (bleeding in a joint cavity) is one of the major complications of bleeding disorders such as hemophilia A (HA), even in patients receiving adequate prophylactic FVIII replacement therapy. Bleeding can occur in the synovia of any of the joints and often manifests from early childhood. The pathogenesis of hemophilic arthropathy is multifactorial, with recurrent joint bleeding causing synovial proliferation and inflammation (hemophilic synovitis) that contribute to end-stage degeneration and arthropathy. If articular bleeding is not treated effectively, it will recur, resulting in a cycle that must be broken to prevent the development of chronic synovitis and degenerative arthritis. Here we tested the efficacy of using a lentiviral vector encoding an optimized FVIII transgene to transduce synovial cells, and the ability of these cells to secrete FVIII protein. We isolated, cultured, and expanded cells from sheep (n=2) synovial tissue. Expanded cells were characterized by flow cytometry to verify that these cells had a phenotype consistent with that of type B synoviocytes (SCB). Next, we investigated whether SCB expressed LDLR, a major entry port of VSV-G-pseudotyped lentiviral vectors. Then we used a VSV-G-pseudotyped lentiviral vector encoding the bioengineered "mco-ET3" FVIII transgene, and subsequently evaluated transduced synovial cells forFVIII production/secretion using aPTT and transduction efficiency by vector copy number using qPCR.The phenotype of the synovial cells we isolated and expanded was consistent with that of type B synovial cells (SCB), exhibiting expression of CD90, CD44, and vimentin and being devoid of CD45. SCB were efficiently transduced, as determinedby a vector copy number of 5.6, and efficiently secreted functional FVIII, as confirmed by aPTT (9.7IU/24h/10^6 cells). Evaluation of levels of mco-ET3 mRNA by qPCR confirmed robust expression of this transgene in the transduced cells. Immunofluorescence analysis using antibodies specific for FVIII, was also used to confirm that the cells were transduced effectively and were producing FVIII protein. In conclusion, type B synoviocytes possess the ability to be efficiently transduced in vitro and to produce and secrete therapeutic amounts of FVIII, and they can therefore

serve as a potential target for *in vivo* gene therapy. *In vivo* studies are underway to ascertain the ability to transduce type B synoviocytes *in vivo* using sheep as a large animal model. Given the proximity of the synovial cells to the synovial capillaries, we postulate that synovial cells are ideally suited for delivering FVIII to the adjacent tissue, and thereby mitigate not only major bleeds but also microbleeds that contribute to the HA arthropathy.

821. Gene Therapy for Rejuvenation of Thymic Function

Reason Reason

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Involution of the thymus is a process of atrophy that progressed throughout adult life, and contributes to age-related immunosenescence via a decline in the supply of mature T cells. Regeneration of thymic epithelial tissue, as well as thymic transplantation, has been demonstrated in rodent and non-human primate models to restore production of naive T cells, CD4+ and CD8+ T cells, and recent thymic emigrants. Regeneration has been achieved in rodent models via castration, sex-steroid ablation, systemic delivery of growth hormone or recombinant KGF, as well as tissue-specific overexpression of FOXN1 and related regulatory genes. Supporting data exists for human trial participants to exhibit the same increase in T cell production following sex-steroid ablation. Systemic delivery of KGF has failed in a human trial that assessed thymic function, but likely because the dose that can be safely applied in patients is too low. That FOXN1 overexpression is well demonstrated to result in thymic regrowth and restored function in aged mice makes it a promising target for gene therapy, with several successful attempts reported in the literature. We here report on the results to date emerging from our development program aimed at the production of a viable modern gene therapy that will improve thymic function and thereby immune function in old individuals. In vitro and in vivo we employed RNA-seq to produce signatures of downstream gene expression changes resulting from upregulation of FOXN1 expression, to complement the existing literature on downstream changes resulting from FOXN1 knockdown. In vivo, in mice, we characterised the behavior of mouse FOXN1 expression in response to AAV-mediated expression of a human FOXN1 transgene, showing variation with age. Further, in aged mice, we delivered human FOXN1 via intrathymic injection of AAV, resulting in upregulation of mouse FOXN1, and favorable increases in size in immune cell populations of interest in the thymus and spleen, as assessed via flow cytometry. Collectively this data forms a promising foundation for the development of a gene therapy treatment for immunosenescence and immunodeficiency.

822. Abstract Withdrawn

823. Highly Precise Single-Stranded Template Repair of a Pyruvate Kinase Deficiency-Causing Mutation in Patient-Derived Lymphoblastic Cell Line

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Pyruvate Kinase Deficiency (PKD) is an autosomal recessive disease, caused by mutations in the *PKLR* gene, which encodes the Erythroid Piruvate Kinase protein (RPK). The defective enzyme fails to produce normal ATP levels and consequently, erythrocytes from PKD patients show an energetic imbalance and die. More than 200 disease-causing mutations have been described up to know and it is well known that RPK expression is highly regulated along erythropoiesis. These characteristics make gene editing a promising therapy for the treatment of PKD. In this work, we have explored the potential of precise gene editing for the correction of mutations causing PKD, using single stranded oligodeoxinucleotides (ssODN) and CRISPR/Cas9 system. First, we generated a PKD patient-derived lymphoblastic cell line (PKD-LCL) from mononuclear blood cells. The patient carried two heterozygous mutations reported in exon 3 (c.359C>T) and exon 8 (c.1168G>A) of *PKLR* gene. Mutation-specific guide RNAs (gRNAs) were designed to target these two PKD mutations. These gRNAs were complexed with Cas9 protein in a ribonucleoprotein format and electroporated into PKD-LCLs. Targeting efficacy was assesed by deep sequencing of the target sequence using *Mosaic Finder*, an in-house developed bioinformatic tool that detects allelic mosaicism on Next Generation Sequencing datasets. In exon 3, the gRNA that presented its PAM sequence closer to the mutation that we aimed to correct, mediated higher cleavage (5.89% in comparison with 0.62% indels). More importantly, targeting was much more specific for the mutated allele than for the WT allele (around 30 times), emphasizing the critical role of nuclease design in allele specific gene editing. Then, we designed a corrective ssODN to restore the wild type amino acid sequence coded by this exon. We introduced a new restriction site in the ssODN sequence without compromising the protein sequence as well, to trace gene editing by *Restriction Fragment Length Polimorphism* (RFLP). When RNP and ssODN were electroporated into PKD-LCLs, we achieved up to 2.5% precise correction maintaining a very high allele-specificity. Our results demonstrate the feasibility of a highly personalized gene editing therapy to treat PKD-causing mutations. New designs in our mutation-specific gene editing tools are being explored to increase efficacy and evaluate the clinical relevance of this approach to correct PKD.

Cell Therapies

824. Therapeutic Effects of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells Encapsulated within Modified-Hyaluronic Acid Hydrogel in Ischemic Tissues

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Background: Cardiovascular Disease is the number one cause of death globally. Drug treatments or surgical interventions do not address the root problems of impaired microcirculation of heart and peripheral limb which is mainly caused by endothelial dysfunction. Biological intervention using human induced pluripotent stem cell (hiPSC) has emerged as a therapeutic approach for increasing neovascularization in ischemic tissues. However, poor survival of engrafted cells and low efficacy with paracrine effects have been major limitations in its application for clinical reality. We aimed to enhance cell survival, vessel formation, and therapeutic potential by encapsulating hiPSC-endothelial cells (hiPSC-EC) within modified-hyaluronic acid (HA) hydrogel in ischemic tissues. **Methods**: EC-lineage cells were differentiated from hiPSCs, followed by sorting for CDH5 (VE-Cadherin) as described in our previous report. We characterized hiPSC-EC with EC-lineage specific markers by flow cytometry, qRT-PCR, and immunostaining, and in vitro EC functional assays. To enhance the survival of engrafted cells in the ischemic area, hiPSC-ECs were encapsulated in modified-HA hydrogel before injection. We evaluated whether the encapsulation of hiPSC-EC within modified-HA hydrogel could enhance long-term cell survival and vascular regenerative effects in cardiovascular disease model of nude mouse by imaging and histological analysis. The recovery of blood flow after hind limb ischemia (HLI) was determined by using Laser Doppler perfusion imager. **Results:** EC-specific markers, such as CDH5, PECAM1, VWF, and KDR were expressed in the highly enriched hiPSC-EC. Encapsulation of hiPSC-EC within modified-HA hydrogel enhanced the engrafted cell survival and retention in ischemic area of both HLI and myocardial infarction (MI) model. Modified-HA hydrogel encapsulated hiPSC-EC improved the recovery of functional microcirculation with better protection from limb loss at 4 weeks after HLI, compared to PBS- or hydrogel-control group. The robust and longer survival of hiPSC-EC encapsulated within modified-HA hydrogel showed contribution to the dynamic neovascularization process in both HLI and MI model. The engrafted cells were re-localized abut to the host vessels, and progressively incorporated into host vessel over 4 -8 weeks after ischemic injury. **Conclusions**: Here we demonstrated that encapsulation within modified-HA hydrogel improved long-term survival of hiPSC-ECs in ischemic tissues. This modified-HA hydrogel mediated transplantation of hiPSC-EC enhanced therapeutic effects via neovascularization at ischemic and adjacent area in both HLI and MI model, suggesting a clinical potential of hiPSC-EC for the treatment of ischemic cardiovascular disease.

825. Optimization of Shielded Encapsulated Cell Therapy for Hemophilia and Beyond

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Cell therapy is an attractive option for treatment of various chronic diseases due to the ability to modify the cell genome to express therapeutic molecules. With allogeneic cell therapy, the main challenge is to avoid the attack by the recipient's immune system. Many biomaterials (e.g. alginate hydrogels) can be used to physically shield the cells from the recipient's immune system. These barriers are effective at protecting the cells, but they themselves can elicit a foreign body response (FBR), resulting in pericapsular fibrotic overgrowth (PFO). The PFO walls off the encapsulated cells from nutrients, leading to cell death. This process typically occurs within weeks, posing a significant challenge to durability, and thus, utility, of this type of therapy. Recently, a group of molecules were identified that significantly diminish the FBR in rodents and non-human primates when conjugated to alginate biomaterials, allowing the encapsulated cells to remain functional long-term (Bochenek Nat Biomed Eng 2018). The resulting spheres containing islet cells have been extensively tested in diabetes. Sigilon Therapeutics has licensed this technology from MIT and developed a category-defining platform to engineer biocompatible, encapsulated cell therapeutics with the goal of applying it to a wide range of chronic conditions. In order to broaden the scope of this technology to other therapeutic areas we optimized and tested various iterations of the platform *in vivo*, via IP administration to mice. First, in order to maximize the expression of therapeutic proteins, we tested 10 human cell lines. The selection criteria included previous clinical exposure, high therapeutic protein expression, contact inhibition and high viability within alginate spheres, resulting in selection of an epithelial human cell line. To improve expression of the engineered construct, we applied codon optimization to stabilize the mRNA and increase protein secretion, driven by a strong promoter chosen specifically for the selected cell line. Next, we developed an alginate sphere consisting of an inner compartment and an outer layer. The goal was to create a material where the cells are completely encapsulated within the center of the sphere. This also allowed for separate optimization of the cellular environment (inner compartment) and the minimization of PFO (outer layer). The inner compartment was modified with varying concentrations of extracellular matrix components conjugated to alginate; we selected the components that yielded the highest cell function and cell adhesion. Building on the MIT technology, the outer layer was also optimized to minimize PFO by varying the level of conjugated small molecule. The most advanced product candidate based on our alginate sphere platform, SIG-001, contains genetically modified allogeneic cells that stably produce human factor VIII (hFVIII) and is intended for application in hemophilia A. SIG-001 produces functionally active hFVIII in a dose-dependent manner, corrects the bleeding phenotype in hemophilia A mice and cells remain viable in vivo through at least 6 months (Carmona ASH 2019). SIG-001 was recently granted orphan designation by the US FDA. To prepare for a first-in-human clinical trial of SIG-001 in hemophilia A, planned for

2020, we developed a manufacturing method to create these spheres on a clinical scale. We are developing a new class of medicines for other rare blood disorders, lysosomal storage disorders, type 1 diabetes and other serious chronic illnesses to provide a long-term solution for patients, and to free them of the burden of their condition.

826. When Measles Virus Vector MET OCT4: A Vector to Study iPSC Reprogramming

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Rapid progress in regenerative medicine has facilitated the development of more sophisticated gene delivery/expression technologies. We have developed Measles virus (MeV) as recombinant viral vector and have previously generated several MeV vectors expressing the four reprogramming factors, OCT-SOX2-KLF4-cMYC, in one single vector (MV4F) to produce induced pluripotent stem cells (iPSC) from somatic cell. Of the four reprogramming factors, OCT4 is a key mediator of reprogramming of somatic cell into induced pluripotent stem cell (iPSC). While its role in mouse reprogramming is clearly elucidated, not much is known about its role in human fibroblasts. Here, we present microRNA (miR) controlled MeV vectors, optimized to study the role of OCT4 during reprogramming and more specifically in the context of mesenchymal-epithelial transition (MET). MET manifests between day 6-12 of the reprogramming and is a critical step in the formation of iPSC. The rationale of our study is to use microRNA naturally upregulated just before (miR371, miR372, miR302a) or during (miR375) MET to eliminate the vector/transgene. The vectors were designed to have three target sequences of miR in the 3' untranslated region of the P gene. After verification that the presence of the miR target sequences did not affect vector propagation or phosphoprotein cofactor activity, the vectors reprogramming efficiency was assessed. We first compared the reprogramming efficiency of MV4F with MV4F^{miR} vectors. While MV4F efficiently reprogrammed human fibroblasts to fully mature iPSCs, MV4F^{miR371}, MV4F^{miR372}, and MV4F^{miR302a} couldn't, indicating that the elimination of all four RFs before MET is detrimental. In contrast, MV4F^{miR375} gave rise to multiples small iPSC-like clones by day 9-12, but these clones never developed into mature iPSC. Supplementation of MV4F^{miR375} reprogramming with three lentiviral vectors (LV), each expressing one of the three factors - SOX2, KLF4, cMYC reprogrammed as efficiently as MV4F, indicating that a continuous expression of exogenous SOX2, KLF4 and cMYC is essential for pushing the iPSC-like clones to maturation phase while exogenous OCT4 might be only required for the initiation of reprogramming. To further test this hypothesis, we produced a MV vector expressing only OCT4, under the control of miR375 target sequence or not, MV(OCT4) and MV(OCT4)^{miR375} and tested their reprogramming efficiency in combination with three LV encoding SOX2, KLF4 and cMYC. Reprogramming using MV(OCT4)^{miR375}+3LV occurs faster and more efficiently than with MV(OCT4)+3LV, indicating that the reduction/elimination of OCT4 after initial induction could enhance reprogramming efficiency. Finally, we next compared the reprogramming efficiency of vector expressing the 4 reprogramming factors, but in which OCT4 was isolated and controlled, or not, by miR375 target sequence, MV(O)(SK)(M) and MV(O)^{miR375}(SK)(M). As previously, the reprogramming efficiency of MV(O)^{miR375}(SK)(M) was superior to that of MV(O)(SK)(M) confirming that elimination of exogenous OCT4 during MET improve the reprogramming efficiency of human fibroblasts. In conclusion, using MeV vector as a tool to study the role of the reprogramming factors during reprogramming, we showed that the expression of the exogenous OCT4 is only required for the initiation of reprogramming and become dispensable for the maturation stage. However, a sustained expression of exogenous SOX2, KLF4 and cMYC during MET is required for the iPSC-like clones to enter the maturation phase and become fully mature iPSC clones.

827. Mitochondrial Augmentation Therapy-Enriched HSPCs for Mitochondrial Deletion Syndromes

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Mitochondrial diseases caused by mtDNA deletions or mutations are debilitating and life-threatening, yet no effective pharmacologic treatments are available and treatment is symptomatic. In mitochondrial augmentation therapy (MAT), being developed to treat these mitochondrial syndromes, patient-derived hematopoietic stem and progenitor cells (HSPCs) are enriched with healthy mitochondria. This augmentation not only allows cells to import organelles harboring full length mtDNA genomes, but to alter their metabolic capacity and mitochondrial function. After augmentation, HSPCs are reinfused into the patient bloodstream, where they may home to hematopoietic and non-hematopoietic organs to exert systemic effects. The conceptual basis for MAT comes from a seminal finding described half a century ago, which demonstrated that cultured cells maintain an inherent capacity to internalize isolated mitochondria. However, in this and subsequent studies, efficacy of mitochondrial internalization and persistence of exogenous mitochondria was dependent on cell type and external selective pressure. MAT allows enhanced dose-dependent mitochondrial uptake by HSPCs. Importantly, we have demonstrated the propensity of mitochondrially augmented hematopoietic cells to maintain exogenous mtDNA over time. Previous preclinical and clinical studies demonstrated that intravenously administered HSPCs have the propensity to home to both hematopoietic and non-hematopoietic tissues. Similarly, we demonstrate preclinically that after reinfusion of augmented bone marrow cells, exogenous mtDNA can be localized to both hematopoietic and non-hematopoietic tissues, possibly enabling rescue of mitochondrial function in multiple tissues.

Pearson Syndrome (PS) is a debilitating rare disease caused by a *de novo* deletion in the mtDNA, beginning with anemia and pancreatic insufficiency and progressing to a systemic disorder with multiple organ involvement and early death. It was previously demonstrated that allogeneic bone marrow transplantation can confer benefit in patients with PS, alleviating both hematopoietic and non-hematologic tissue dysfunction, but is associated with severe adverse events. We reasoned that MAT, in which autologous HSPCs would be augmented with normal mitochondria from the patient's mother, may be used for treatment of PS, balancing the safety of autologous cell therapy with potential systemic efficacy. Under a compassionate use program, we treated three PS patients. Improvement in general health and quality of life, fatigue and muscle function were observed. In addition, specific improvements per patient were noted based on different organ involvement, including improvements in growth and kidney function. We are currently investigating whether the mechanism by which MAT exerts clinical efficacy involves HSPC homing to non-hematopoietic tissues, intercellular mitochondrial transfer, systemic alterations in cytokine or metabolite levels, or immunomodulation.

828. Bacterial Nano-Cellulose: Human Muscle Stem Cells Culture Strategy for Improving Gene-Editing Approaches

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Skeletal muscle ability to regenerate relies on satellite cells (SCs), muscle stem cells. In a steady state, they are quiescent cells localized in a defined stem cell niche. In the presence of damage, they exit the niche, proliferate and hence generate a committed progeny (myoblasts) that differentiate and fuse into multinuclear myofibers, ultimately resulting in repair of the damaged muscle. The regenerative capacity of SCs makes them the optimal cell type for cell-based therapies, if they can be isolated from patients, manipulated *in vitro* and expanded while preserving their stem cell characteristics. However, once out of the niche, SCs quickly age and lose their regenerative potential. Therefore, muscle stem cells have not been successfully used for therapeutic purposes yet. We aim to develop a cell culture method that will enable us to perform *in vitro* manipulations (i.e. gene editing) on human primary myoblasts while avoiding extensive cells propagation, which is known to contribute to exhaustion of stemness. For this purpose, we use a bacteria nano-cellulose (BNC) substrate that allows preserving myoblasts for many weeks at a very low proliferation rate without evidence of terminal differentiation. A characterization of hPMs on the BNC substrate revealed changes in morphology, marker expression and proliferation rate compared to standard culture conditions on plastic. The low dividing state of hPMs on BNC was further confirmed by transcriptome sequencing. Therefore, BNC is a well suitable and innovative new method for the maintenance of slow-dividing cells in *in vitro* cultures.Gene editing is a powerful tool to repair disease-causing mutations, however, the repair of non-dividing cells remains a major challenge. We used human primary myoblasts on BNC as a tool for testing gene-correction-strategies in non-dividing cells. First, we chose NCAM1 as a model gene. Human primary myoblasts were transfected with a plasmid encoding eCas9::Venus and a sgRNA targeting NCAM1 exon 3. After enrichment for Cas9::Venus positive cells, a sub-sequent analysis of the NCAM1 locus on DNA-level revealed insertions and deletions (indels) at the targeting locus, suggesting successful gene editing on BNC substrate. Loss of NCAM1 protein expression was shown by FACS-staining. These data suggest that CRISPR/Cas9 based editing is possible in slowly- or non-dividing cells cultured on BNC, which makes it an excellent tool for optimizing gene manipulation strategies. Once verified that CRISPR/Cas9 based genome editing can be successfully used to target NCAM1 in human primary myoblasts, we aim to repair muscular dystrophy-causing mutations. Limb girdle muscular dystrophy type 2B (LGMD2B) is an autosomal recessive muscular dystrophy caused by mutations in the dysferlin gene (*DYSF*). Loss of dysferlin causes severe muscle weakness and atrophy of the pelvic and girdle muscles. We are comparing homology-directed repair and the more recently developed method of Prime Editing in primary human myoblasts under dividing and non-dividing conditions. Under dividing conditions, gene repair is observed with very high frequency and accuracy.

829. *In Vitro* Characterisation of Spontaneously Immortalised Non-Human Primate (NHP) Müller Glia Cell Lines as a Potential Source for Cell Replacement Therapies for Retinal Degenerative Eye Disease

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Cell replacement therapies for ocular diseases characterised by degeneration of the light-sensitive cells of the retina, the photoreceptors, are challenging due to poor survival of primary cells in culture. Therefore, a stable source of cells within the retina to replace lost photoreceptors is potentially promising for cell replacement therapies. Müller glia cells, which are astrocyte-like radial cells that play a pivotal role in maintaining retinal homeostasis, are considered to be the main glial cells in the retina. Their expression spans along the retina providing general structural support to retinal neurones and blood vessels. Among several other functions, they prevent aberrant photoreceptors migration into the subretinal space and facilitating facilitate glutamate uptake to keep its extracellular concentration below toxic levels. In lower vertebrates such as fish and amphibians injured retina show a regeneration potential which is believed to stem from Müller glia cells, such a phenomenon has not been shown in the human retina for unknown reasons. Investigations of Müller cells have proven to be laborious in the past due to the difficulty in obtaining a pure cell population and the tendency of these cells to differentiate rapidly in culture. Although various Müller glia cell lines have been described in the literature including those derived from human and rat, to our knowledge no non-human primate Müller glia cell line is currently available. Here we report a four spontaneously immortalised Müller glia cell lines, which were isolated from rhesus macaque monkeys, grown under normal culture conditions, responded to epidermal growth

factors and could be expanded indefinitely without the presence of growth factors. Cell lines 1, 2 and 4 exhibit normal morphology and express Müller glia markers such as Glutamate synthetase (GS), cellular retinaldehyde binding protein (CRALBP) and epidermal growth factor receptors (EGF-R) among other Müller glia specific markers when examined with immunocytochemistry, Reverse transcription PCR and western blotting. As well as expressing Müller glia specific marker, they also express stem cell specific markers. Our NHP Müller glia cell lines retained Müller glia characteristics after several passages *in vitro* and can be frozen and thawed without losing their viability. Our observations indicate that non-human primate retina harbours a population of cells that express both Müller glia and stem cell markers and suggest that those cells will potentially be useful for cell based therapies to restore retinal function in human.

830. Mesenchymal Stem Cells (MSCs) Generated Using mRNA Reprogramming Show Enhanced Growth Potential, Secretome, and Therapeutic Efficacy in a Demyelinating Disease Model

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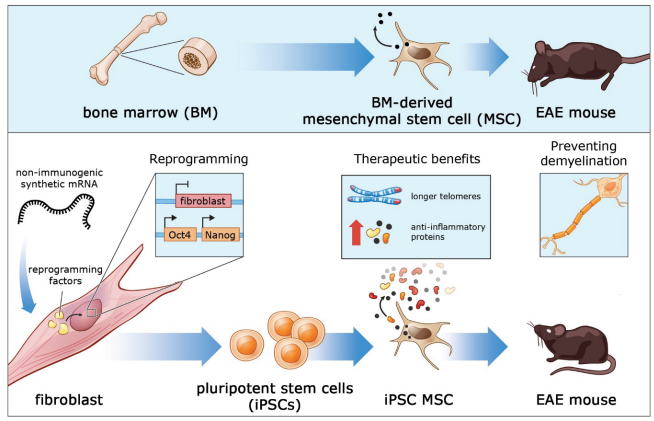
MSCs have undergone extensive clinical testing for many diseases and have consistently demonstrated safety. While the immunomodulatory properties of MSCs have been well characterized, adult-tissue-derived MSCs have shown limited therapeutic efficacy as well as significant variability among samples and limited proliferative capacity. Induced pluripotent stem cells (iPSCs) are being explored as an alternative source of MSCs to potentially overcome these limitations. We generated iPSCs from adult human dermal fibroblasts using a high-efficiency, immunosuppressant-free mRNA-based protocol. iPSCs were then differentiated into MSCs using a 21-day high-yield monolayer protocol. rtPCR analysis showed downregulation of Nanog and Oct4 and upregulation of CD73 and CD105 in the differentiated MSCs. Multipotency was confirmed by differentiation into adipocytes, osteoblasts, and chondrocytes. iPSC MSCs had approximately 13kb long telomeres compared to 7kb long telomeres of bone marrow-derived MSCs (BM MSCs), as measured by Southern analysis of terminal restriction fragments. When serially passaged, iPSC MSCs underwent >70 population doublings before senescence, compared to <20 population doublings for BM MSCs. Comparative secretome analysis showed overexpression of multiple neuroprotective and anti-inflammatory factors, including CXCL1, VEGF-A, and CXCL5. When administered to an experimental autoimmune encephalomyelitis (EAE) mouse model, iPSC MSCs delayed disease progression and improved clinical score ($p < 0.001$), while BM MSCs showed no difference from the disease-only control. Hierarchical clustering analysis revealed greater similarity between BM MSCs and fibroblasts than iPSC-derived MSCs, suggesting that the limited therapeutic efficacy of BM MSCs may be due to loss of an undifferentiated MSC gene-expression pattern.



831. Abstract Withdrawn

832. Stx2-Producing-*E.coli*-Associated Encephalopathy by Intravenous Injection of Muse Cells of Muse Cells

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Shiga toxin-producing *Escherichia* coli (STEC) causes hemorrhagic colitis, hemolytic uremic syndrome, and acute encephalopathies that may lead to sudden death or severe neurologic sequelae. Current treatments, including immunoglobulin G (IgG) immunoadsorption, plasma exchange, steroid pulse therapy, and the monoclonal antibody eculizumab, have limited effects against the severe neurologic sequelae. Multilineage-differentiating stress-enduring (Muse) cells are endogenous reparative non-tumorigenic stem cells that naturally reside in the body and are currently under clinical trials for regenerative medicine. When administered intravenously, Musecells accumulate to the damaged tissue, where they exert anti-inflammatory, anti-apoptotic, anti-fibrotic, and immunomodulatory effects, and replace damaged cells by differentiating into tissue-constituent cells. Here, severely immunocompromised non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice orally inoculated with 9×10^9colony-forming units of STEC O111 and treated 48 h later with intravenous injection of 5×10Muse cells exhibited 100% survival and no severe after-effects of infection. Suppression of granulocyte-colony-stimulating factor (G-CSF) by RNAi abolished the beneficial effects of Muse cells, leading to a 40% death and significant body weight loss, suggesting the involvement of G-CSF in the beneficial effects of Muse cells in STEC-infected mice. Thus, intravenous administration of Muse cells could be a candidate therapeutic approach for preventing fatal encephalopathy after STEC infection.

833. Leukapheresis Guidelines and Tisagenlecleucel Manufacturing in Patients Less Than 3 Years of Age with Relapsed/Refractory Acute Lymphoblastic Leukemia

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Tisagenlecleucel is a CD19-directed autologous chimeric antigen receptor (CAR)-T cell therapy. Patients (pts) <3 years of age (yo) at screening were excluded from tisagenlecleucel clinical trials in pts with relapsed/refractory B-cell acute lymphoblastic leukemia (r/r ALL; NCT02435849 and NCT02228096). We report feasibility of leukapheresis and commercial manufacturing of tisagenlecleucel for pts <3 yo with r/r ALL. Eligible pts were <3 yo with manufacturing data after August 30, 2017 (first FDA approval). Thirty-one pts with r/r ALL underwent non-mobilized leukapheresis of mononuclear cells (MNC). Median age was 15 months (range, 3-35 mo) and median body weight was 10.2 kg (range, 6.0-15.6 kg; 14 pts <10 kg and 17 pts ≥10 kg). Successful leukapheresis in pts with low weight was possible using guidance for safe and efficient collection, including: 1) Blood prime leukapheresis instrument tubing with irradiated and leukoreduced packed red blood cells (PRBCs) for pts <25 kg; 2) Transfuse PRBCs prior to leukapheresis to raise hematocrit (HCT) to 40% in pts <10 kg (preferably <15 kg) to maintain hemodynamic stability during procedure; 3) Prevent hypothermia using warming blanket or in-line blood warmer; 4) Anticoagulate with mixture of anticoagulant citrate dextrose solution-A (ACD-A) and heparin to minimize risk of ACD-A-induced hypocalcemia; 5) Observe pts for hypocalcemia, hypomagnesemia, and alkalosis (clinical signs and symptoms of irritability, inconsolable crying, and heart rate and blood pressure instability) by monitoring Ca^{2+}, Mg, and blood gases and replacing as needed and consider prophylactic IV Ca and Mg supplementation during procedure; 6) Monitor rate and color of in-line collection for non-mobilized MNC collection (maintain in-line salmon color consistent with a leukapheresis product, HCT at 3%-4%, and rate of ~0.8-1.2 mL/min or slower in small children); 7) Monitor collection volume (not to exceed 10 mL/kg per AABB standards) and perform partial rinse back at the end of each leukapheresis day; 8) Ensure adequate venous access with appropriate size central venous catheter to accommodate required inlet flow rate and potential need for adjustment during leukapheresis; 9) Verify adequate absolute lymphocyte count and/or CD3+ cell count on day prior to leukapheresis; and 10) Allow for >1 day of leukapheresis if needed to meet acceptance criteria in small pts. A median of 1 leukapheresis day (range, 1-6 d) was required to collect sufficient cells, including 2 pts who underwent repeat leukapheresis for second manufacturing attempt. Acceptance criteria for tisagenlecleucel manufacture were met in 29/33 leukapheresis materials; 2/4 that did not meet acceptance criteria were successfully manufactured. The first manufacturing attempts were successful in 26/30 (86.7%) pts, and 4/30 experienced manufacturing failure (2 pts <10 kg and 2 pts ≥10 kg). Among the 4 manufacturing failures, 2 successfully underwent repeat leukapheresis and a second manufacturing attempt, and 2 did not undergo a second attempt. Leukapheresis material did not meet acceptance criteria for 2/4 manufacturing failures. The median manufactured cell dose was 4.0×10^6 CAR+ viable T cells/kg (range, 0.37×10^6 to 4.0×10^6), median percent cell viability was 87.6% (range, 66.7%-95.7%), and median CAR+% expression was 10% (range, 2.7%-25.6%). Adhering to guidance for safe and efficient leukapheresis ensured successful leukapheresis in pts with r/r ALL <3 years of age and with low weight.

834. Engineering Natural Killer Cells by an Alpharetroviral SIN Vector to Target ALK in Neuroblastoma

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Neuroblastoma (NB) is a neural crest-derived malignancy, accounting for ~15% of childhood cancer mortality. The majority of NB tumors express or overexpress full-length anaplastic lymphoma kinase (ALK) oncogene or ALK gain-of-function mutations. Given its low or absent expression in normal tissues beyond embryonic development, ALK may be an excellent target for chimeric antigen receptor (CAR) cell therapy. Compared to CAR T cells, CAR-Natural Killer (NK) cells retain intrinsic capacity to recognize and target tumor cells through native receptors, are not HLA-restricted and do not mediate graft-versus-host disease (GVHD). Compared to lentivirus vectors (LVV), alpharetroviral SIN vectors (α-RVV) have been shown to efficiently transduce NK cells, demonstrate nearly neutral genomic integration profile in transduced cells and can generate sustained high titers in cloned packaging cells. We designed CAR constructs containing humanized ALK (hALK) single-chain variable fragment (scFv) with signaling domains containing human CD3ζ/CD28 or CD3ζ/NKG2D/DAP10. We transduced the human NK-92 cell line with the anti-ALK CD28 CAR (CD28 NK-92), anti-ALK NKG2D/DAP10 CAR (NKG2D/DAP10 NK-92) and a GFP control (GFP NK-92). ALK transduced HT1080 cells were used as a positive control and we targeted NB cell lines NB-1, IMR-32, SKNBE and SH-SY5Y (Table 1). NK cell activation has been shown to lead to IFN-γ secretion upon specific antigen stimulation. We co-cultured engineered NK-92 cells with ALK+ target cells at a 1:1 effector/target (E/T) ratio for 24 hours and analyzed IFN-γ levels; CD28 NK-92 and NKG2D/DAP10 NK-92 cells produced significantly higher IFN-γ levels than GFP control NK-92 cells when incubated with ALK+ HT1080 (p=0.04 and 0.03), NB-1 (p<.01 and p=0.03), and IMR-32 cells (p=0.02 and 0.02). To assess the direct killing effects of CAR NK-92 cells, we measured the surviving target cells after co-culture with effector NK cells (Figure 1). Against ALK-expressing HT1080 cells, NKG2D/DAP10 NK-92 cells demonstrated a killing effect that was superior to CD28 NK-92 (p=0.01,

NKG2D/DAP10 vs CD28). CD28 NK-92 and NKG2D/DAP10 NK-92 cells effectively killed NB-1 cells with amplified expression of ALK, as well as IMR-32 and SKNBE cells, which have WT ALK expression. In SH-SY5Y cells that carry an F1174L ALK mutation with low ALK expression the effective killing was only seen with increased E/T ratio of 5:1 (CD28 NK92 and NKG2D/DAP10 NK 92 vs control, p=0.04 and 0.05, respectively). Our data suggest that using α-RVV-transduced NK cells may represent a potential new strategy for treating neuroblastoma and other ALK-positive cancers.

Table 1. ALK and MYCN expression and IFN-γ secretion levels.

			IFN-γ (pg/ml)			
Cell line	ALK	MYCN	NK-92	GFP NK-92	CD28 NK-92	NKG2D/DAP10 NK-92
ALK HT1080	Wild-type, Amplified	Not amplified	306.86± 99.27*[1]	281.93± 57.92**[1]	801.50± 242.36**[1,2,3]	866.70± 299.39**[1,3]
NB-1	Wild-type, Amplified	Amplified	205.89± 36.67**[1]	243.08± 41.67*[2,**1]	1001.05±149.42***[1,2,3]	978.38± 288.38**[1,2,3]
IMR-32	Wild-type High expression	Amplified	182.09± 42.41*[1]	222.01± 43.14*[1]	588.85± 129.99*[2,3,**1]	613.83± 141.15**[1,2,3]
SKNBE	Wild-type, High expression	Amplified	274.45± 95.67*[1]	476.53± 212.37	558.97± 134.06*[2,**1]	555.58± 146.75*[1]
SH-SY5Y	F1174L, low expression	Not amplified	319.64± 139.85	428.16± 204.40	605.16± 180.31*[1]	513.34±168.014*[1]

n=3; *p≤0.05 , **p≤0.01, ***p≤0.005; [1]: compared to target cell group, [2]: compared to NK-92 cell group, [3]: compared to GFP NK-92 cell group;

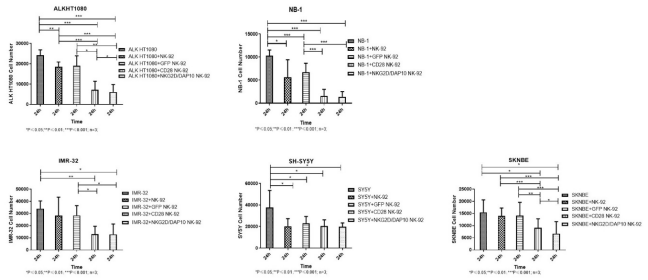

Figure1. Cell numbers of ALK+ HT1080, NB-1, IMR-32, SKNBE, SH-SY5Y cells co-cultured with NK-92, GFP NK-92, CD28 NK-92 and NKG2D/DAP10 NK-92 cells for 24 hours.

835. Ex Vivo Expansion of Umbilical Cord Blood Hematopoietic Stem/Progenitor Cell Populations Cultured in Serum-Free Medium QBSF-60

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The use of umbilical cord blood for adult transplants has been hampered by the relatively low number of hematopoietic stem and progenitor cells (HSPC). We have developed a method to culture and expand non-enriched human cord blood derived CD34+ hematopoietic stem cells in an ex vivo system using our proprietary serum free medium QBSF-60 (www.qualitybiological.com). The culture conditions support the maintenance and a 23-fold expansion of the long-term engrafting HSPC after 14 days of culture. A 50-fold increase expansion of CD34+ cells in culture for four weeks was achieved with short-term engrafting progenitor cells. We evaluated several combinations of early acting cytokines for their ability to support the ex vivo culture of cord blood CD34+ cells while maintaining their long-term engrafting ability. For in vivo engrafting studies in the fetal sheep xenograft model, we selected cells cultured in the presence of the cytokine combinations, FS6, FST6 and FST3611, based on their ability to yield significant numbers of the primitive progenitors without excessive differentiation. The expansion occurs without the need for a feeder layer, making this culture method highly translational with potential for scale up. The primary recipients transplanted with CD34+ cells from each group including the uncultured control cells all engrafted and underwent multilineage differentiation, indicative of short-term engraftment. Secondary recipients transplanted with cells cultured in the cytokines FST6 combination maintained their long-term engrafting ability and underwent multilineage differentiation. These results demonstrate that it is feasible to culture and expand non-enriched cord blood CD34+ cells under clinically relevant conditions for the development of ex-vivo gene therapy applications. The expanded grafts retain both phenotypic markers and the ability to support early engraftment in a sheep model.

836. Clinical Evidence for Immune Reprogramming with Extracorporeal Mesenchymal Stromal Cell Therapy

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Background and Aim: Acute kidney injury (AKI) involves damage to renal epithelial cells, podocytes, and vascular beds that manifests into a self-perpetuating immune response and peripheral organ dysfunction. Such a complex injury pattern requires a multifaceted therapeutic to address the systemic, immune-mediated inflammation. Mesenchymal stromal cells (MSCs) are a unique source of secreted factors that modulate an inflammatory response and enhance the repair of injured tissue. Sentien has created a novel delivery approach to enable sustained exposure to MSCs and their secreted factors, overcoming limits of cell transplantation/infusion while preserving the broad acting and dynamically responsive properties. Our lead product, SBI-101, contains allogeneic human MSCs inoculated into a hollow-fiber hemofilter, which facilitates contact with patient blood via the semi-permeable membrane, while preserving MSC viability. SBI-101 is designed to restore balance to the immune system by reprograming the molecular and cellular components of blood in patients with severe organ injury. SBI-101 is being studied in a Phase I/II clinical study in critically ill patients with Dialysis-Requiring Acute Kidney Injury (AKI-D). Methods, Results & ConclusionIn the initial cohort analyzed, patients were randomized to receive SBI-101 (250x106 cells) or sham control for up to 24 hours of continuous treatment (n=4 per group). Over 200 exploratory biomarkers were measured to characterize the pharmacokinetic and pharmacodynamic effects of SBI-101. Samples were collected for the measurement of immune cell populations, kidney injury biomarkers, and biomarkers of inflammation at pre dose, conclusion of treatment, days 1, 3, 7, 14, 21, and 28 post-treatment with SBI-101 or sham control.Data showed a measurable PK response that is dynamic and appears to be specific to each patient. Trends in PD response were also observed that were consistent with known MSC biology. Inflammatory (anti- and pro-) markers were shown to

be modulated, suggestive of a shift from a pro- to anti- inflammatory state. In addition, innate and adaptive cellular immune changes were also observed. Finally, a reduction in markers of kidney injury were also seen at effect sizes greater than one.While the study was not powered for significance, the results demonstrate a dynamic biological response from the MSCs with potentially beneficial effects in the immune system as well as in the kidney of these critically ill patients. Moreover, it supports the therapeutic hypothesis of SBI-101.

837. Use of Click-Chemistry to Make Bivalent Antibodies for Redirection of T Cells: Proof of Concept in an In Vitro HIV Model

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Neutralizing HIV gp120 Ab-coated T cells may play a role in providing cytotoxic targeting of HIV-infected cells and thereby reduce or eliminate the HIV reservoir. Using recombinant DNA methods, bispecific T-cell engager (BiTE) antibodies have been designed and FDA approved for treatment of cancer. In this study, we used a simpler method of production, namely hinge region-specific click-chemistry to form a dual-specific, bivalent BiTE (dbBiTE). This was done by joining an intact (150 kDa) anti-gp120 antibody 3BNC117 in high yield (30- 50%) to an intact (150 kDa) anti-human CD3 (OKT3) antibody. The resultant dbBITE was able to bind to both T cells and HIV gp120-positive HEK cells as shown by flow staining. To assess specific cytotoxic responses against HIV gp120-positive cells, we used the anti-gp120/CD3 dbBITES to coat activated human T cells, derived from PBMCs, after anti-CD3/CD28 activation and IL-2 expansion, and co-incubated them with target cells including HIV Lab strain NL4-3 infected PBMC, or cells expressing HIV gp120 and control cell lines. T cells coated with anti-gp120/CD3 dbBITES showed anti-HIV effect in HIV-infected PBMC at various effector to target ratios ranging from 53% to 27%, as measured by p24 Elisa testing, and showed target specific killing ranging from 96% to 36% killing of gp120-positive HEK cells as measured by incuCyte imaging analysis. This methodology has the potential to be developed into a cellular immunotherapy for HIV/AIDS.

838. Machine Learning-Based Immunological Synapse Quantification to Predict CAR T Efficacy

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Human immune system consists of a network of billions of independent, self-organized cells that interact with each other to form a highest intelligence. Artificial intelligence (**AI**) is being used in immunological research. Machine learning (**ML**) is an **AI** tool that can process huge data and generate models for immune system and immunological research. One of the most exciting, recent breakthroughs in immunological research is cancer immunotherapy. Specifically, one such therapy involves engineering immune cells to express chimeric antigen receptors (**CAR**), which combine tumor antigen specificity with immune cell activation in a single receptor. Recent clinical trials testing cancer immunotherapies have shown promising results for treating various cancers. The adoptive transfer of these CAR-modified immune cells (especially T-cells, CAR T) into patients has shown remarkable success in treating multiple refractory blood cancers. To improve their efficacy and to expand their applicability to other cancer types, scientists are optimizing different CARs with different modifications. However, predicting and ranking the efficacy of different CAR T cell products with identical antigen specificity, selection of responders with an identical CAR T construct, and identification of the optimal CAR for a translation researcher to further develop potential clinical application are limited by the current, time-consuming, costly, and labor-intensive conventional tools used to evaluate efficacy. Particularly, T cell efficacy is not only controlled by the specificity and avidity of the tumor antigen and T cell interaction, but also it depends on a collective process, involving multiple adhesion and regulatory molecules, spatially organized at the T cell immunological synapse (**IS**). Optimal function of cytotoxic lymphocytes depends on IS quality. Recognizing the inadequacy of conventional tools and importance of IS in T cell functions, we investigate a new strategy for assessing CAR T efficacy. Previous studies in our lab show evidence that: 1) CAR T-cell immunological synapse (IS) quality (structure, function, and signaling) varies between CAR T-cells, 2) CAR co-stimulatory endodomains influence IS quality, 3) CAR T IS quality correlates with antitumor activity both *in vitro* and *in vivo,* and 4) CAR IS quality assay developed here can distinguish a responder from non-responders. However, current IS quality image data analysis was quantified manually, which is time-consuming and labor-intensive with low accuracy. Here we develop a machine learning method to quantify thousands of synapse images with enhanced accuracy. The ML-based, automated algorithms to quantify CAR T IS can develop fast and easy tools to predict CAR T cell efficacy, which provides guidelines for designing and optimizing CARs for clinical cell therapy.

839. Ex-Vivo Cell Therapy Platform for Immune Reprogramming in Autoimmunity

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Mesenchymal stromal cells (MSCs) are a unique source of secreted factors that modulate an inflammatory response and enhance the repair of injured tissue. Sentien has created a novel delivery approach to enable sustained exposure to MSCs and their secreted factors, overcoming limits of cell transplantation/infusion while preserving their broad acting and dynamically responsive properties. Our lead product, SBI-101, contains allogeneic human MSCs inoculated into a hollow-fiber hemofilter, which enables contact with patient

blood via the semi-permeable membrane, while promoting MSC viability. Through this interplay SBI-101 is hypothesized to restore balance to the immune system by reprogramming the molecular and cellular components of blood in patients with severe organ injury. Sentien's Phase I/II clinical study of SBI-101 in critically ill patients with Dialysis-Requiring Acute Kidney Injury (AKI-D) has produced data to support the therapeutic hypothesis of SBI-101 as a potent immunotherapy. Consistent with MSC biology inflammatory (anti- and pro-) markers, such as IL-10 and TNFa, were shown to be modulated, suggestive of a shift from a pro- to an anti- inflammatory state in treated patients. We have developed a miniaturized version of our lead product to assess immune reprogramming ex vivo. We previously showed that this platform can shift activated lymphocytes from healthy donors from a pro to an anti- inflammatory state. In the present study we describe results obtained using this platform on PBMCs from patients with autoimmune conditions. We first tested multiple sclerosis (MS) and systemic lupus erythematosus (SLE) for ex-vivo responsiveness. PBMCs were isolated from donor blood and perfused through R&D scaled SBI-101s seeded with either 0 or 9x10^6 MSCs. Following 5 days of perfusion the PBMCs were then immunophenotyped and the perfusion media assayed for cytokine output. The results indicate that, as also seen in SBI-101 treated AKI-D patients, MSCs drive a large decrease in specific pro-inflammatory markers elevated in diseased donors (e.g. IL-17A). Further work to characterize SBI-101's immunomodulatory capacity in these and other indications may broaden clinical applicability of the product and improve patient treatment options.

840. High Throughput Screen Optimization of Surface Topographies with Media Additives to Prime Human Mesenchymal Stem Cell Transfection

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Introduction: Human mesenchymal stem cells (hMSCs) are under study for applications in cell and gene therapies due to their ease of isolation and expansion, and regenerative and immunomodulatory potentials. Though nonviral genetic modification of hMSCs could safely endow them with improved therapeutic properties, nonviral gene delivery is limited by toxicity and inefficiency. To advance hMSC therapeutic potential, strategies that allow for more efficient and less toxic hMSC transfection are needed. Although micro/nanoscale culture surface topographies have been identified that improve endocytosis and intracellular trafficking of transfection complexes, finely tuned optimization of hMSC transfection across a broad continuum of multiscale structures has not been demonstrated. We used femtosecond laser surface processing (FLSP) to fabricate continuous gradients of micro/nanoscale surface features within wells, to high-throughput screen for structures that promote hMSC transfection with and without the glucocorticoid dexamethasone (DEX), which has recently been shown to prime transfection of hMSCs on polystyrene culture surfaces via mitigation of transfection toxicity. **Methods:** Gradient topography wells, fabricated via FLSP of titanium slides for alignment with clip on well devices, exhibited submicron periodic ripples and nanostructure over microscale mound-like structures, that were further etched via alkaline hydrogen peroxide, producing pores ranging from 3 to 21 μm in depth, characterized via SEM and confocal laser profiling (**Fig.1A**). Adipose derived hMSCs were seeded in gradient wells, primed with 150 nM DEX (DEX+) or vehicle control (DEX-), then transfected with plasmid encoding enhanced green fluorescent protein (EGFP) via Lipofectamine 3000. Nuclei and EGFP expression were imaged 24 and 48 hrs post-transfection, then quantified by image processing in seven 2x4 mm regions per well.

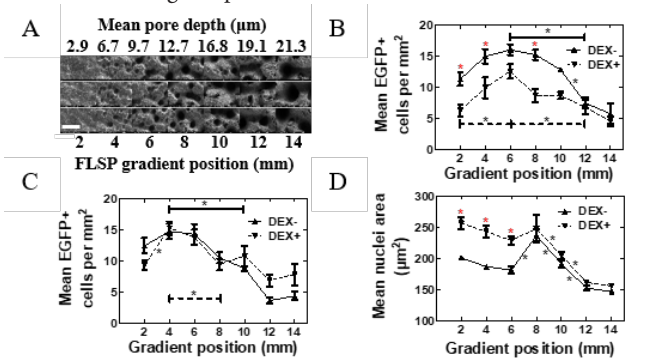

Figure 1. A) Pore depths and SEM micrographs along etched FLSP Ti gradient wells, scale 10 μm. **B)** EGFP+ hMSC density quantified 24 hrs post-transfection. **C, D)** EGFP+ hMSC density and nuclei area quantified 48 hrs post-transfection. Significant differences between DEX+ (solid) and DEX- (dashed) at gradient positions denoted (red *) and between regions (*). **Results:** Density of hMSCs expressing EGFP (EGFP+) inversely correlated with pore depth, with ~2-3-fold greater EGFP+ observed over pore depths of less than 10 μm, relative to the minimum EGFP+ observed over ~20 μm pore depth (**Fig.1A-C**). At 24 hrs, DEX+ resulted in decreased EGFP+ relative to DEX- (**Fig.1B**), while at 48 hrs, there were no significant differences in EGFP+ between DEX+ and DEX- transfection (**Fig.1C**), At 48 hrs, nuclei area was increased by ~50 μm^2 in DEX+ transfection over pore depths less than 10 μm (**Fig.1A,D**), implicating DEX modulation of cell cycle and nuclei morphology via cytoskeletal tension, as potential mechanisms for the transfection delay and recovery observed in DEX+ hMSC transfection. **Conclusions**: Screening hMSC transfection on gradient FLSP topographies revealed optimal surface pore depths within a continuous range and provided insight into potential mechanisms for modulating transfection dynamics via combinatorial tuning of surfaces and DEX. Ongoing investigations include screens in multiple donors of additional topographies, DEX doses, and other priming compounds, towards optimization of hMSC *ex vivo* transfection for cell therapies.

841. Luciferase-Modified Urine-Derived Stem Cells Home to Sites of Injury

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Urine is a practical and painless source of cells for gene and cell therapy applications. Urine-derived stem cells (USCs) are adult human cells of renal origin that propagate in tissue culture in media containing growth factors on gelatin-coated plates. In order to assess their potential for regenerative gene and cell therapies, we have isolated, expanded, transfected, and tracked these cells. FACS characterization revealed that they expressed the characteristic marker panel (CD44, CD73, CD90, CD146+/CD31, CD34, CD45-). They are multipotent as evidenced by their differentiation along the osteogenic, chondrogenic, and adipogenic lineages. Transfection was optimized to achieve 61% transfection via non-viral methods. Five different *piggyBac* luciferase transposons were compared with the CMV and EF1-alpha giving the highest luciferase signals. When luciferase-modified urine-derived stem cells were injected directly into the renal pelvis of healthy mice, they migrated to the surgical wound scar rather than staying in the uninjured kidney. Luciferase-modified USCs also migrated to the surgical wound scar if a sham operation was performed with an IP injection of cells, suggesting that the cells are highly mobile and able to home to sites of injury. Because the cells are renal in origin, we hypothesized that they may be able to treat acute kidney injury. Acute kidney injury is a major cause of morbidity and mortality that is present in nearly 1 in 4 intensive care unit admissions. The current therapies for acute kidney injury are supportive rather than regenerative so new gene and cell therapies are needed. Renal ischemia-reperfusion injury mouse models are highly dependent on the intersection of the mouse strain with the ischemia time. The ischemia time is the period during which the renal vessels are clamped to stop blood flow to the kidney and induce acute kidney injury. We optimized the ischemia time together with unilateral nephrectomy in the immunocompromised NSG mouse strain. We found that 22 minutes of renal pedicle occlusion was ideal, with 83% of mice having elevated creatinine, developing a model of ischemia-reperfusion injury in immunocompromised mice. Urine-derived stem cells transfected with *piggyBac* transposons expressing luciferase were injected into the peritoneum of NSG mice on Day 3 post-ischemia-reperfusion injury. Traditional luciferase imaging on the IVIS Spectrum produces 2D images that do not give a high degree of confidence regarding the organ specificity nor quantitative information of the luciferase-expressing cells. In contrast, the InVivoPLOT plug-in for the IVIS enables quantitative tomographic optical live animal imaging. We used this technique to quantify the luciferase signal within the individual organs of the live mice. We found that the luciferase-labeled USCs homed to the injured kidney, with lower levels found in the spleen. No significant signal was detected in liver or bladder. This signal appeared within two hours following IP injection, suggesting that homing of the USCs to sites of injury occurs rapidly. In conclusion, USCs are an easily isolated, clinically relevant cell type that can be manipulated with non-viral genetic tools. Based on InVivoPLOT 3D luciferase imaging data, the USC quickly migrate from their injection site to the injured kidney. In future studies, we will quantify recovery of kidney biomarkers and stain for immunohistochemical evidence of the transplanted cells.

842. Development of a Novel Endometrial Stem Cell-Laden 3D Artificial Endometrium: Successful Pregnancy and Live-Birth

In Sun Hong

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Serious injuries of endometrium (the innermost lining layer of the uterus) or naturally thin endometrium can cause uterine dysfunction and subsequently predispose to infertility or miscarriage. In this context, many stem cell researchers effort have been devoted toward regeneration of injured or thin endometrium by administrating various types of stem cells. Despite some promising initial results, regenerating the endometrium with stem cells is very challenging due to their lack of 3D microenvironment and subsequent low therapeutic effects of transplanted stem cells. Therefore, as an alternative, we developed a novel endometrial stem cell-laden 3D artificial endometrium by combining various endometrial cellular components and natural biodegradable polymers to mimic multilayered endometrial structure and its microenvironment. The artificial endometrium constitutes a triple-layered structure to recapitulate the structural and physiological features of human endometrium. Cell viability and biological characteristics of various types of encapsulated endometrial cells are well maintained within artificial endometrium. It also exhibits similar behavior to human endometrium by properly responding to steroid hormones and actively secreting various growth factors. Remarkably, severe degenerative changes were significantly relieved by artificial endometrium transplantation. More importantly, the successful pregnancy and subsequent successful live birth can be archived by transplanting our artificial endometrium into endometrial ablation mice without any phenotypic or genetic abnormalities.

843. Abstract Withdrawn

Vector and Cell Engineering, Production or Manufacturing

844. Impact of PEI to DNA Ratio and Further Transfection Enhancing Agents on Yield of Transient Gene Expression for Production of rAAV Particles

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Currently about 7,000 rare diseases are known worldwide, about 80% of them originate from genetic diseases which often impairs the lives of those affected congenitally. Gene Therapy is one of the most encouraging concepts to address the unmet needs of these patients. We envision that recombinant adeno associated virus (rAAV) which emerged during the last years as a highly promising vector for Gene Therapy has the strong potential to alleviate numerous of these diseases in the near future. For the generation of rAAV capsids a widely used approach is transient gene expression in HEK293 cells using polyethyleneimine (PEI) as a transfection reagent. PEI polyplexes with

the plasmid DNA that hosts the genes needed for the proliferation of rAAV in the cells which results in the formation of complexes. These complexes are positively charged due to the original charge of PEI. They interact with the negatively charged host cell membrane and ultimately release the DNA into the host cell. The amount of PEI used is crucial to the success of the transient gene expression and to the AAV yields that are gained. Usually the amount of PEI loosely correlates to the amount of plasmid DNA that is used, though different ratios can be beneficial. In the present study we show the impact of various PEI to DNA ratios on the yield of rAAV capsids produced by transient transfection in HEK 293 cells. With the adaption of the PEI to DNA ratio, we could obtain a yield increase of up to 90% compared to the ratio that was used in the prior setting. To further investigate the efficiency of transient transfection, several other techniques that are known from literature were tested in small scale systems. Both successful and unsuccessful modifications to the transfection mix will be presented in the poster at hand.

845. Cell Counting Method Validation and the Complexities of an Autologous Cell Product

Caitlin Wint

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Cell therapy has become increasingly prevalent in the world of regenerative medicine. The complexity of cell-based assays is not always represented within industry standards. Performance characteristics are assessed during validation to ensure the method is performing as intended. This abstract will focus on the validation of a cell-based analytical method used to determine the strength of an autologous muscle derived cell product. An automated flow cytometer is used for the cell count method. Detection for physical characteristics include forward scatter and side scatter parameters which are used to distinguish free nuclei and debris from cells. During execution, an operator applies a proprietary reagent directly to the cell sample. The reagent contains two DNA-binding dyes capable of staining both viable and non-viable cell populations. The instrument automatically analyzes and quantitates the presence and concentration of viable and non-viable cells, while debris are excluded based on settings. The most difficult characteristic to design testing for was Accuracy, as this is the closeness of agreement between the value observed and that of a reference standard. The issue with a cell-based method and an autologous cell sample, is that a reference standard does not exist, and therefore, accuracy is often inferred. In order to avoid inferring accuracy, a Batch Record was prepared to manufacture a small cell bank using donor tissue to represent an internal standard. Following manufacture, characterization testing was completed to statistically determine the internal reference standards' "known value" which was then used to directly assess the accuracy of the method, where a comparison between the actual and theoretical values was completed; the acceptance criterion was based on an allowable % Bias between the actual and expected cells/mL. Viability % was approached in a similar fashion, however, following manufacture of the standard and prior to testing, the cells were manipulated to create both viable and non-viable cell populations. Mixtures of the populations were formulated to represent 80-120% of the assay range. Following the formulations, qualification was performed in order to determine the "known value" of the standards; a similar approach to acceptance, % Bias, was used. In addition to the direct comparison to the internal standard, additional studies were designed surrounding the accuracy of the flow cytometry method. Not only would the assay outputs require complex testing, but the test dilution used must prove to be appropriate for the intended use of the assay and the flow cytometer as a holistic system. For example, the flow rate, cells/µL, and final dilution factor all contribute to the methods validated state. Linearity testing focused on the dilution factor, dependent on the expected cells/mL of the cell sample. These dilution factors were designed by considering the expected cells/mL and the instrument capabilities, which can impact the accuracy of the instrument for anything >300 cells/µL. The study was designed to show that across the assay range, the method was linear using the predetermined dilution factors, which were designed to stay within instrument capabilities (i.e. determine if the method is being impacted by the instrument constraints). To fully understand the performance of a method, an appropriate sample size must be tested. Although replicates of the sample are essential, often with a cell-based assay the largest variability is attributed to the sample itself, specifically in the use of autologous cells. For purposes of this design, a much larger sample size was used to assess the method performance than typically recommended by industry guidance; this was done to show that even with an autologous sample (i.e. subject to subject variation), the method would still perform as intended, which is ultimately the same goal of all method validations.

846. Critical SF9 Metabolites for Baculovirus Infection and AAV Production

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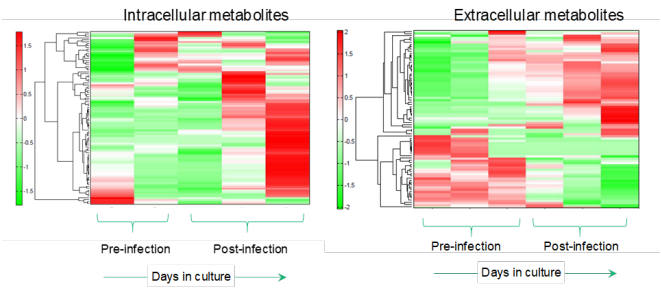
The baculovirus expression vector system using SF9 cells is a convenient method for manufacturing recombinant adeno associated virus (rAAV) used for gene therapy applications. The process has seen little optimization since it was first introduced. Overall productivity is limited since the process only produces AAV at relatively low cell densities. This productivity limit is attributed to a nutrient or metabolic deficiency during baculovirus infection at high cell densities. To overcome this bottleneck and shed light into the SF9 metabolism during growth and infection, we quantified the intra- and extracellular metabolites of Sf9 cells. Experimental conditions included infection at low and high cell densities; different growth media with increasing nutritional strength; and multiple time points pre- and post-infection. Intracellular metabolites from several Sf9 metabolic pathways were quantified using CE-MS. Cellular redox and energy status were monitored and spent media metabolites quantified for consumption and secretion. Preliminary results have demonstrated a range of nutrient consumption rates corresponding with different cell densities and media backgrounds. These changes identified critical media components necessary to replenish anaplerotic pathways. Activation of certain salvage pathways and changes in cellular redox and energy status were also seen. Results of this study are being used to develop strategies to sequentially promote Sf9 cell growth and increase AAV titers. Stable isotope studies are continuing to further investigate the observed metabolic differences.



847. CDMO Challenges in the Viral Vector Market

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It is widely recognised that not only the manufacturing of viral vectors is a critical bottleneck in the supply of viral vector for clinical studies, but also the development of the manufacturing processes to produce them. The development of manufacturing platforms for viral vectors is inevitably challenging due to the size and nature of the product themselves, but is it further complicated not only by the nature of the selected vectors, but also by the therapeutic payload the vectors are carrying. This is especially true for AAV vectors, where productivities, product location, aggregation and full to empty vector ratios are impacted not only by the serotype, but also to a less degree by the therapeutic product the vector codes for. To address these challenges a number of different manufacturing approaches have been proposed and adopted for clinical supply including the use of both human and insect cell lines, and a variety of recovery and purification approaches being used. Consequently, all of these processes have their own nuances, making the establishment of standardised development platforms and manufacturing processes and the requirement investments into development and production equipment and facilities highly challenging. Whilst some groups are establishing in-house process development and manufacturing capabilities, accepting the costs and risks associated with this approach; other groups prefer to use CDMO's (Contract Development and Manufacturing Organisations) that are able to access existing facilities and skill bases for generation of clinical material and potential in market products. For those seeking to develop in-house manufacturing processes, the selection of the manufacturing process and recovery and purification process will be tailored to the specific needs and predicted manufacturing requirements of the selected vectors. For CDMO's wishing to meet the needs and expectation for multiple clients with widely different requirements it is less clear what optimal manufacturing strategies should be adopted to meet customer requirements around capacity, cost, time and quality. The development of successful production processes is not "a given" in terms of amounts or product quality or timelines, and the impact of failure is very high to developers and CDMO's alike. Therefore as the viral vector market evolves and expands CDMO's need to carefully assess potential manufacturing platform to adopt. To be successful they will need to identify ones which meet a wide range of manufactures' needs, whilst reducing risks within development and manufacturing processes, whilst having the basis for longer term vector production and the investment in facilities, equipment and staff that will entail. This will require a detailed understanding of the costs, time and potential investments required to support the planned programme of works and to establish longer term manufacturing processes.

848. Overcoming Challenges for Developing AAV Purification Process for Large-Scale GMP Manufacturing

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Adeno associated virus (AAV) is one of the most prominent vectors for gene therapy. The need for production and purification of large quantity of AAV according to cGMP for clinical and commercial applications is paramount. Large-scale AAV purification processes suffer from several well-known challenges, such as: low recovery in the first purification step, instability of AAV at low pH conditions, aggregation of AAV during purification or formulation process, clearance of (potentially) contaminating virus from AAV and enrichment of full vector particles from the empty particles. This presentation will address the scientific insights on these challenges.

849. Generation of a DuoBac Expression System for Robust and High Quality AAV Production

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Vector Development, uniQure, Amsterdam, Netherlands

Scalable and robust production of clinical grade AAV is a critical step for delivering successful AAV based gene therapies to patients. AAV expression cassettes comprising Replicase (Rep, DNA replication and packaging proteins) and Capsid (Cap, Structural proteins) encoding genes are delivered to the producer cell alongside a to be packaged transgene flanked by AAV2 ITRs. The BEVs production system presents a scalable platform for rAAV production as baculoviruses can be amplified on site and insect cells can be grown without serum in suspension. The most frequently used method for producing rAAV in insect cells is via the co-infection of three separate baculoviruses. These baculoviruses comprise Rep, Cap and Transgene expression cassettes, however, the number of baculovirus seeds needed for rAAV production can be reduced by creating baculoviruses containing double expression cassettes (either Cap-Rep or Cap-Trans). Advantages of using such a double baculovirus (DuoBac) infection system can be 1) lower risk of contamination, 2) higher AAV yields in cell lysates, 3) a more robust MOI response 4) increased flexibility for up-scaling and 5) lower cost of goods as one less seed virus is required for production. These advantages arise because all the molecular components required for AAV production are more likely to be present in the cell at the correct time during infection. In this study we devised and tested multiple molecular designs in context of the BEV system which express Rep, Cap and Trans in the correct spatiotemporal profile resulting in high yield and quality AAV production.

850. Accelerating Advancement in Gene Therapy by Enabling Large Scale Development of AAV Vectors

Orjana Terova[1], Jessica D. de Rooij[1], Alejandro Becerra[1], John Li[1], Pim Hermans[2], Frank Detmers[2]

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Major progress has been made in the area of gene therapy and due to improved safety and efficacy, the Adeno-Associated Virus (AAV) has emerged as one of the most important vectors for the development of gene therapies. With a growing pipeline of clinical trials, it is evident that scalable manufacturing technologies (i.e. downstream purification) are needed by this rapidly growing industry. Affinity chromatography resin development, specific for AAV downstream processing, has gone through considerable improvements. In addition to being a scalable purification solution, these unique affinity resins have demonstrated they can serve as a platform for the purification of a large variety of serotypes achieving high purity and high yield in a single step. Here we outline the benefits of implementing POROS™ CaptureSelect™ AAV affinity resins into the downstream process of AAV vectors, including scalability, reducing purification steps, increased productivity and process consistency. In conjunction, results of a viral clearance study are shown, demonstrating the AAVX resin could serve as an effective viral clearance removal step in the downstream manufacturing of these vectors at industrial scale.

851. A Robust, Scalable Ultracentrifuge-Free Method for Generating Purified High-Titer Lentiviral Vectors

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While there has been significant progress in scaling up the manufacture of viral vectors, bottlenecks remain at the preclinical stage particularly for replication deficient lentiviral vectors. Common issues with the testing and development of lentiviral vectors in a research setting include the inability to generate large quantities of high-titer vectors without a special skillset and significant investment in equipment. Another issue is the low transduction efficiency of crude virus preparations. Here, we present an optimized process that consistently generates purified lentivirus with a titer ranging from mid E8 to E9 IFU/ml depending on scale. We show that a third-generation lentiviral vector can be produced at a significantly higher yield with a lipid polymer transfection reagent when compared to $CaPO_4$-based transfection and PEI. We also demonstrate a downstream processing pathway involving Tangential Flow Filtration (TFF), and centrifugal concentrators. We use an easily assembled TFF system that requires no prior expertise, coupled with diafiltration (DF) to further purify the virus. TFF/DF simultaneously concentrates and purifies the vector, demonstrably removing host cell protein and serum contaminants, leading to more efficient transduction of cells. This process eliminates the need for high-speed (or ultra) centrifuges, and is easily performed in a biosafety cabinet, with yields and purity sufficient for *in vitro*, *in vivo* and *ex vivo* studies. The biggest advantages it offers are the flexibility to generate vector at scales ranging from T75 flasks to cell factories with minimal alterations, and increased functional titers with the elimination of ultracentrifugation. This makes it an attractive solution for efficient generation of vectors as needed for pilot studies.

852. Development of a qPCR Method for Quantification of Adeno-Associated Virus Based Product

Pushpendra Singh, Arun K. Upadhyay

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Objective: To develop a quantitative polymerase chain reaction (qPCR) method to quantify vector genome (vg) titer of an adeno-associated virus (AAV) based OCU400 product. **Introduction:** Recombinant AAV vectors have shown great promise to cure several genetic disorders by delivering functional copy of the affected gene. Ocugen is developing OCU400, an AAV vector consisting human Nuclear Receptor Subfamily 2 Group E Member 3 (*NR2E3*) gene, for the treatment of underserved retinal degenerative diseases. Quantification of AAV vectors is challenging but needed for process development, vector production and the clinical dose determination to maintain quality control. The lack of accurate method can significantly impact the clinical trial outcome may lead to serious safety concerns. In this study, a qPCR method was developed to accurately determine the titer of OCU400. **Method:** In order to optimize a robust qPCR method, various parameters such as primer design, conformation (linear *vs.* circular) of plasmid standard, diluents, method run conditions (temperature and time), different fluorophore chemistry (e.g., SYBR and TaqMan) based detection methods were evaluated during development stage. To that end, several primers were designed and evaluated targeting different regions of the OCU400, such as promoter, transgene, and polyA. The qPCR run method conditions were developed and optimized by examining the priming and amplifying capability of different primer sets to amplify 70-150 bp target regions. First, the amplification efficiency of 14 different primer sets targeting different regions of OCU400 was determined using optimized SYBR method. Thereafter, the optimized SYBR method conditions were implemented to TaqMan based qPCR where primer and probe concentrations were further improved to achieve accurate and reproducible results. Further, the DNA extraction methods to determine accurate and precise titer of OCU400 were developed and optimized. **Results:** Through systematic investigations, it was learnt that annealing temperature below 5 degree than melting temperature (Tm) of primer pair, is optimal to achieve consistent PCR readouts. For OCU400, a longer denaturation time (~15 sec) was needed to achieve reproducible results, probably due to its high GC content. Primer concentration from 100 to 900 nM range was evaluated and it was observed that 200-500 nM concentration range resulted in optimal amplification. In addition, linearized plasmid exhibited slightly higher copy number consistently compared to circular plasmid. Three primer sets targeting 3 different regions of OCU400 were selected based on their excellent sensitivity, specificity and reproducibility in qPCR reaction. These primer sets exhibited high dynamic range

from 10^8 to 10^2 copies per reaction with amplification efficiency of ~ 95-105% and linear correlation (R^2) of >0.98. In addition, limit of detection (LOD) of 1 copy/reaction and limit of quantification (LOQ) of 100 copies per reaction was achieved in the optimized condition. A DNA extraction method consisting pluronic PF-68 surfactant during DNaseI and proteinase K treatment conditions yielded consistent and accurate measurement of the vg titer for OCU400. **Conclusions:** A qPCR method was developed and optimized for quantification of OCU400 drug product. Optimized method showed reproducible and accurate quantification of OCU400 in the dynamic range from 10^8 to 10^2 copies/reaction with excellent performance.

853. Pilot-Scale Production, Purification, and Concentration of GMP Lentiviral Vectors Using Single-Use, Disposable Supplies

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Lentiviral (LV) vector-mediated gene delivery has shown clinical promise in the gene and cell therapy field. LVs offer a high rate of transduction into many cell types, the ability to transduce non-dividing cells, stable gene integration, and a reduced risk of insertional mutagenesis when compared to gamma-retroviruses. However, the production and purification of high-quality LVs for clinical trials remains challenging. The MCW/BCW GMP Vector Production Facility's mandate is to produce high-titer, clinical-grade LV for use in early-stage trials at the Milwaukee Regional Medical Center (MRMC). Herein we describe a method to scale-up production of high-titer LV that are capable of transducing multiple cell types under GMP conditions. We package our LV using HYPER technology, a commercially available, fully closed and disposable system composed of multiple layers of a gas-permeable, tissue culture-treated, growth surface. Following packaging, we purify LV by post-harvest clarification and Mustang Q anion-exchange membrane chromatography, resulting in a 1000-fold volume reduction with excellent viral recovery (60-70% of starting input LV). The chromatography eluate is subsequently diluted and concentrated using tangential flow filtration (TFF) and treated with Benzonase endonuclease (50 U/ml) at 37°C. TFF is then repeated to further concentrate the LV down to 15-20 ml prior to final product fill and finish. To evaluate compliance with current FDA guidance for clinical application, we analyzed samples from our production and purification process to assess LV purity and titer. We report functional titers between 2-6 x 10^7 TU/ml for a clinically relevant vector. Our purification method results in minimal endotoxin detected (<100 EU/ml). Furthermore, post purification residual plasmid DNA, host-cell DNA, and host-cell protein were reduced >98%, >99%, and >98%, respectively. The HYPER LV packaging strategy, Mustang Q chromatography, and TFF purification regimen described here is a practical method for LV scale-up to early-stage clinical trial volumes using affordable disposable laboratory supplies.

854. Optimization and Scale-up of Treg Expansion from CD4+ Cells in G-Rex6M plates

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Regulatory T Cells (Tregs) are a small subset of $CD4^+$ cells and play a crucial role in treatment of autoimmunity. Thus, research or patient treatment with these cells requires scalable and efficient expansion methods. Current products on the market use plate bound antibody or antibody-coated magnetic beads for expansion. These methods pose challenges regarding scalability and the removal of magnetic beads before downstream applications, respectively. Cloudz™ Human Treg Expansion Kit, utilizing CD3/CD28 antibody-conjugated dissolvable hydrogel microspheres (Cloudz Treg CD3/CD28), addresses these challenges by simplifying the scale and collection of cells for downstream applications. Herein, we have demonstrated a working dose of Cloudz Treg CD3/CD28 that produces similar growth and FoxP3 expression levels as market competitors. Furthermore, we show the scalability of Treg expansion by using Cloudz Treg CD3/CD28 in the G-Rex6M platform. A working dose of Cloudz Treg CD3/CD28 (from Cloudz Treg Expansion Kit) was determined by titrating the activation and expansion capabilities in culture. Human $CD4^+$ cells were seeded (Day 0) at 0.5x10^6 cells/well in 24 well plates (n = 3) in Excellerate Human T Cell Expansion Media supplemented with 10% FBS and 20 ng/mL Recombinant Human (rh)IL-2. Cloudz Treg CD3/CD28 was added to wells at several different doses to determine the best working dose. Flow cytometry was used to study the viability, growth & phenotypes - $CD4^+/CD25^+/CD127^-$ and $CD4^+/CD25^+/FoxP3^+$. We then used the working dose to activate and expand Tregs from human $CD4^+$ T cells in G-Rex6M well plates. Human $CD4^+$ T cells from 2 different donors were seeded (Day 0) at 5x10^6 cells/well in G-Rex6M well plates in Excellerate T Cell Expansion Media supplemented with 20 ng/mL rhIL-2. Cloudz Treg CD3/CD28 was added at 750 µL per 5x10^6 cells. On Day 3 and Day 6, an additional 20 ng/mL rhIL-2 was added to each well. Following expansion, flow cytometry was used to assess viability and growth, along with expression levels of CD4, CD25, and FoxP3. Activation and expansion capabilities of the Cloudz Treg Expansion Kit was compared to the Dynabeads Human Treg Expander and Miltenyi Treg Expansion Kit. All doses of Cloudz Treg CD3/CD28 increased growth of Treg cells and FoxP3 levels after 9 days. 75 uL of Cloudz/well gives the best compromise between growth (Figure 1) and FoxP3 expression levels (data not shown) while also being comparable to current market controls. Using this working dose, we studied the capabilities of expanding Tregs from CD4+ cells in G-Rex6M well plates. All activation reagents stimulated expansion of the $CD4^+$ cells into $CD4^+CD25^+FoxP3^+$ cells. Cloudz Treg CD3/CD28 and Dynabeads produced similar growth (Total viable $CD4^+CD25^+FoxP3^+$ cells (x10^6): 39.0± 8.1 and 38.5 ± 2.9, respectively), while Miltenyi lagged (17.8 ± 3.1). All three reagents caused a comparable increase in the expression levels of FoxP3. Cloudz Treg CD3/CD28 (79.8% ± 6.5%) fell in between Dynabeads (73.4% ± 0.1%) and Miltenyi (87.2% ± 1.2%). This demonstrates the working dose of Cloudz can be used successfully within the G-Rex platform to expand Treg cells in serum-free media conditions. Together these results suggest that the Cloudz Treg Expansion Kit can be used to scale up the expansion of Treg cells for clinical scale manufacturing. The working dose of Cloudz Treg CD3/CD28 yields similar or better results than both market competitors. Additionally, the dissolvable aspect of this platform allows the use of Cloudz™ Treg CD3/CD28 to seamlessly integrate in a Treg expansion platform.

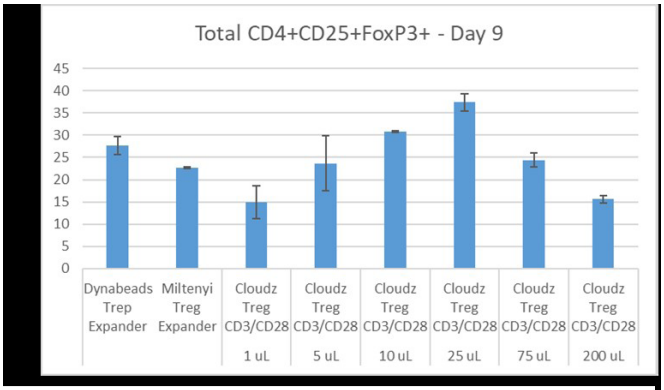

Figure 1: CD4+ cells were expanded with several volumes of Cloudz Treg CD3/CD28 particles. Other commercial expansion reagents were used as a control. Total Treg growth increasees as more Cloudz Treg CD3/CD28 is added, with maximum expansion observed using the 25 uL dose.

855. Abstract Withdrawn

856. Optimization Strategy and Process Economics of DNA Digestion Step in Gene Therapy

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Benzonase® is employed to reduce the levels of host cell nucleic acids and has seen an increased adaptation in the gene therapy industry. The use of this enzyme can in some cases significantly reduce the levels of DNA by more than 100,000-fold while also reducing viscosity and protecting downstream equipment from DNA fouling. The enzyme activity is strongly influenced by the matrix of the process intermediate and optimization of its use should be considered a mandatory and often crucial step in process development. This talk will shed light on general guidelines of the enzyme usage and design of experiments to maximize the value proposition of the unit operation. Examples of cost modelling insights using Benzonase® for DNA digestion in a typical gene therapy manufacturing process will also be discussed.

857. HPLC Detection of Empty and Full Capsids by Right-Angle Light Scatter in Complex Sample Matrices

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Bioreactor cell supernatants or post lysis materials entering the downstream purification of AAV vectors are generally heterogeneous mixtures of empty, full and misassembled AAV particles embedded in a complex mixture of host cell proteins, genomic DNA, chromatin complexes and other host cell-related components. Needless to say, assessing information about the empty and full AAV capsid ratio in these harvests is an intractable process that requires labor-intensive and time-consuming AAV purification that sometimes results in skewed empty-to-full capsid ratio measurements. Monolith-based analytical HPLC assays provide fast, accurate, and meaningful characterization of cell culture harvests and in-process samples. However, detection is traditionally limited to UV absorbance and fluorescence detectors. These detectors have major limitations with respect to their abilities to discriminate AAV capsids from various contaminant classes. An approach to overcome the limitations of UV and fluorescence detection of empty and full AAV capsids in complex sample matrices is presented in this poster. Chromatographic separation of empty and full AAV capsids, as well as partial separation of complex matrix components, was achieved on a CIMac™ AAV full/empty analytical column. Whereas the UV absorbance (proteins, nucleic acids, and small molecules) and the tryptophan fluorescence (proteins) render intricate chromatographic profiles containing numerous peaks, right-angle light scatter, characteristic of high molecular weight complexes, is exploited to enhance AAV capsid detection above the contribution of lower molecular weight components in the chromatographic profile. Therefore, the addition of a right-angle light-scatter detector allows rapid empty-to-full AAV capsid ratio estimation in complex matrices like vector harvests. Simultaneously, UV and fluorescence chromatographic profiling (fingerprinting) provides a valuable PAT tool for impurity characterization and enables efficient monitoring of downstream unit operations.

858. Scalable Single Use Solution for Chemical Lysis in Plasmid DNA Production

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Plasmid DNA (pDNA) extraction from microbial cells using a chemical lysis step is a relatively easy process when performed at the very small laboratory scale. However, at the much larger scale demanded by gene therapy developers it is a very technically challenging operation which if performed incorrectly can lead to shear damage of the pDNA or elevated levels of residual cDNA (chromosomal DNA) and batch failures. The visco-elastic properties of the chemically lysed cells do not fall under conventional scale up and sale down approaches for the mixing processes. Cobra's current approach for cell lysis is based on a proprietary stainless steel mixing vessel, operated at a set process scale suitable to deliver 1-5g of pDNA. This process was developed over 15 years ago and currently no other operational models exist to support smaller or larger scale batches. Moreover, conventional approaches in design and production of new vessels at different scales from glass or steel are inflexible, costly and time-consuming. In addition, regulatory pressure and customer requirement to reduce and potentially eliminate the risk of cross-contamination push the market towards single use technologies and closed-processes. This project focused on the application of 3D-printing and single use technology to design and deliver unconventional and fully customised single use lysis vessels at various scales which can be rapidly manufactured from robust and compliant materials using 3D-printing. The advantage of the approach is that any design can be quickly scaled up or down at the push of a button, enabling near off-the-shelf supply of previously established designs at a range of scales. Moreover, it is a perfect tool for evolving

the design to develop and improve mixing strategies and develop new platforms such as closed system manufacturing. The single-use vessel is easy to operate, can be adapted to GMP and can be automated. The possibility to horizontally scale up the production by a modular approach enables the feasibility studies at various scales reducing the cost of capital equipment and de-risking process scale-up. We have developed and assessed lysis vessels at three scales testing mixing performance, lysis efficiency and the levels of cDNA in the produced lysates. The process parameters such as stirrer speed, cell density and flow rates of the lysis buffers have tested.

859. Optimizing Suspension HEK293 Transfection Processes for AAV Productivity

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[1]Process Development, MassBiologics of the University of Massachusetts Medical School, Fall River, MA,[2]Apic Bio, Cambridge, MA

The development of a robust and scalable production process is critical as gene therapies transition from pre-clinical to later phase clinical and commercial production. HEK293 cells grown in suspension are frequently used for the production of Adeno-Associated Virus vectors (AAV) and allow for rapid vector, serotype or process changes to be implemented from a single Master Cell Bank. To efficiently manufacture and scale-up AAV production processes using HEK293 suspension cells, process parameters affecting productivity must be better understood. The productivity of transfection processes can be affected by multiple factors. In this study, a two-plasmid AAV system and PEI-mediated transfection in shake flasks were used to investigate the effect of cell density at transfection, amount of DNA per cell, PEI:DNA ratio and post-transfection feed on productivity. Initial optimization consisted of a design of experiment (DOE) study with several conditions comprised of cell densities, DNA amounts, and PEI:DNA ratios, to identify factors determining optimal productivity. This was followed by evaluations of different complexation media and feeds. While there was no difference between different complexation media, we identified an optimal cell density, DNA:PEI ratio and feed condition that improved productivity.

860. Automated Manufacturing of Human T Cells with Non-Viral Gene Delivery: Cocoon® System Integrated with Large Volume (LV) Nucleofector ™

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With the increasing demand for automation and non-viral method for gene delivery in the cell therapy field, Lonza's enabling innovative technologies Cocoon® system (automated manufacturing platform) and LV Nucleofector™ provide solutions to reduce cost, and maximize process efficiency and quality. Cocoon® automated manufacturing platform is an end-to-end system that addresses the key process challenges in autologous cell therapies including cell isolation, activation, transduction, expansion, cell washing and collection with real-time biofeedback in a single-use cassette with a small footprint. The 4D-Nucleofector™ LV unit enables closed and scalable transfection capabilities for a wide range of cell types and applications, delivering proven and published transfection with the highest quality control and cell viability. Cocoon® with LV Nucleofector™ integration will address the market demand and trend for automating the manufacturing process and moving away from retro or lenti-based transduction to non-viral method to reduce cost of goods (COGs). The objective of this study is to connect the Cocoon® system and LV Nucleofector™ in a closed system and automate transfection as a unit of operation. The addition of LV Nucleofector™ will expand capabilities of the Cocoon® system to address market need for non-viral gene delivery technology. A series of experiments were designed by using leukopak derived cryopreserved or fresh human peripheral blood mononuclear cells (PBMCs) as our starting material. Briefly, 900 million cells were transfected with pmaxGFP™ vector using LV Nucleofector™ in a maximum volume of 20 ml. Post nucleofection, cells were then transferred through a closed system connection via designed fluid paths to Cocoon® for 7 or 10 days' expansion. The cells were activated by TransAct beads and expanded in X-VIVO 15 media supplemented with 5% human AB serum and 20 IU/ml of IL-2. Cell washing and harvesting were performed in the Cocoon®. Cells were analyzed for GFP expression, CD3, CD4 and CD8 via flow cytometry and cell counts were performed with NC-200. The nucleofected cells were successfully transferred to Cocoon® cassette in a closed tubing system. 50% to 70% transfection efficiencies were observed for both cryopreserved and freshly isolated PBMCs on day 1 post transfection. The GFP expression in T cells peaked on day 1 with gradual loss of GFP protein over the culture days due to the transient nature of the vector. However, the GFP expression and trend of gradual loss in Cocoon® system is similar to that of manual controls not in Cocoon® system. Lower cell loss post-transfection was observed with freshly isolated PBMCs compared to cryopreserved PBMCs. There was a lag phase till day 4 before cell expansion was observed in both groups. Furthermore, we observed over 6-fold expansion of cells between harvest and day 1, and achieved over 90% $CD3^+$ T cells by the end of expansion process. The ratio of CD4 to CD8 in expanded T cells varied from donor to donor. In summary, we demonstrated the feasibility of connecting LV Nucleofector™ to Cocoon® system to automate non-viral end-to-end cell and gene therapy processes. Our next step is to test a CAR coding plasmid to demonstrate clinical relevance. This automated and connected Nucleofector™ and Cocoon® system provides an alternative non-viral gene delivery system without the need of virus supply chain to address market needs, allowing scalability, high yield, reduction of manufacturing cost, minimizing operator error, and better process control to generate high quality CAR-T cells.

861. Development of Improved Transient and Stable Platforms for the Scalable Clinical Manufacture of Lentiviral Vectors

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Engineered viral vectors are effective tools for the development of cell and gene therapies targeting a range of diseases. Current vector manufacturing approaches primarily use fully transient, adherent processes, bringing associated issues of robustness, scalability and high cost-of-goods. To address these issues, we set out to improve the lentiviral (LV) plasmid systems and develop enhanced cGMP compliant, suspension, HEK293 cell lines for both transient and stable manufacture. Via the genetic optimisation of the lentiviral packaging plasmids, we demonstrate 20-100% increase in infectious titres compared to competitor products. Subsequent high-throughput screening of HEK293 clonal cell lines for transient manufacture also identified a clone (SP7D5) showing improvement in yield compared to the parental line. Combined with our modified lentiviral packaging plasmids, the clonal cell line consistently achieves infectious titres of 1-2E8 TU/mL in our preferred manufacturing process, optimised via the study of transfection conditions and feeding strategies in both shake flask format and bioreactor systems. In order to reduce the requirements for large plasmid quantities in the manufacturing process, we also developed doxycycline inducible HEK293 LV packaging and producer cell lines, using a fully traceable, animal component-free, process. The LV packaging cell lines require a single plasmid transfection and achieve yields of 1E7 TU/mL. LV producer cell lines, which have been developed through subsequent modification of the packaging cell line, require no plasmid transfection, thereby enabling higher cell culture densities and therefore higher LV yields in a transfection free process. Combined, these systems provide both transient and stable solutions to improve the scalability and cost effectiveness of manufacturing lentiviral vectors for clinical applications.

862. Update on the SecNuc™ System: Optimisation of Secreted Nuclease Helper Cells to Eliminate Residual DNA During Lentiviral Vector Production

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Process development for viral vector-based products is complex, time-consuming and resource-heavy. Optimisation of any given process towards GMP manufacturing varies between vector platforms, and can often vary within the platform depending on the vector product. This variability can also lead to different levels of residuals through the downstream process and potentially in the final product. Elevated residual DNA levels may attenuate efficiency of capture/purification, and significant levels within the final product are not desirable, especially for direct *in vivo* administration. The standard approach to minimize DNA impurities is the use of GMP-grade recombinant nucleases (e.g. Benzonase®, SAN-HQ) to treat crude vector harvest material. However, salt/pH conditions at vector harvest may not be optimal for these commercial nucleases, and increased input may not be economical. To address this, a second nuclease treatment during downstream processing of lentiviral vectors (LVs) is typically required. We previously developed a highly efficient alternative approach that uses modified secreted nucleases based on endonuclease I from *vibrio cholera*, co-produced with AAVs and LVs. This nuclease 'vcEndA-1glc' performs optimally at physiological salt and near-neutral pH. Here we report advances in use of secreted nuclease Helper cells generated in parallel to suspension, serum-free LV production cultures. We present data from optimisation experiments identifying the proportion/timing of Helper cells added to the main LV production culture to achieve extremely efficient clearance of residual DNA, without impacting on the titre of LVs. Finally, we show that the improved feed quality imparted by the SecNuc™ approach can lead to increased recoveries during downstream purification.

863. Regulatory-CMC Considerations for Successful Gene/Cell Therapy Approval

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In the recent years, a significant increase is seen in the discovery, clinical development and expedited regulatory approval of Cell and Gene Therapy (CGT) products due to their enormous potential to treat life threatening diseases. Gene therapy modifies or manipulates the expression of a gene(s) for therapeutic use and offers the potential to address significant unmet clinical needs. Novel cellular therapy products include cellular immunotherapies, cancer vaccines, and other types of both autologous and allogeneic cells for certain therapeutic indications, including hematopoietic stem cells, adult and embryonic stem cells. However, these novel therapies present unique challenges due to unknown safety profiles, complex manufacturing technologies and the use of cutting-edge testing methodologies. With respect to CMC issues, these novel CGT products present the following challenges during the early development stages that need to be carefully managed to ensure quality (identity, strength, purity and potency) of the product and patient safety: 1. Sourcing and safety/quality concerns of manufacturing components. 2. Process optimization challenges leading to a scalable process which is robust and consistent. 3. Small scale manufacturing and the associated challenges with early analytical method qualification and stability studies. 4. Lack of clear understanding of critical manufacturing process steps of a continuous process. 5. Lack of or limited early understanding of CQA's and CPPs of manufacturing process which can impact assessments of process change controls. Judicious application of science, risk-based approaches and phase appropriate control strategies are appropriate to manage the CMC challenges and ensure quality and assure patient safety of CGT products. FDA had suggested that sponsors discuss their programs with them very early in the form of an INTERACT (Initial Targeted Engagement for Regulatory Advice on CBER product) meeting. EMA has a similar program of Advanced Therapy Medicinal Products (ATMPs). Using case studies, Syner-G's presentation will illustrate the science and risk-based approaches and phase appropriate control strategies to manage the early CMC challenges associated with the initiation of clinical studies using CGT products.

864. Scalable Biotransport-Mediated Platform (Proxim) Synergizes with Transduction-Enhancing Strategies to Maximize Gene Transfer

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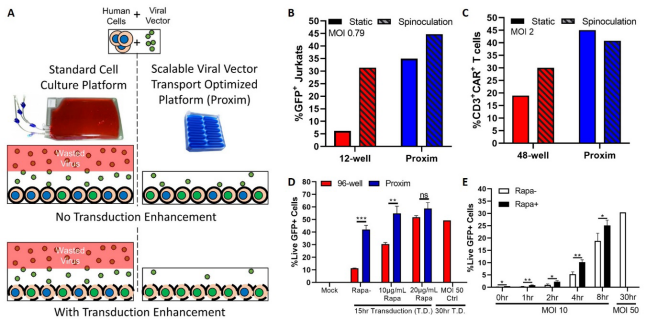
Years after the approvals of the first gene-modified autologous cell therapies, manufacturing remains a major bottleneck impeding further widespread clinical translation and commercialization. Methods and small molecules that make cells more permissive to viral infection have been identified, though high vector utilization and long transduction times are still needed for therapeutic gene transfer levels. We previously demonstrated that Proxim, our scalable transduction platform designed to optimize the biotransport of lentiviral vector (LV) to target cells, reduces LV usage by 4-5 fold, shortens transduction times by at least 50%, and accommodates $\geq 1x10^9$ cells of clinical interest. This platform was designed for transduction, and is easily adaptable in any process without requiring external equipment. In this study, we investigate the combination of transduction-enhancing methods with Proxim to maximize transduction while shortening transduction times and reducing LV usage in T cells and hematopoietic stem cells (HSCs). Jurkats and purified human T cells were spinoculated in well plates and Proxim devices at the specified MOI. T cells were incubated for 5 hrs more after spinoculation. Addition of Rapamycin (Rapa) to mobilized human CD34$^+$ HSCs were compared in 96-well plates and Proxim devices at the specified MOI and transduction times. All cells were transduced with a GFP-LV or GFP/CD19 CAR for T cells. Transduction efficiency was assessed by flow cytometry after culturing cells for at least 72 hrs. Primary human cells have innate restriction factors that inhibit LV infection. Transduction enhancement methods in cell culture platforms still use exorbitant amounts of vector and long transduction. Proxim significantly improves LV biotransport to cells and, combined with transduction-enhancing methods, maximizes gene transfer **(A)**. Non-additive physical methods of transduction enhancement, such as spinoculation, synergize with Proxim in both Jurkats **(B)** and primary human T cells **(C)** compared to well plates. Additive small molecule enhancers, such as Rapa, also synergize with Proxim to increase transduction in primary human CD34$^+$ HSCs **(D)**. Although Rapa synergizes with Proxim to increase gene transfer, a kinetic effect was observed. Thus, other enhancers that are added to cells prior to transduction may be more effective for shorter transduction times **(E)**. Proxim is a simple, but elegant platform that synergizes with transduction-enhancing methods to overcome transport and biological barriers that commonly inhibit gene transfer. The reduction in viral vector and transduction times enabled by this approach significantly impacts vein-to-vein time and may improve cell quality in addition to simplifying supply chain logistics and reducing costs of goods for these complex and expensive therapies. Ongoing work will include characterization of other combination strategies such as pretreatment of cells with Cyclosporine H or UM171.



(A) Schematic of Proxim synergy with enhancers to overcome transport and biological barriers with minimal LV. Well plate and Proxim spinoculation of **(B)** Jurkats and **(C)** CAR T cell manufacturing. Comparison of Rapa treatment on CD34$^+$ transduction **(D)** and Proxim + Rapa kinetics **(E)**.

865. Investigation of Cell Line and Baculovirus Inocula Attributes for AAV Recombinant Vector Production in the ambr250 Automated Mini-Bioreactor System

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Adeno-associated virus (AAV) gene therapy vectors hold great promise for the treatment of a wide variety of diseases. In recent years, several AAV based biologics have gained regulatory approval, with many more potential approvals on the horizon. Current AAV-based drugs on the market include LUXTURNA for RPE65-deficiency mediated retinal dystrophy, and ZOLGENSMA, for spinal muscular atrophy in patients less than 2 years old. Despite these approvals, widespread use of AAV gene therapy is limited due to the low yield of current manufacturing techniques. Thus, optimized strategies for AAV manufacture would be highly desirable. We sought to investigate the ambr250 automated mini-bioreactor system (Sartorius) as a scale down model for AAV production using the baculovirus/insect cell binary system. Different parameters including cell line, MOI and type of inoculum were investigated to determine optimal conditions for AAV production. For infection of insect cells, free baculovirus inoculum was compared to Baculovirus Infected Insect Cell (BIICs) inoculum. The latter inoculum was shown to possess superior stability over long storage durations. Overall, free baculovirus and BIICs inoculum performed similarly in terms of product quality attributes such as qPCR titer but BIICs inoculum yielded AAV vectors with ~2-fold improved infectivity compared to free baculovirus inoculum. To our knowledge, this is the first report of the parallel optimization of baculovirus/insect cell

recombinant AAV production utilizing the ambr250 mini-bioreactor system. Process optimizations utilizing automated bioreactors for greater throughput may pave the way for further improvements in AAV manufacturing.

866. Empty-Full Capsid Separation of AAVS Using an Optimized Anion Exchange Chromatography Method

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Adeno-associated virus (AAV) vector production presents novel analytical challenges compared to traditional biopharmaceutical production. One area requiring significant workflow improvement is the separation of non-genome containing empty AAV capsids from the therapeutic genome containing capsids. Currently, ultracentrifugation provides a means for the elimination of empty capsids. However, there is little room for increasing throughput in this time consuming and tedious method. Luckily, biology has given us a handle to work with; one observable difference between empty and full capsids is the isoelectric point (pI) imparted by the negatively charged nucleic acids found in the genome-containing capsids. Harnessing this physiological difference, anion exchange chromatography stands as a viable method for the efficient and robust separation of empty and full AAV capsids. Due to the relatively small difference in pI, as well as the presence of partially filled capsids, optimization is required. Herein, we present an efficient empty-full separation using an optimized anion exchange chromatography method.

867. Osmolality Provides Valuable Control in Advanced Therapy Processing

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In gene and cell therapy, manufacturing capacity, production yields, and supply chain logistics continue to be an area of focus. The increased need for robust process control strategies requires a broad and versatile toolset. Osmolality testing provides the concentration of any liquid solution and has several potential applications within cell and gene therapy development and manufacturing. Here we explore cell expansion, transduction efficiency, cryopreservation, and potency/stability. Osmolality testing becomes critical during cell expansion and cultivation, when media conditions and concentration must be optimized for cell growth and yield. We will provide examples of osmolality as an indicator of out-of-range media components. Furthermore, to preserve the integrity and quality of cells, it is standard to freeze them at breakpoints throughout processing. Introducing cryoprotectants and freezing cells has a considerable impact on their osmotic pressure, and repeating this process becomes increasingly complex. Testing the osmolality of the cryopreserves before freezing ensures that the composition and compatibility are correct and provides confidence in the upcoming preservation steps. We measured the osmolality of various homemade and commercially available cryopreservatives. Despite the fact that freezing point technology was used, the tests showed repeatability and consistent performance across the samples, again serving as an indicator of deviations. These data, along with previous outside works, support the role of osmolality testing in cell expansion and further processing. Implementing this process control in a cell therapy workflow will support improved product quality and yield.

868. Integrative and Non-Integrative Lentiviral Production Platform Allowing a Continuum from Discovery to Therapy for Immune Cell Engineering

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<Safe and efficient cancer therapies using adoptive transfer of engineered immune cells are very challenging but promising approaches. Various immune cell types can be engineered to express CARs that drive efficient and specific killing of tumor cells. However, such results are only obtained when good CAR expression is observed at the surface of the effector immune cells. It's possible to achieve this through the use of delivery tools, which allow highly efficient gene transfer while maintaining transduced cell viability and phenotype. Here we show that lentiviral vector composition, in terms of titer, specific activity, and most importantly purity, is essential to ensuring efficient transduction of human T cells. Furthermore, we display that transduced T cells exhibit their original phenotype, maintaining both viability and specific markers expression. Thus, this data demonstrates that lentiviral vector composition is essential to guaranteeing T cell functionality. Moreover, in the case of allogenic approaches using T cells, endogenous TCR expression must be suppressed. This can be achieved using gene editing technologies, ensuring efficient knock-out of targeted genes. Since gene editing systems require low and short-term expression in order to avoid off-target effects, RNA delivery is favored over DNA delivery. RNA delivery presents major advantages compared to DNA delivery, being that it is completely safe and devoid of any recombination events in the host genome. It is actually the most versatile, flexible, and safe mean for human therapy. Here, we present an innovative RNA delivery vector, called LentiFlash, based on a chimeric lentiviral technology that allows RNA delivery into target cells without integrating into the host genome. In comparison with current existing systems, this new vector is an ideal example of break-through technology. This lentiviral system efficiently delivers, in a transient manner, non-viral coding and/or non-coding RNA into any cell type. Furthermore, since the LentiFlash system allows for the packaging of multiple RNA species, it is capable of delivering any gene editing system, including CRISPR/Cas9, with one or several sgRNAs and Cas9 simultaneously in one single vector batch. Here we present gene-editing data on hT cells using LentiFLash, illustrating the efficiency of the LentiFlash technology in the editing of various target genes. All together, these major technological leaps allow for the precise control of the expression of specific receptors in immune cells, and can be applied to various applications, including gene-addition and gene-editing strategies. This technology, in combination with Flash Therapeutics' high quality lentiviral vector production platform, is paving the way for the design of innovative tools and the development of cell-based cancer therapies and responds to

accelerating therapeutic demands. Finally, our lentiviral production platform is expanding its development by providing cGMP compliant lentiviral vector production for clinical applications, incorporating an incremental quality control plan in compliance with regulatory requirements. Flash Therapeutics thus proposes a global offer for the production of integrative and non-integrative lentiviral vectors with cutting-edge vector design, state-of-the-art in-house expertise in vector manufacturing and vector/cell analytics.>

869. Single-Use Platform for Scalable Purification of a VSV-G Lentiviral Vector

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Recent advances in viral vector based cell and gene therapies have opened the door to bringing life-saving, curative treatments to many patients and families. Many of these treatments are based on recombinant lentivirus (LV) vectors for gene transfer. Large scale manufacturing of these vectors requires scalable upstream production as well as efficient downstream purification. Purification of these vectors is complicated by their large size (120 nm) and fragile lipid envelope. Efficient purification of these vectors can be achieved using scalable, single-use technologies. The purification strategy achieves harvest clarification using bioburden reduction filters (0.45 µm), vector purification by membrane based, anion-exchange chromatography using Mustang® Q membrane, concentration and diafiltration by flat-sheet tangential-flow filtration (TFF), and final sterile filtration (0.2 µm sterilizing grade). Here we demonstrate a single-use platform for purification of a VSV-G lentiviral vector using all downstream materials from Pall Biotech. Preliminary results indicate each downstream unit operation can be optimized for >80% infective particle recovery as determined by Lentivirus Transduction Assay (TU).

870. Development of a Perfusion Bioreactor Platform for High-Density Suspension Hek293 Cells

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The emergence of gene therapy for genetic diseases has rapidly evolved with the approval of several gene therapy products. New research and clinical developments suggest that ex-vivo transfection may deliver corrected genes more efficiently and safely than historical direct transfection systems. Cell lines such as HEK293 and HT1080 produce high quality and reproducible viral vectors for this purpose. These cell lines, however, are often adherent cultures that require dated and labor-intensive techniques to produce these vectors. In contrast to gene therapy, monoclonal antibody (mAb) production utilizes suspension cells such as CHO (Chinese hamster ovary) to efficiently produce well-characterized biologics. Suspension cell lines for gene therapy are being evaluated as an alternative to adherent cultures to improve infective titers and produce high quality viruses. Additionally, adapting high density cell culture production from mAb technology will likely increase output with the potential of treating more patients with less equipment, personnel, time, material waste, and cost. Using tangential flow perfusion (alternating and single pass), we have successfully and reliably grown suspension HEK293 cell cultures up to 80 x 10^6 viable cells/mL (vc/mL). This platform sustains high density bioreactor cultures that do not become nutrient depleted during transfection and can continually harvest to ensure high quality viral vector production. This next generation gene therapy platform has potential to transform gene therapy manufacturing into a cost-effective, low waste, and superior technology.

871. Comparisons of Recombinant Adeno-Associated Viral Vectors Manufactured in HEK293T Cells Versus Sf9 Cells

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The major drawback of the Baculovirus/Sf9 system for recombinant adeno-associated viral (rAAV) for large-scale manufacturing is that recombinant baculovirus(rBEV) is not stable when passaging several generations and with lower biological potencies. Furthermore, the ratio of full-to-empty particles is lower compared with HEK293T cells using three-plasmid transfection system. Here, we describe a new Baculovirus/Sf9 system named Bac4.0 to produce rAAV with stable cell capacity and rBEV titers. We tested different passages' titer of rBEV with Q-PCR and TCID50. In the head-to-head comparision of rAAV9-EGFP manufactured in HEK293T cells suspension system to Baculovirus/Sf9 system, the yield of Baculovirus/Sf9 system was higher by Q-PCR, the purity of two systems were comparable without obvious impurity proteins and VP1:VP2:VP3 ratio was nearly 1:1:10. Higher infectious titer at the same genomic titers, and higher ratio of full-to-empty particles were observed in the Baculovirus/Sf9 system. rAAV9-EGFP from both systems were further tested in C57BL6 by Stereotactic injection of brain. Thus, the described Bac4.0 system of rAAV vectors with superior infectivity and higher ratio of full-to-empty particles provides a scalable platform for GMP-grade vector production.

872. Development of Large-Scale Downstream Processing for Lentiviral Vectors

Anniina J. Valkama[1,2,3], Eevi M. Lipponen[1,2], Igor Oruetxebarria[1,2], Hanna M. Leinonen[1,2], Piia Käyhty[1,3], Heidi Hynynen[1,2], Vesa Turkki[1,2], Joonas Malinen[1,2], Tuukka Miinalainen[4], Tommi Heikura[3], Nigel R. Parker[3], Seppo Yla-Herttuala[3], Hanna P. Lesch[1,2]

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Lentiviral vectors (LVs) are an increasingly popular alternative in gene therapy, and clinical applications are advancing towards commercialization. However, large-scale manufacturing of the

LVs has remained challenging. In this study, we developed and optimized a downstream purification and concentration protocol for a 178L LV harvest produced with the iCELLis 500 bioreactor system. Chromatographic purification has been one of the main bottlenecks in LV downstream, and thus we concentrated mostly on this step. We tested different types of anion exchange columns as well as several other conditions in order to obtain maximum recoveries. Strong AEX membrane-based adsorbers yielded the best results in terms of functional particle recovery and scalability, however, binding efficacy seemed to depend on the column design and preceding purification level. Elution with salt gradient revealed only partial separation of LV particles and cell-derived DNA impurities. Therefore, step-wise elution with optimized salt concentration resulted in sufficient recovery of the LV and removal of the DNA impurities. We performed two large-scale (166L and 178L harvests) purification runs, of which the first was partly divided into smaller development experiments, and the second focused on scaling up the whole downstream with the optimized parameters. Our final process includes also clarification of harvest material with depth-filtration (approx. 0.6-1.1 μm) and concentration and buffer exchange with tangential flow filtration (100 kDa) before and after the chromatographic purification step. In addition, we screened different buffers for the best cryopreservation of the final product, revealing that sucrose and magnesium chloride assist in maintaining the stability of the final product. From the optimized large-scale downstream process, we obtained a total of 1.12×10^{12} transducing units (1.97×10^{9} TU/ml, C8166 cells) free of replication competent LV. Impurity removal from harvest to final product was notable. The future steps in development of this protocol towards clinical-grade manufacturing include final sterile filtration optimization in large-scale, and specific assessment of host-cell and process-related impurities in the final product.

873. The iCELLis® Nano Bioreactor Provides a Reliable Method to Produce Animal Vaccines with High Titer

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Pall Corportation, Westborough, MA

In the past five years, Pall's iCELLis bioreactors have emerged as the leading bioreactor technology for clinical manufacturing of viral vectors. In the Americas, most of this technology adoption has occurred in the United States and within the human gene therapy niche of biopharmaceuticals. However, the advantages of the iCELLis bioreactor - scalability, fixed-bed cell retention, closed-processing, advanced process control, single-use ease-of-implementation, and footprint reduction - that make the iCELLis bioreactor well suited for viral vector production also make it well suited for production of live-virus vaccines. In this poster, Pall has partnered with a Brazilian animal health company to demonstrate successful viral vaccine production in the iCELLis bioreactor for rabies virus, distemper virus, and parvovirus. For each of these case studies, the titer observed experimentally in the iCELLis Nano bioreactor was either equivalent to or higher than the legacy flatware process, with little to no process development effort required.

874. Considerations and Approach to Selecting a Suitable AAV Manufacturing Platform

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With the explosion in number of AAV based gene therapy getting into clinical, manufacturing has been recognized as a bottleneck to successful clinical development and regulatory approvals. Companies are also racing to be the first-in-clinic and move much more quickly to commercial in gene therapy compared to traditional biologics. It becomes critical to balance speed-to-clinic and market while mitigating critical risks impacting scale-up and operation during GMP manufacturing. Decisions made in early process development can have long lasting impact - both good and bad in meeting manufacturing and supply requirements. We present case study in our evaluation of adherent vs suspension cell culture using transient transfection systems and discuss the advantages and limitations of each of the approaches. Critical starting materials such as plasmids, raw materials used in manufacturing, scalability, manufacturing yield and costs were some of the factors considered in the decision making process. Actual small scale data across both adherent and suspension culture was generated and analyzed for product quality and yield in the process.

875. Risk Evaluation of Residual Recombinant Lentiviral Vector Capsid Protein p24

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Recombinant lentiviral vectors (RLVs) are often used in the production of gene-modified cell drug products, such as CAR T cell and hematopoietic stem cell therapies. Residual components of RLVs may be present in the final drug product. The purpose of this assessment is to identify, evaluate, and summarize the potential toxicological hazards associated with the RLV capsid core protein, p24, that may be present in a final drug product and derive a permissible exposure limit for p24. The capsid core of the gammaretrovirus family, which includes HIV and lentivirus, is derived from cleavage of the gag protein into a 24-kDa protein, also called p24. The capsid core p24 appears to be important for viral transduction and delivery of the genetic components. Public databases were searched for relevant toxicology data for p24. One *in vitro* study, four *in vivo* nonclinical studies, and two clinical studies involving p24 were identified. These studies were conducted to evaluate p24 as a peptide vaccine candidate against HIV. The nonclinical data suggest that p24 has limited cellular bioavailability and is not toxic following repeated intradermal or intraperitoneal injections at microgram quantities in the presence of immunogenic agents. Similar findings were reported in a Phase 2 clinical trial where p24 was administered to HIV+ patients as 10 intradermal injections over 26 weeks with GM-CSF. Only mild local reactions were reported. On intradermal provocation with p24, local dermal delayed-type hypersensitivity (DTH) reactions were observed in the majority of subjects by the end of the study. In the setting of gene-modified cell therapy, DTH reactions would not be expected in patients without prior exposure to p24. From these data, a permissible exposure limit for residual p24 per dose of drug product is proposed.

876. Generation and Optimization of Insect Based Stable AAV Production Cell-Line

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Recently there has been a rapid increase in the number of gene therapy trials and products based on Adeno-associated virus (AAV)-derived vectors. This rapid increase poses a challenge for industry to cope with the high vector demands. Therefore, AAV-production systems that can produce large amounts of high quality AAV in a cost-effective and robust manner are needed. The most common production method for AAV vectors relies on transient transfection of mammalian host cells, however, this cell platform is not suitable for the development of a scalable and robust process. The use of baculovirus expression vectors (BEV) for AAV production represents a well-established alternative for the mammalian production system and has been approved to bring the first AAV gene therapy product, Glybera, to the market. Although the BEV system can provide several advantages over the transient transfection method, it also conveys some limitations. The holy grail for an AAV production platform, from a process point of view, would be a simple and scalable platform supporting a common process routinely used in the production of other biologicals, such as vaccines. The first effort to answer this challenge was the OneBac platform creation, which nevertheless still has its own issues. By applying molecular baculovirology and insect cell engineering approaches we have developed a flexible and scalable expression system that can be further translated into a simple insect cell platform to produce AAV. This proprietary technology would potentially create a system capable to yield high titer and quality batches of different AAV serotypes.

877. Rapid Lentiviral Vector Titration Using a Novel Droplet Digital PCR Assay

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Precise determination of functional vector titers is of central importance in the manufacture and quality control of lentiviral gene therapies. Due to the complex nature of the lentiviral life cycle, particle-based measurements of vector titers (p24 levels, vRNA levels) are often inaccurate and do not meaningfully represent the number of successful transduction events. In a laboratory setting, fluorescent proteins are often included within the vector cassette as a means to measure functional vector titers, but clinical lentiviral therapies do not typically include a reporter gene and generally cannot be titered by conventional flow cytometry methods. We have developed a droplet digital PCR (ddPCR) method to rapidly and precisely quantify reverse transcription (RT) products. Unlike particle-based metrics, formation of RT products require progression through several steps of the viral life cycle and more closely represent the number of functional virions. The high dynamic range, high sensitivity, and direct quantification make ddPCR well suited for lentiviral titration assays. By simultaneously quantifying associated DNA contaminants and non-productive RT products (carry-over plasmid DNA, 2 LTR vector circles) we are capable of detecting RT products destined for stable integration. Further, by targeting a commonly-used sequence in the lentiviral packaging signal, this assay can be used to titrate a wide variety of lentiviral vectors in a transgene-independent manner.

878. New Insights on the Optimization of the T Cells Manufacturing Process

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Over the last decades, Advanced Therapy Medicinal Products (ATMPs) have led a real revolution in medicine and healthcare, offering cures for many unmet medical needs. The translation of the research achievements from academia into clinical products requires several steps of development addressing "manufacturability" and "sustainability" of the product, balancing: critical quality attributes, cost of goods, needle-to-needle logistic and scale. As a **Biotech Manufacturing Organization** (**BMO**), we have been exposed to multiple ideas in need of process development and a multi- parameter approach was often required. In order to optimize the end-to-end process, including looking at workflow simplification and scalability of production, we have been evaluating different parameters and we observed that culture media and manufacturing platforms are often the most critical ones. Here we present the results of testing different media formulation to develop a robust, efficient and GMP compliant process for T cells expansion *ex vivo,* paving the way towards the improvement of adoptive T cell therapies manufacturing and overcoming the challenges that are associated with their industrial production.

879. Optimization of ddPCR Assays: Minimizing Capsid Loss During Sample Preparation for ddPCR Titering and Cell-Based Assays

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Recombinant Adeno Associated Virus (rAAV) vectors are promising vehicles for the delivery of therapeutic DNA into humans. Measuring the biophysical and biological attributes of rAAV such as the genomic titer, infectivity, and potency are integral to the successful manufacturing and application of rAAV as gene therapy products. Accurate genome titering is essential for the design of *in vitro* potency assays as well as for dosing with *in vivo* studies. Droplet digital PCR (ddPCR) is an approach to genome titering that removes the dependence of a DNA standard curve and reduces assay variability. During sample preparation, the rAAV capsid may bind to the surface of materials thereby influencing the genome titer. This can manifest with inaccurate multiplicities of infections (MOIs) and doses used for animals during pre-clinical studies. Here we show that inclusion of poloxamer 188 (Pluronic® F-68) in the diluent used for preparing rAAV for titering via ddPCR ameliorates the loss of rAAV for parental and novel capsids. The impact of pluronic F-68 appears process and formulation independent since it was observed with three separate manufacturing processes each with different formulation buffers.

Using the optimized titering method, *in vitro* infectivity ($TCID_{50}$) and potency assays of rAAV parental and novel capsids are presented. Results support the assessment of different diluents to test for rAAV loss during sample preparation to ensure accurate titration of rAAV, the determination of infectivity, and quantification of potency.

881. Efficient Cell Isolation, Washing, and Concentration Using Rotea™ in Closed and Automated Cell Therapy Workflows

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The processing and delivery of cell and gene therapy products is one of the major obstacles to bringing treatments to patients. Rotea's counterflow centrifugation technology enables cell separation, washing, and concentration of many cells types and can be easily programmed to serve many additional workflows. The single use kit and instrument are able to capture and sustain billions of cells in a stable, fluidized bed with flexible inputs and output volumes as low as 5 mL. Rotea is capable of consistent lymphocyte separation from leukapheresis products with greater than 80% recovery. Similar to manual isolation methods, cells isolated and cryopreserved using Rotea exhibit high viability and robust expansion in culture for over 14 days. Protocols can be designed to wash and concentrate virtually any cell type, including T cell and others used in cell and gene therapies by adjusting the centrifuge and pump rate. Rotea has the ability to support multiple cell therapy workflows and the scalability to take a therapy from process development to manufacturing.

882. Residual DNA Detection Using Digital PCR

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Continuous cell lines are used in the production of a variety of products including recombinant biopharmaceuticals, vaccines and products for gene therapy. Cellular DNA which remains following the purification of these products has the potential to induce tumour formation. To ensure product safety, regulatory authorities stipulate that residual DNA must be removed to a maximum allowable level of 100 pg or 10 ng per dose (U.S Food and Drug Administration (FDA) and the World Health Organisation (WHO) respectively). Quantification of residual DNA can be performed by qPCR analysis of DNA purified from product material. However, extraction of DNA from the sample matrices is often inefficient and the purified DNA can exhibit inhibition in the PCR reaction, each of which can impact the accuracy and sensitivity with which the residual DNA may be quantified. Digital PCR is a molecular technology which potentially provides a number of key advantages over qPCR for detection of residual DNA. More specifically, the method is not impacted by differences in the relative efficiency of standards versus samples and is more tolerant to PCR inhibitors, enabling accurate quantification of DNA derived from an inhibitor rich matrix. There is also the potential for quantification of DNA directly within product material with no prior extraction of DNA. We have investigated the potential of droplet digital PCR to quantify residual DNA within drug product material without the need to perform prior purification of DNA. Preliminary results indicate the method will enable detection of residual DNA to meet regulatory guidelines.

883. Automation in Hemopoietic Stem Cell Gene Therapy: Results of a Head to Head Comparison of a Manual vs an Automated Procedure

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The recent successes in gene therapy for the treatment of rare blood disorders such as primary immunodeficiencies and hemoglobinopathies have highlighted the need to generate robust and scalable manufacturing processes. Here we compare large scale lentiviral transductions of hemopoietic stem cells performed on the CliniMACS Prodigy® to manual processes in terms of efficiency of procedures and viability of the end product. In our first series of experiments, we used a low, non-saturating MOI of 30 and the results showed that transduction efficiency on the CliniMACS Prodigy® was significantly increased from an average of 26% (manual) to 39% (CliniMACS Prodigy®). An MOI of 100 resulted in an average transduction efficiency of 62% for the CliniMACS Prodigy® compared to 60% for the manual steps. Moreover, the total viability of the cells cultured on the CliniMACS Prodigy® remained unaffected after two days of cultivation with an average recovery of 106% but with significantly less variability (SD: 12.7) compared to the manual steps (SD: 33.5). Finally there was no difference to the average VCN which was 1.9 and 2.2 (MOI 30) and 3.4 and 3.2 (MOI 100) from the manual steps and from the CliniMACS Prodigy®. Overall, our results indicate that the CliniMACS Prodigy® generates higher transduction rates, combined with high viability compared to the manual process, but most importantly, with significantly lower variability, suggesting that it represents a closed system able to automatically perform complex processes as successfully as when manual handling steps are performed, but with higher predictability, efficiency and with minimal user interaction.

884. The Cocoon® Platform: An Automated, Closed System for Manufacturing Patient-Scale Cell Therapies

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Autologous chimeric antigen receptor T-cell (CAR-T) immunotherapies have generated significant enthusiasm due to their robust therapeutic efficacy in hematological malignancies and culminated with FDA-approved Kymriah™ and Yescarta™. Although, immunotherapies for blood cancers have dominated research pipelines, treatments for

solid tumors are beginning to emerge. However, significant challenges remain including high manufacturing costs due to complex workflows conducted within open, manual cell culture systems, thereby limiting the full utilization of cell therapies. Moreover, as additional cell therapies are approved for indications with larger patient populations, the need for scalable platforms to manufacture patient cell therapies at commercial-scale is essential. Enabling cell therapy clinical and commercial success will entail employing closed, automated platforms to produce cost effective, robust therapies. One solution is the Cocoon® Platform and this abstract highlights a study detailing the step to translate a CAR-T therapy process from an open, manual method to the fully automated, closed method on the Cocoon® Platform. The Cocoon® Platform automates cell seeding, activation, transduction, real time process monitoring, feeding, washing/concentration, and harvesting, within a functionally closed system. Manual research scale processes were optimized, scaled up, and programmed to run without manual intervention in the Cocoon® Platform. In this process, 200 million positively-selected T cells from fresh leukopaks were activated with TransAct™ beads. The following day, cells were transduced with a lentivirus vector that modifies the selected cells to express an engineered gamma delta (γδ) TCR. Following transduction, cells were expanded in cytokine and human serum supplemented medium with a pre-defined feeding strategy until they were harvested at Day 8. After harvest, cells were analyzed for cell yield, viability, transduction efficiency, and cell phenotype via flow cytometry. Parameters including cell density during transduction, surface area, and utilization of cryopreserved or freshly isolated cells were optimized and applied to generate an optimized manufacturing protocol for the Cocoon® Platform. Transduction efficiency was optimized by keeping cells in a high density as well as large surface area. While no significant difference in cell yield or transduction efficiency between cryopreserved and fresh cells was observed, cell numbers were elevated when using fresh T cells. The automated Cocoon® Platform employs a proliferation chamber with a large surface area (260 cm^2) to optimize cell culture conditions. Two independent Cocoon runs were conducted using freshly isolated T cells transduced at high cell density with an average cell yield of $1.82x10^9$ cells and viability of greater than 90%. Transduction efficiency averaged 62.5% in the $CD3^+$ $\gamma\delta TCR^+$ T cell fraction, with $CD4^+$ and $CD8^+$ T cells at a 1:1 ratio, and transduction efficiencies of 71% $\gamma\delta TCR^+$ and 56.5% $\gamma\delta TCR^+$, respectively. By optimizing the feeding strategy and utilizing integrated biofeedback, the nutrients and metabolites were maintained within the desire specifications throughout the 8-day manufacturing process (lactate of < 1 g/L, ammonium of < 0.3 mmol/L, glucose of ~3 g/L, glutamine of < 0.3 mmol/L, and ~7 pH). In summary, we were successful in translating an open, manual process to an automated process running on the Cocoon® Platform which demonstrated the capability of producing a patient-scale CAR-T cell therapy. The Cocoon® Platform is a viable solution to translate labor-intensive cell therapy processes to an automated system allowing scalability, high yield, reduction of manufacturing cost, minimizing operator error, and improved process control.

885. Benefits to Standardizing Helper and Packaging Plasmids for Viral Vector Production

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Success in gene and cell therapy has increased demand for plasmid DNA used to produce viral vectors, both in quantities for commercial products as well as the breadth of different vectors for the expanding number of programs in development. Our data and analysis indicate that standardization and large-scale production helper plasmids represent an opportunity to significantly reduce timeline, cost, and risk. We have developed processes to quickly produce a set of helper and packaging plasmids that consistently produce functional viral vectors, these are immediately available for research and clinical production, and are free of any royalties or future payments. These plasmids include the envelope and packaging plasmids for lentiviral production, and the adenoviral helper and AAV2, AAV5 and AAV6 serotype plasmids for rAAV production. To meet production scale requirements, our technical operations team has developed and deployed a platform based around a single-use, 300-liter fermentation device and a process train capable of purifying up to 100 grams of a plasmid in a single processing event. The manufacture of the output from an individual fermentation process can take as little as seven days. Scaling work done in the early engineering phase for this train was integral to the design of a new 70,000 square foot manufacturing plant. In conclusion, the availability, cost, freedom to operate, and consistency of these plasmids will help address the growing demands of cell and gene therapy.

886. Development of Analytical Methods for Ancestral Adeno-Associated Virus

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One of the challenges associated with gene therapy AAV production is to develop optimized processes for AAV viral vectors. The iterative optimization of both upstream and downstream processes requires robust analytical tools to characterize and quantify in-process samples. Current analytical methods for the in-process samples, such as quantitative PCR (qPCR) and droplet digital™ PCR (ddPCR™), mainly focus on parsing out the packaged viral DNA, which cannot provide the information regarding the titers of the virus without genomes (empty capsids). To support the process development of our ancestral AAV manufacturing platform, we have developed analytical methods for in-process samples, including high-performance liquid chromatography and enzyme-linked immunosorbent assay.

887. Industrial Lentiviral Vector Manufacturing Using Advanced Process Analytical Technologies

Lee Davies[1], Thomas Williams[1], Rui Sanches[1], Carol Knevelman[1], Nicholas Clarkson[1], Laura McCloskey[1], Robert Law[1], Kyriacos Mitrophanous[1], James Miskin[1], Greg Blanchard-Emmerson[2], Sam Watts[2], Isobelle Evie[3], John Churchwell[3], Oliver Dewhirst[3], Marc-Olivier Baradez[3], Damian Marshall[3], James Rutley[4], James Arpino[4], Allen Shaw[4], Markus Gershater[4], Peter Jones[1]

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Oxford Biomedica (OXB) is a pioneer in gene and cell therapy with a prominent global position in lentiviral vector research, development and production. Utilising its proprietary LentiVector® delivery platform, OXB has produced gene and cell therapy candidates for a wide variety of therapeutic applications in the fields of oncology, neurology and ophthalmology. Faced with the challenge of consistently manufacturing lentiviral vectors for gene therapy products at commercial scale within rigorous tolerances for titre, purity and potency, OXB has developed a scalable, serum-free suspension manufacturing process utilising stirred tank bioreactors and state-of-the-art bioprocessing facilities. As part of ongoing process development activities, OXB is evaluating a range of novel Process Analytical Technologies (PATs) in order to enhance process control during vector manufacture and to support future process intensification. As part of an Innovate UK funded project, OXB has been working with collaborators to evaluate Raman spectroscopy and refractive index profiling for real-time process evaluation during lentiviral production. A multi-channel Raman Analyser was utilised to assess media components in multiple 5 L stirred tank bioreactors during production of a VSV-G pseudotyped HIV-1 lentiviral vector expressing GFP. Vector was generated either via transient transfection or from a novel stable producer cell line. Data obtained from Raman spectroscopic analysis was correlated with contemporaneous offline chemometric analyses and utilised to generate predictive models to allow real-time evaluation of key process elements during vector manufacture. Using this approach, viable cell concentration, vector titre, and the concentration of up to 80 independent metabolites could be accurately predicted using preprocessed Raman spectra. Models developed in 5 L bioreactors were subsequently evaluated at larger scale in 50 L single use bioreactors.Refractive index profiling utilising the Ranger™ RI system was used to detect real-time changes in culture composition related to cellular metabolism. The probing of various bioprocess parameters in real-time enabled the application of automated adaptive process control, potentially leading to the rapid optimisation and fine-tuning of bioreactor operating parameters for individual cell lines and enhancing overall process performance.

888. Recombinant Adeno-Associated Virus (AAV) Production in the ExpiSf™ Expression System

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Adeno-associated virus (AAV) is among the most commonly-used viral vectors, currently representing ~23% of the gene therapy market. To date, there have been three approved AAV products for the treatment of disease, and there are currently greater than 300 development projects and clinical trials ongoing worldwide. A roadblock faced by researchers and clinicians is the production of suitable amounts of viral vectors for their pre-clinical and clinical trials, as well as for patient treatment. Production of recombinant AAV in insect cells is poised to address material demand, as AAV production runs at the 2000L bioreactor scale have been reported with overall volumetric yields in the range of 10^{16} - 10^{17} total genomic (VG) particles. The ExpiSf™ Expression System is a complete chemically-defined baculovirus-insect cell protein expression system that delivers superior yields and consistent performance run after run using a fast, streamlined workflow. Here, we demonstrate the versatility of the ExpiSf system beyond its use for expression of recombinant proteins. Using a dual-infection approach, recombinant adeno-associated virus (rAAV) for two serotypes was produced in the ExpiSf system. Production of rAAV particles is scalable from shake flask to bioreactor scale, yielding high viral genome titers (vg/mL) of infectious rAAV, and high rAAV yields were obtained following purification using POROS™ GoPure™ AAVX affinity resin.

889. Platform-Ability of the AAV Purification Process and Membrane Chromatography Polishing

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The approval of gene therapies delivered by Adeno-Associated Viruses (AAVs) has resulted in an increased commitment to adopt AAV as the vector of choice to address a broad range of diseases. The increased demand for AAV is a challenge, which will probably only be fulfilled by adoption of platform processes, similar to those developed for monoclonal antibodies. An affinity capture step can be applied to most AAV serotypes and appears to be part of a nascent platform purification process. However, a post affinity step, most often ion exchange chromatography, is required to remove the remaining host cell proteins and DNA, and to separate full capsids, containing DNA payload, from empty capsids. These chromatography steps are difficult to develop as a robust process and as dilutions must be performed after the affinity step, volumes to load can be large compared to the titer. To address these challenges, we have investigated the performance of membrane chromatography as a polishing step for purification of AAV serotypes 2, 5 and 9. We compare the performance for contaminant removal compared to commonly used resin based approaches to show the potential of membrane chromatography as a platform-able polishing step for AAV purification. Membrane chromatography can

be loaded 40 times more quickly than conventional chromatography resins, which can lead to much higher productivities and greatly speed the purification step.

Pharmacology/Toxicology Studies or Assay Development

890. Reference Materials for Viral Gene Vectors

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Reference Materials for Viral Gene Vectors Starting with the development of an adenovirus, serotype 5, reference material in 2001, there has been a serious regulatory and product quality focus on the use of viral reference materials (RM). This first RM evolved from the need acknowledged by FDA, NIH, and RAC when a patient at the Children's Hospital of Pennsylvania (CHOP) died in 1999 after receiving increasing doses of an adenoviral vector. And even though the patient (Jesse Gelsinger) was immuno-compromised, the number of viral particles administered created a fatal immune response that was considered unacceptable. It was then agreed that the amount of antigen a patient would be given had to be related to a reference of some sort. Therefore, the industry, FDA, and academia formed a Working Group to produce an Ad5 RM that could be used by all adenoviral product developers to validate their internal reference materials and assays. This way, all adenoviral product candidates could be compared to the RM for both total particles and infectious titer. Today, additional viral RMs have been produced for AAV2 and AAV8, and now an RM is under development for a lentiviral vector. This presentation will explain the process involved in producing, characterizing, and distributing viral reference materials.

891. Development of a One-Step RT-ddPCR Method to Determine the Expression and Potency of AAV-Based Gene Therapy

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Robust assays to measure AAV vector potency, biodistribution, and vector expression are essential for gene therapy preclinical development. Results from these assays inform the efficacy, safety, and the pharmacokinetics and pharmacodynamics (PKPD) profiles of AAV development candidates. In addition, for gene therapy using gene downregulation approaches (RNAi, gene editing, etc.), knockdown levels of endogenous genes reflect the mechanisms-of-action of the development candidates. Therefore, the method to quantitate the target mRNA knockdown is also necessary for measuring vector potency and efficacy in both *in vitro* and *in vivo* models. Quantitative PCR (qPCR) and reverse-transcription quantitative PCR (RT-qPCR) have been the methods of choice for analyzing AAV biodistribution, vector expression, and endogenous gene knockdown. Herein, we report the development of a one-step reverse-transcription droplet digital PCR (RT-ddPCR) method to quantify vector expression and endogenous gene knockdown levels for a vectorized RNAi approach. This one-step method of RT-ddPCR significantly simplifies the workflow, allows for duplexing of target gene and housekeeping gene in a single reaction, and enables absolute quantification of transcripts. Using an RNAi AAV vector, we demonstrated the utility of this RT-ddPCR method in quantifying both vector expression and gene knockdown. Data from RT-qPCR, ELISA of the target gene product, and functional readout of the target protein demonstrate correlation with RT-ddPCR results. Using a gene augmentation vector, we demonstrated the application of RT-ddPCR in quantifying vector expression. In addition, we will discuss how to use RT-ddPCR as a platform technology to develop potency assays for gene downregulation programs and gene expression assays for both *in vitro* and *in vivo* sample analyses. In conclusion, we developed a new platform method for the preclinical studies of AAV gene therapy programs. This RT-ddPCR method can be applied to expression analysis and potency measurements. Coupled with ddPCR-based vector titering assays, this RT-ddPCR method allows for robust analysis of the pharmacology profiles of AAV development candidates.

892. Quantification of Enzyme Activity for Lysosomal Storage Disorders Using LC-MS

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Introduction: Lysosomal storage disorders (LSDs) are caused by the deficiency of lysosomal enzymes associated with an accumulation of mucopolysaccharides, oligosaccharides, and sphingolipids. These disorders are monogenic, thus the concept of existing therapy largely depends on genetic strategy. Newborn screening is a part of public health program aiming to identify affected individuals during asymptomatic period. To achieve this, a sound assay needs to be developed. One such assay for LSD includes LC-MS-based assay using a dried blood spot (DBS) that measures enzyme activity in acidic conditions in the presence of deuterated internal standard. Materials and Methods: Assay reagents for LSD enzyme activity and QC DBS were purchased from PerkinElmer (Yokohama, Japan). A 3-mm punch was reacted with enzyme assay cocktail (30 uL) at 37°C for 20 h. Then, the enzyme reaction products were extracted into ethyl acetate. These are dried under nitrogen stream followed by solubilized into acetonitrile/water (20/80) with 0.2% formic acid. An aliquot was injected onto a CSH C18 column (2.1 mm x 30 mm, 1.7 um) using a gradient elution of mobile phase A (acetonitrile/water = 20/80 with 0.2% formic acid) and mobile phase B (acetonitrile/water = 80/20 with 0.2% formic acid). A tandem mass spectrometer Quattro Premier XE equipped with a liquid chromatograph Acquity (Waters) was used for quantification. Results: To validate assay conditions, we first quantified the enzyme activity of QC DBS that contained 5 and 100% enzyme activity. The R2 value for correlation equation was greater than 0.9, demonstrating that this assay is applicable to clinical specimens. When we quantified the LSD enzyme activity of several batches of QC DBS, a small intra-assay CV value was obtained. Conclusions: Identification of affected individuals for LSDs may be performed several methods including LC-MS-based assay. Gene therapy for identified individuals is considered as a promising treatment for LSDs.

893. Effects of Different Myeloablative Conditioning Methods on Hematology Parameters in NHP Models

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Non-human primates (NHP) are important models for cell therapies when the product attributes or cell editing process dictate the need to test the efficacy or safety of the therapy. As NHP models can be important in addressing pertinent translational concerns in cell therapies such as immunogenicity, long-term safety, and the functional integration of engrafted cells, it is imperative to implement a suitable and low-impact myeloablative conditioning method to ensure successful implantation and engraftment post treatment. Data was compiled from studies in which NHP models underwent myoablative conditioning via either gamma irradiation at different doses (from 100cGy to 1210cGy), fractionated radiation regimens at different doses (from 500cGy to 700cGy per day), or carboplatin treatment (infused at 42.5 mg/kg). Anti-thymocyte globulin (ATG) was evaluated for T cell depletion and could be considered as a combination therapy. Retrospective data evaluation included clinical observations and hematology parameters, such as absolute lymphocyte and neutrophil counts. Single dose gamma irradiation at ≤400cGy and carboplatin treatment induced very limited clinical signs such as decreased appetence, with limited severity and incidence in NHP models. At radiations doses of above 400cGy, regardless of whether the dose was fractionated, clinical signs were apparent in all animals including decreased activity and appetence, abnormal feces, retching and/or pallor. These observations were characteristic of the expected symptoms from animals that are exposed to higher irradiation doses, and typical of gastro-intestinal and/or hematopoietic changes in acute radiation syndrome (ARS). A sharp and dose-dependent decline in absolute neutrophil ($1.63x10^9$/L and $0.63x10^9$/L) and lymphocyte counts ($1.17x10^9$/L and $0.36x10^9$/L) on Day 7 post irradiation was also found as early as Day 2 in animals receiving a single radiation dose of 100cGy and up to 400cGy, respectively. While the decline in both neutrophil ($0.63x10^9$/L and $0.02x10^9$/L) and lymphocyte ($0.36x10^9$/L and $0.06x10^9$/L) counts remain dose-dependent at higher radiation doses (between 400cGy and 1210cGy, respectively) with or without fractionation, the magnitude of the decrease is markedly lower compared to the decreases in both counts between radiation doses of 100cGy and 400cGy. In comparison, carboplatin treatment also mildly lowered neutrophil and lymphocyte counts, but the kinetics of the effects were abrogated compared to radiation conditioning, with both counts reaching nadir on Day 5 and recovery to baseline values on Day 8 post carboplatin treatment. The results from NHP studies indicate that myeloablative conditioning with total body irradiation (single dose or fractionated) at a dose of ≤400cGy and carboplatin infusion are generally associated with low incidences of adverse side effects. In general, robust and reliable preconditioning regimens in NHPs will provide a current unmet need to de-risk certain immunologic cell therapies.

894. A Rapid Alternative to Culture Based Mycoplasma Detection

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Per regulatory requirements, cell-culture based therapies must be free of mycoplasma. Manufacturers have traditionally outsourced testing to labs that specialize in the 28-day culture-based test method. For manufacturers of gene and cell therapy products, as well as other low-dose and short shelf-life therapeutics, it is not feasible to wait 28 days for test results. Thus, the need for rapid mycoplasma test results has also increased. Real-time PCR based assays provide a viable alternative to the culture based method and provide results in hours while meeting the required sensitivity. Following validation, regulatory filing and review, users across multiple therapeutic modalities have received regulatory acceptance to use the MycoSEQ assay for lot release testing.

895. Viral Vector Characterization to Support Early Product Development

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Manufacturing changes may be necessary as product development proceeds. As such it is important to sufficiently characterize the product early in the development cycle. The establishment of Critical Quality Attributes (CQAs) and how the impact of manufacturing changes, even minor changes, on product safety and quality is an integral part of process development and managing the life cycle of the product. Here we present the analytical characterization of an AAV product to support early process development and platform selection. Traditional biochemical, molecular and biophysical techniques, including orthogonal methodologies, in conjunction with a cell-based bioassay were used to evaluate the impact of both small and large process changes have on the CQAs of the product.

896. Neonatal Administration of scAAV9.CLN8 Gene Therapy Prevents Disease Pathology and Behavioral Deficits in a Mouse Model of CLN8-Batten Disease

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CLN8-Batten disease is a fatal autosomal recessive, neurodegenerative lysosomal storage disorder affecting children. Patients experience motor, visual, and memory/learning decline, and generally succumb to the condition in adolescence. At the cellular level, the disease is caused by mutations in *CLN8*, leading to altered ER-to-Golgi trafficking, lysosomal storage accumulation, glial activation, and neuronal loss in the central nervous system. At present, there are no treatments or cures available to patients with CLN8-Batten disease. Building on success in developing gene therapies for other NCL proteins, namely CLN3- and

CLN6-Batten diseases (NCT03770572, NCT02725580), we sought to determine if similar gene therapy strategies would be effective in a mouse model of CLN8-Batten disease. We show that a single neonatal, intracerebroventricular (ICV) injection of scAAV9.CLN8 provides long term transgene expression out to 24 months of age and is sufficient to prevent cellular pathologies throughout the central nervous system of *Cln8*^{mnd} mice, including prevention of lysosomal storage material accumulation and glial activation. Additionally, this single dose had a major impact on behavioral phenotypes associated with the disease including motor abnormalities and tremor presentations. Moreover the treatment also prevents premature death, normalizing *Cln8*^{mnd} survival from 10 months of age to 24 months of age (the natural life span of mice in our colony). Taken together, this single dose, neonatal ICV administration of scAAV9.CLN8 provides very promising therapeutic potential for the treatment of CLN8-Batten disease.

897. Development of an Immunohistochemistry Assay for the Detection of CS-1 Expression in Multiple Myeloma Patients

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CS-1 (also known as SLAMF7, CD319, CRACC) is a receptor initially identified on NK cells, that is also expressed on other immune cell subsets at low levels. In Multiple Myeloma (MM), CS-1 has been observed to be a robust marker of malignant plasma cells, with 95% of MM cases reporting over expression of CS-1 on MM cells. Thus, CS-1 is a promising target for immunotherapy. Mustang Bio has acquired an autologous CS1- CAR T cell therapy to be used in clinical trials for patients with CS-1 positive recurrent/refractory MM. To enroll patients, it is necessary to screen subjects for CS-1 positive tumor expression prior to treatment. Here we have developed, optimized, and validated and anti-CS-1 immunohistochemistry assay for the assessment of bone marrow core biopsy samples as well as plasmacytoma solid tumor samples from MM patients to be used for enrollment into our CS-1 CAR T clinical trials. Methods: For the selection of the most appropriate antibody, we utilized three different commercially available anti-CS-1 clones. Tonsil tissue was sectioned and stained with the different clones followed by a hematoxylin counter stain. Three different dilutions of each antibody were performed based on manufacturer's recommendations. Slides were then cover slipped and images were captured on a Leica slide scanning system using the standard 20x brightfield imaging template. Slides were assessed by a pathologist to evaluate staining parameters. The chosen antibody was then optimized and validated with additional tonsil tissue samples. To ensure staining between the positive control sample and patient samples of interest, samples of bone marrow core biopsies (normal, as well as MM patient samples) and plasmacytoma solid tumors were tested utilizing the optimized protocol. All samples were then evaluated by a pathologist. Results: The CS-1 immunohistochemistry assay was assessed for consistency and reproducibility across a cohort of normal as well as diseased samples. slides were reviewed by a pathologist and evaluated individually for stain quality, characteristics and positivity. Across all tissue sample slides, CS-1 staining was found to be concordant with staining in tonsil positive control tissues and acceptable for use for the evaluation of positive CS-1 expression on MM patient samples. Thus, we present for the first time, a robust and analytically validated IHC based assay to determine CS1 positivity for MM patient enrollment for CAR-T trial.

898. Model Characterization: Myeloablative Conditioning with Total Body Irradiation in NOD/SCID/IL2Rγnull (NSG) Mice

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The NOD/SCID/IL2Rγnull (NSG) mouse is a relevant model for in vivo toxicology, biodistribution and tumorigenicity studies to evaluate human cell therapies. For certain cell types such as hematopoietic cells, additional conditioning of the NSG mouse is required to facilitate the necessary engraftment. Data was compiled from GLP compliant safety studies in NSG mice which underwent myeloablative conditioning via gamma irradiation at 200cGy (160cGy/min) (n=41 males and 68 females) and control animals which were not irradiated (n=10 males and 10 females). Animals were subsequently dosed with vehicle (phosphate buffered saline (PBS) or 0.1% human serum albumin in PBS) via a single intravenous injection. Retrospective data evaluation included common mortality, body weights, clinical observations, hematology, external and internal macroscopic observations. No mortality was observed in any of the 129 vehicle-treated (irradiated and sham-irradiated) animals up to 20-weeks post-irradiation. The irradiated group presented a minor average decrease in body weight - with nadir around Day 9 - compared to the sham-irradiated group following 30 days post-irradiation, with female animals presenting an overall greater decrease in body weight than male animals (-7.67% vs. -2.45%, respectively). However, the overall body weight changes from 1 to 30 days post-irradiation were similar between the two groups. While irradiated male animals appeared to have slightly increased incidences of radiation-related clinical signs such as dehydration (i.e. slow skin turgor) compared to all other subsets of animals, clinical signs in irradiated animals were generally low in incidence and severity and did not differ significantly from sham-irradiated animals. Hematology parameters were comparable between irradiated and sham-irradiated animals at Weeks 13 and 20, confirming bone marrow recovery. Selected parameters such as mean platelet ($1153.6x10^9$/L vs. $1577.8x10^9$/L), white blood cell ($0.80x10^9$/L vs. $1.13x10^9$/L), neutrophil ($0.55x10^9$/L vs. $0.71x10^9$/L), and lymphocyte counts ($0.16x10^9$/L vs. $0.23x10^9$/L) were only slightly decreased in irradiated compared to sham-irradiated male animals, respectively. In addition, all four parameters were mildly elevated in males compared to females at Week 13 regardless of irradiation status, with elevated trends returning to comparable levels at Week 20. Finally, there were no irradiation-related differences in macroscopic observations with lymphoid atrophy identified comparably in irradiated and sham-irradiated groups across sexes. Taken together, these results indicate that myeloablative conditioning with total body irradiation has generally low incidences of adverse side effects in both male and female NSG mice, and supports the use of gamma irradiation for immunoconditioning prior to dosing in cell therapy studies using NSG mice in the context of regulatory toxicology, biodistribution and tumorigenicity studies.

899. Immunogenic and Toxicology Profiles of Liposomal Doxorubicin and Recombinant Adenoviral Protein in Macaca Fascicularis

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Liposomes are extensively used as amphiphilic nanoparticles capable of delivering a variety of payloads including chemotherapeutics and genetic information. Therapeutic liposomes often contain polyethylene glycol (PEG) on the surface for the purposes of chemical stabilization and cargo conjugation. While traditionally thought to be non-immunogenic, some previous studies indicate the possibility of PEGylated liposomes triggering an immune response and enhanced clearance. We investigated the immunogenicity and toxicity profiles of Doxil®, a PEGylated nanoparticle containing doxorubicin, in combination with an experimental recombinant adenovirus-3 protein, Junction Opener (JO). JO may offer clinical benefits in cancer patients as it enhances penetration of therapeutics and immune cells into a solid tumor by compromising its tight junctions. The combination of Doxil® and JO was tested in *Macaca fasciularis* in a series of four injections (4 weeks apart) with and without immunosuppression, as immunogenicity may result from both the liposomal formulation and the recombinant adenoviral JO protein. We found that while antibody levels to JO increase upon administration, this does not lead to anaphylactic shock and response is characterized by a transient leukopenia. The anti-JO response was sufficiently suppressed by cyclophosphamide/steroid pre-treatment. As for Doxil®, no detectable IgM or IgG antibodies to PEG were found. The Doxil clearance half-life was approximately three days. No changes in electrocardiograms were observed in response to Doxil® or JO. No toxic reactions to Doxil® were noted at the dosage (40mg/m2) used throughout the duration of the experiment. With the widespread use of liposomal formulations of gene and chemotherapeutic delivery, it seems that PEGylation does not trigger additional immune response. The combination of the Doxil® and JO may be used safely in clinical settings.

900. Establishment of Assays to Characterize Human Monoclonal Antibody Expression by DNA, RNA and AAV Delivery Platforms in Cynomolgus Macaques

Daniel Flores[1], Patrick McTamney[1], Richard Roque[1], Nicole Kallewaard-LeLay[1], David Lowe[2]

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AstraZeneca and the Defense Advanced Research Projects Agency (DARPA) have partnered to evaluate three nucleic acid delivery platforms that enable host cells to express a monoclonal antibody (mAb) of choice and may provide protection in the case of a pandemic. As a model mAb, we selected MEDI8852, a human broadly neutralizing, anti-influenza A mAb, to investigate the following platforms: plasmid DNA delivered via electroporation, RNA delivered via a lipid nanoparticle and viral vector delivery via adeno-associated virus (AAV). In preparation for non-human primate studies, assays lacking cynomolgus/human cross-reactivity were established to evaluate MEDI8852 serum levels, anti-drug antibody (ADA) levels and confirm functionality. The most important assay developed is a universal human IgG quantification ELISA assay with a 10 ng/ml lower limit of detection and uses antibodies devoid of cynomolgus macaque cross-reactivity. To confirm MEDI8852 functionality, an ELISA based hemagglutinin antigen (H1 and H3) binding assay was created with a minimum required antibody input of 500 ng/ml, using the same anti-human antibody from the IgG quantitation assay for detection. Lastly, detection of MEDI8852 ADA was quantified by an MSD sandwich assay that has broad species specificity and can detect MEDI8852 ADA at a minimum input dilution of 1:50. These now established assays enable a direct comparison of our three nucleic acid platforms and are essential in determining which nucleic acid platforms successfully express functional MEDI8852 and may proceed into clinical trials. Funding source: This research was developed with funding from the Defense Advanced Research Projects Agency under HR011-18-3-001. The views, opinions and/or findings expressed are those of the author and should not be interpreted as representing the official views or policies of the Department of Defense or the U.S. Government. Approved for Public Release, Distribution Unlimited

901. Monoclonal Antibodies for AAV Neutralization Assays

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A growing number of academic and industrial labs are using AAV vectors for the development of gene therapies leading to an increase in the demand for effective and reliable analytical AAV tools for R&D and manufacturing. Serum antibodies against many of the natural serotypes of AAV can be found in a significant proportion of the population representing a critical factor in the selection of patients for gene therapy. Testing of sera from potential patients for the presence of neutralizing antibodies against a specific AAV serotype used for gene therapy is an essential step for clinical studies and therapeutic application. PROGEN's unique panel of AAV antibodies, recognizing only intact assembled capsids, have been shown to be neutralizing and can therefore be used as positive controls in neutralization assays. Here we present data of cell-based neutralization assays for most of the widely used AAV serotypes (AAV1, 2, 3, 5, 6, 8, 9, rh10). HeLa cells were incubated with each of the different AAV serotypes containing a GFP construct in the presence of increasing amounts of PROGEN's well-known capsid-specific antibodies. The decrease of the number of GFP-positive cells proved the neutralizing activity of these antibodies. New variants and serotypes of AAV are already in use and likely to increase in number in the future. The wide range of serotypes that PROGEN's AAV antibodies cover could be used to identify neutralizing antibodies also for these new variants. Since cell-based assays tend to show higher variability than biochemical assays in general PROGEN's AAV antibodies can help to verify the reproducibility and improve inter-assay comparability of neutralization assays.

902. Discussion of a Framework for Risk Analysis in Gene and Cell Therapies

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Understanding the risks related to new therapeutic product candidates helps to determine the safe conditions of human use, but also the path forward to the clinic, through the establishment of risk mitigation measures. The increasing emergence of gene therapy products triggered the development of new regulatory guidelines. For Advanced Therapy Medicinal Products (ATMPs), a risk-based approach has been proposed by regulatory authorities. This approach consists of elaborating preclinical packages based on anticipated risks to be identified, evaluated, and characterized for Gene Therapy Medicinal Products (GTMPs). Science-based, case-based programs may, therefore, be defined considering the disease, the targeted cells, the vehicle, the route of administration, the nucleic acid sequence, the mechanism of action of the treatment and the potential side effects. As the gene therapy field is rapidly growing and the domains of application are diversifying, it becomes clear that safety concerns and risk mitigation will have to be managed based on the specificity of the programs. The requirement will differ, for example, from *in vivo* gene therapy based on the delivery of AAV-vectors to *ex vivo* gene therapy approach using lentiviral gene transfer in cells to be re-injected. Managing risk will also differ from rare monogenic diseases to multifactorial complex frequent conditions. A framework for risk analysis and tools available to evaluate safety concerns will be discussed based on regulatory agency guidelines and case studies.

903. Abstract Withdrawn

Presidential Symposium & Presentation of the Top Abstracts

905. Generation of Islet-Specific Gene-Edited Regulatory T Cells (edT_{reg}) for Restoration of Immune Tolerance in Type 1 Diabetes

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Adoptive transfer of regulatory T cells (T_{reg}) represents an emerging therapeutic approach to promote immune tolerance in the setting of stem cell or solid organ transplantation, and in autoimmune diseases including type 1 diabetes (T1D). To overcome the limited purity, quantity, and stability of ex vivo expanded T_{reg}, we previously developed gene-edited T_{reg} (edT_{reg}) from conventional $CD4^+$ T cells as a novel source for T_{reg} cell therapies. edT_{reg} are generated by co-delivering a designer nuclease and an AAV donor template resulting in homology directed repair (HDR) and enforced expression of FOXP3 by the integrated MND promoter. Stable and high-level FOXP3 expression in edT_{reg} derived from human and murine $CD4^+$ T cells redirects the transcriptional landscape to mimic natural T_{reg} (nT_{reg}) with immunosuppressive function equivalent to nT_{reg}. edT_{reg} manufactured using this approach will soon be utilized in a Phase I/II clinical trial for Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX), a fatal autoimmune disease due to T_{reg} deficiency. While polyclonal edT_{reg} are likely to be beneficial in GvHD and IPEX, edT_{reg} with antigen (Ag)-specificity are predicted to enhance potency in autoimmune diseases driven by known autoantigens. In mouse models, Ag-specific T_{reg} exhibit superior function in preventing diabetes in comparison to polyclonal T_{reg}. Thus, we aimed to develop islet-specific edT_{reg} to treat or prevent T1D. In murine T cell studies, polyclonal or islet-Ag-specific edT_{reg} were generated using $CD4^+$ T cells from NOD and NOD.BDC2.5$^+$ mice, respectively. Following adoptive transfer, islet-specific edT_{reg} homed to pancreas and persisted. Both islet-specific edT_{reg} and nT_{reg} blocked diabetes triggered by islet-specific T_{eff} in immunodeficient recipient mice while polyclonal edT_{reg} or nT_{reg} failed to do so. Based on these findings, we next established a platform to generate islet-Ag specific human edT_{reg}. Candidate islet Ag- specific TCRs derived from clonally expanded $CD4^+$ T cells in T1D subjects were introduced using lentiviral delivery in parallel with *FOXP3* HDR-editing. The resulting islet-specific edT_{reg} exhibited a T_{reg}-like phenotype and suppressed proliferation and cytokine production by islet-specific T_{eff} with matched TCRs. Consistent with bystander suppression, islet Ag-specific edT_{reg} also suppressed polyclonal islet-reactive T_{eff} derived from autologous PBMC; demonstrating impact across multiple specificities. Notably, edT_{reg} expressing TCRs with higher avidity had superior suppressive capacity to those with lower avidity. Next, dual-HDR-editing strategies targeting the *TRAC* locus were established to permit concurrent expression of FOXP3 and the islet-specific TCR in parallel with endogenous TCR disruption. Finally, as edT_{reg} must compete with both nT_{reg} and T_{eff} for engraftment and expansion in vivo, we engineered HDR donors to simultaneously introduce a heterodimeric, chemically-induced, signaling complex (CISC) that mimics IL-2 signaling; enabling selection and enrichment of the dual-edited, islet-specific edT_{reg}. Collectively, these engineering platforms generate edT_{reg} specific to pancreatic islets that efficiently suppress islet-antigen responses. This approach has the capacity to deliver targeted immunosuppressive therapy to treat or prevent T1D and could be adapted to target other tissue-specific autoimmune diseases.

906. Validation and Long-Term Follow Up of CD33 Off-Targets Predicted In Vitro and In Silico Using Error-Corrected Sequencing in Rhesus Macaques

Aisha A. AlJanahi[1], Cicera Lazzarotto[2], Shirley Chen[1], Taehoon Shin[1], Stefan Cordes[1], Isabel Jabara[1], Yifan Zhou[1], Kyung-Rok Yu[1], Yuesheng Li[1], Bradley Toms[3], Sogun Hong[1], Lauren Truitt[1], Julia Klermund[4], Miriam Y. Kim[5], Toni Cathomen[4], Saar Gill[5], Shengdar Q. Tsai[2], Cynthia E. Dunbar[1]

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Accurate and sensitive methods for off-target characterization are crucial prior to applying CRISPR/Cas9 therapeutically. Here, we utilized a rhesus macaque (RM) model to ask whether CIRCLE-Seq (CS), an in vitro off-target (OT) prediction method, more accurately identifies OTs compared to in silico prediction (ISP) based solely on genomic sequence comparison. We optimized and validated the reproducibility of CS on primary RM DNA for a clinically-relevant CD33 gRNA, which we have used to create CD33 knockout hematopoietic stem and progenitor cells (HSPCs) to reconstitute hematopoiesis following autologous transplant (Kim et al, Cell, 2018). We ran a current ISP algorithm to identify predicted OT sites for the same gRNA. We then designed a custom AmpliSeq HD error-corrected targeted sequencing panel of the top 500 sites predicted by CS and by ISP for the CD33 guide. Of the selected sites, only 66 overlapped between CS and ISP. This panel allows for high-throughput and extremely sensitive edit detection down to allele fractions of 0.1%. Using this panel, we sequenced the infusion product and blood samples for up to 2 years post-transplant in 4 RMs following transplant with CD33 CRISPR/Cas9-edited HSPCs. In granulocytes, dependent on ongoing production from HSPC, 17 bona fide OT sites were detected, with VAFs of as high as 9.4% in animals with on-target editing at the same time points ranging from 95.6% to 1.4%. As with on-target editing, OT sites were detected at higher levels immediately following engraftment, presumably due to more efficient editing of short-term repopulating cells compared to long-term repopulating stem cells. None of the animals demonstrated clonal expansions of cells containing OT edits over time. In order to ask whether OT edits were linked to perturbations in hematopoiesis, we analyzed T, B and NK cells at multiple time points. We found one additional OT site in vivo in T cells of 2 of the 4 RMs at low levels of 0.2% or lower. We found that other OT sites were present in multiple lineages with no evidence for lineage bias. Of the 18 bona fide off-targets, 8 were found in the top 500 sites from both CS and ISP. However, 7 bona fide sites were identified by CS only and 3 by ISP only, therefore each methodology provides non-overlapping valid OT identification. Lastly, we used CAST-Seq to identify engrafted cells with translocations resulting from editing of on and off-target sites. In blood cells 2 weeks post-transplantation from 1 RM. A translocation between the on-target site and the most highly edited OT site was detected. We will expand this CAST-Seq analysis to all 4 RMs and validate and follow any translocations detected over time via translocation-specific primers. In conclusion, this represents the first longitudinal, multi-lineage and highly sensitive analysis for off-target editing following HSPC CRISPR/Cas9 editing in a relevant large animal HSPC transplantation model.

907. Chemical Engineering of Therapeutic RNAs for Allele Specific Gene Silencing In Vivo

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Chemically engineered siRNAs are capable of sustained and potent silencing of mutant proteins *in vivo*. Maintaining wild type protein function via allele-selective silencing of the mutant mRNA allele may be necessary for various genetically based disorders. Targeting patient SNP heterozygosities provides a pathway for therapeutic siRNA-guided allele selection. Using a previously identified, fully chemically stabilized divalent siRNA scaffold, we selected sequences that specifically target SNP site rs362273 of the *htt* gene *in vivo*. This site is heterozygous (A/G) in ~35% of the HD patient population. By combining sequence selection techniques with chemical engineering we discovered an siRNA that provide significant discrimination between mutant and wild type HTT in the BAC97-HD mouse brain. Walking the sequence of the siRNA around the site of interest, and introducing an additional purposeful mismatch, allowed us to identify a compound that provides 100 fold discrimination *in vitro*. The same compound provided an average of 70% silencing of the targeted mutant allele across brain regions *in vivo*, with no impact on the wild type. By testing this method with an additional SNP site, rs362307, we showed that the best position for placement of the SNP and additional mismatch site depends on the sequence of the mRNA target. We also found that manipulating the pattern of 2'ribose modifications completely changed the behavior of the siRNA. Enrichment of 2'-OMe around the secondary mismatch site decreased silencing of the non-target allele, while 2'-F enrichment around the SNP site eliminated discriminating power. This method of development for SNP-specific siRNAs allows for the design of allele-specific treatments for individual HD patients.

908. Chimeric Antigen Receptor T Cell Therapy and Env Boosting Enables Durable SHIV Remission in Nonhuman Primates

Blake Rust[1], Leslie Kean[2], Lucrezia Colonna[1], Katherine Brandenstein[1], Nikhita Poole[1], Willimark Obenza[1], Colby Maldini[3], Gavin Ellis[3], James Riley[3], Hans-Peter Kiem[1,4], Chris Peterson[1,4]

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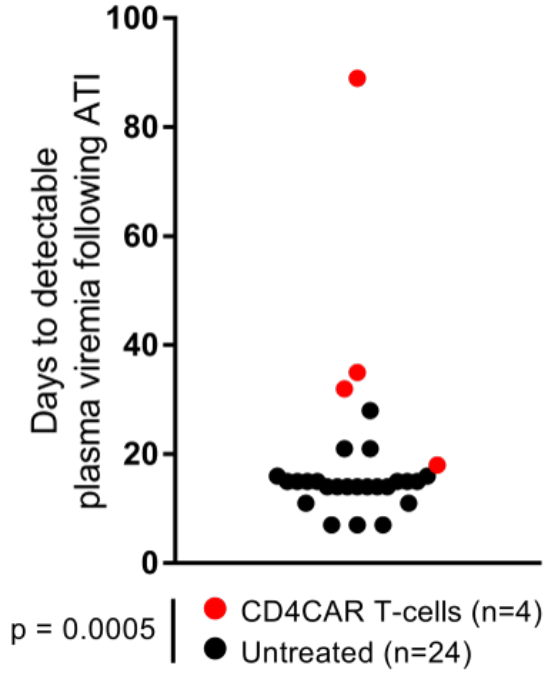
Chimeric antigen receptor (CAR) T-cells have proven extremely effective for the treatment of hematological malignancies. In contrast, retargeting these cells for infectious diseases such as HIV-1 has proven more challenging. We hypothesized that this difference was due to abundant expression of tumor antigens, relative to extremely low levels of HIV-1 antigen (Envelope protein), namely in patients on suppressive antiretroviral therapy (ART). To test this hypothesis, we combined an optimized CD4-based CAR molecule specific for HIV-1 Envelope with an antigen boosting strategy designed to mimic high levels of B-cell antigens such as CD19 and CD20. This approach was modeled in nonhuman primates (NHPs) infected with simian/human immunodeficiency virus (SHIV) and suppressed long-term on antiretroviral therapy (ART). Our goal was to quantify the impact of ΔCCR5 CD4CAR T-cells on viral rebound following ART treatment interruption (ATI). Rhesus macaques (n = 4) were infected with SHIV and suppressed by ART for at least 1 year prior to intervention. Autologous T-cells were CCR5 gene-edited to protect against infection, modified with lentiviral vectors expressing HIV/SHIV-specific CD4CAR, and infused without a conditioning regimen. To boost the persistence of CD4CAR T-cells *in vivo*, transplanted animals subsequently received a single dose of cell-associated HIV-1 Envelope antigen. Flow- and PCR-based assays were used to characterize the T-cell infusion product and to track the engraftment of these cells in blood and tissues. SHIV plasma viral loads were monitored weekly before and after T-cell infusion and ATI. Our optimized manufacturing protocol augmented the ratio of CD4 to CD8 CAR T-cells, increasing the persistence of CAR^+ CD4 lineages *in vivo*. Infusion of cell-based Envelope antigen was well-tolerated, led to significant increases in the percentage of CAR^+ T-cells in peripheral blood prior to ATI, and potentiated these effectors to respond to infected cells that arose following ATI. Post-ART withdrawal viral rebound was significantly delayed in all animals as compared to a control cohort (p=0.0005). Currently, plasma viral loads remain undetectable in one animal and at extremely low levels in a 2^{nd} animal at 172 days post-ATI. To our knowledge, ours is the first study to boost virus-specific CAR T-cells in infected, suppressed hosts, and to delay/control viral rebound via CAR T-cell therapy. We are continuing to monitor this cohort to i) determine if our treatment is consistent with a functional cure in 2 out of 4 animals, and ii) test whether immune checkpoint blockade may reactivate CD4CAR T-cells in 2 animals that did not control SHIV viremia. Due to the lack of a cytotoxic conditioning regimen, the safety profile of our approach is highly favorable. Our data reinforces the promise of ΔCCR5 CD4CAR T-cell therapies for viral reservoir reduction in HIV^+ individuals, including a Phase I clinical trial underway at the University of Pennsylvania (NCT03617198).



Viral Vector Development: RNA Virus Vectors

909. Human CD3 as a Target Receptor for Simultaneous Activation and Cell-Specific Transduction by Lentiviral Vectors

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Conventional lentiviral vectors (LVs) pseudotyped with the vesicular stomatitis (VSV) envelope require activation of human T lymphocytes for efficient gene transfer. Commonly, activation is achieved by cross-linking of the CD3/TCR complexes by CD3-specific, agonistic antibodies in presence of costimulatory signals by CD28 antibodies and supportive cytokines. In combination with this activation protocol, VSVLVs have been frequently used for T cell modification *in vitro*. However, the vectors cannot modify resting or minimally stimulated cells, which may be beneficial for some applications, such as efficient *in vivo* modification of T lymphocytes. Here, we have generated novel CD3-targeted LVs (CD3-LVs) capable of genetically modifying T lymphocyte subsets without prior activation. For CD3 attachment, agonistic CD3-specific single-chain variable fragments were chosen, allowing simultaneous activation and transduction of resting primary human T cells. Activation, proliferation and expansion mediated by

CD3-LVs were less rapid compared to conventional antibody-mediated activation owing to lack of costimulation. Upon addition of CD28 costimulation, T cell proliferation and expansion with CD3-LVs were comparable to conventional T cell activation. Remarkably, all CD3-LVs allowed efficient transduction of resting T cells by CD3-LVs at rates even similar to fully activated cells. In this non-activated setting, the CD3-LVs clearly outperformed the clinical benchmark vector VSV-LV.Following transduction with CD3-LVs, we observed strong downmodulation of the CD3 target receptor, which was likely caused by vector-mediated T cell activation. Interestingly, inhibition of endocytosis using the V-ATPase inhibitor Bafilomycin A1 or NH_4Cl substantially increased the transduction efficiency of CD3-LVs on cell lines, but not on primary cells, indicating that CD3 downmodulation does not pose an obstacle for CD3-LV gene transfer into primary human T lymphocytes. To assess the potential of CD3-LVs in an "*in vivo*-like" situation, we added the vector particles to human blood without the addition of any further stimuli. With VSV-LV, transduced cells were barely detectable under these conditions. Remarkably, up to 6% of the CD3+ cells were transduced with the CD3-LVs, probably due to their activating capacity. Hence, CD3-LVs seem to be ideally suited not only for T cell transduction under minimally activating conditions, but also for *in vivo* applications. Indeed, upon administration of CD3-LV into NSG mice intraperitoneally transplanted with human peripheral blood mononuclear cells, efficient and exclusive transduction of $CD3^+$ T cells in all analyzed organs was achieved. Taken together, the data strongly support implementation of T cell activating properties within T cell-targeted vector particles. Such particles may be ideally suited for the genetic modification of T lymphocytes *in vivo*.

910. Lentiviral Gene Therapy for $p47^{phox}$ Deficient Chronic Granulomatous Disease: One Step Closer to the Clinic

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Chronic Granulomatous Disease (CGD) is a genetic primary immune disorder with an incidence of 1:200,000 live birth. The disease is caused by defective components of the phagocytic NADPH oxidase, the enzymatic complex responsible for the production of superoxide involved in pathogen killing. Patients affected by CGD are susceptible to recurrent bacterial and fungal infections and chronic inflammation. A phase I/II clinical trial of lentiviral gene therapy is currently underway for the most common X-linked form of CGD (caused by mutation in the CYBB gene encoding for the gp91phox protein), with encouraging results. We propose to use a similar strategy to tackle p47phox-deficient CGD (p47-CGD), caused by mutations in the NCF1 gene which encodes the p47phox cytosolic organizer of the enzymatic complex. p47-CGD accounts for approximately 25 % of all CGD cases in western countries while its frequency rises in regions with high degree of consanguinity. For our gene therapy protocol, we have developed a lentiviral vector 'the pCCLChim-p47' that drives high levels of p47phox expression in myeloid cells. The lentiviral vector, contains the chimeric cathepsin G/c-fes myeloid promoter and a codon-optimised version of the human NCF1 cDNA. When tested in vitro in granulocytes derived from hematopoietic stem and progenitor cells (HSPCs) taken from p47-CGD patients, the vector was able to restore up to 70% of functional neutrophils. Furthermore, transplantation of corrected p47-CGD HSPCs into NSG mice led to the rescue of p47phox expression in a high percentage of human neutrophils. In vitro immortalization assay and in vivo genotoxicity studies using the p47phox knock-out mouse model showed that the vector is not mutagenic, and does not alter blood parameters or lineage distribution in hematopoietic organs. Overall, our results support the clinical development of a gene therapy approach using the pCCLCHIM-p47 vector.

911. Investigating the Stability of Lentiviral Vector Targeted Liver Cells During Post-Natal Growth for In Vivo Gene Therapy Applications

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Liver-directed gene therapy with adeno-associated viral (AAV) vectors delivering a clotting factor transgene has shown successful results in adults with hemophilia. However, because AAV vectors do not actively integrate into the host cell genome, they are diluted upon liver growth, thus challenging their proficient use in pediatric patients. In contrast, lentiviral vectors (LV) integrate into the target cell chromatin and are replicated with the cell genome. We have developed LV that achieve stable transgene expression in the liver of adult mice and dogs, following systemic administration. We recently generated improved phagocytosis-shielded LV, which, upon intravenous (i.v.) administration to non-human primates (NHP), showed selective targeting of liver and spleen and enhanced hepatocyte gene transfer, providing up to supra-normal activity of both human coagulation factor VIII (FVIII) and factor IX (FIX) transgenes, the genes mutated in hemophilia. These studies support further pre-clinical assessment and clinical evaluation of liver-directed LV gene therapy for hemophilia. In view of a potential use of LV in pediatric patients, we treated 3 hemophilia B puppies of 2-4 months of age by i.v. administration of LV expressing canine FIX. Reconstituted FIX activity initially decreased over the first months post LV, then it remained stable for up to 3.5 years post LV. To better understand this finding, we turned to mice and performed longitudinal studies with LV encoding luciferase or FIX. In mice treated as newborns, we observed a stable luciferase total signal over time, which decreased around 3 weeks if normalized on mouse weight, similarly to what observed in dogs. By contrast, when we treated newborn mice with LV-GFP we observed a continuous increase in size of GFP+ clusters of cells by 3D imaging analysis,

showing local proliferation and long-term maintenance of transduced hepatocytes, thus suggesting a decrease in transgene expression *per* cell rather than reduction of targeted hepatocytes during growth. We confirmed the same pattern in LV-FIX treated newborn mice, while administration in 2-week old mice resulted in stable and 3-fold higher FIX output than that observed in mice treated as neonates. We evaluated clonal proliferation of LV-transduced and untransduced hepatocytes in Alb-Cre/Rosa26-Confetti mice during growth and showed that only a fraction of cells generates continuously growing clusters, while most of the others appear quiescent. Our data show that timing of LV administration impacts on gene therapy efficacy and suggest that different hepatocyte subpopulations contribute to liver growth. Our work will inform about the mechanism underlying long-term maintenance of LV-transduced hepatocytes in newborn mice and may provide a rationale for application of LV-mediated liver gene therapy to pediatric patients. *Equal contribution #These authors share senior authorship

912. Development of Cas9 Protein Delivery Non-Integrating Lentiviral Vectors for Gene Correction in Sickle Cell Disease

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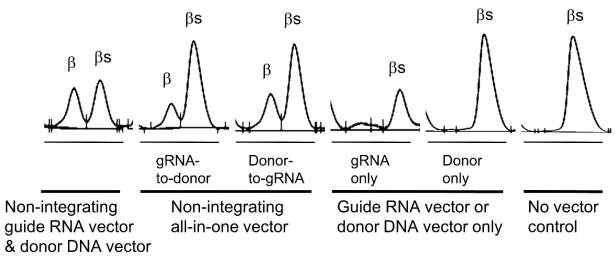
Gene correction is an ideal gene therapy for hereditary disease, including sickle cell disease (SCD). Recently, the CRISPR/Cas9 system was developed to allow site-specific DNA breakage, which enhances gene correction with donor DNA; however, efficient delivery remains crucial. We previously developed Cas9 protein delivery non-integrating lentiviral vectors encoding both guide RNA (gRNA) and donor DNA, demonstrating efficient gene conversion (up to ~30%) using a GFP-to-YFP gene correction cell line model (ASGCT 2016). In this system, Cas9 protein is delivered with lentiviral particles that don't integrate Cas9 DNA, possibly decreasing their genotoxicity and improving vector delivery by removing the need for large Cas9 DNA (4.3kb). Here, we hypothesized that Cas9 protein binds to the gRNA sequence encoded in the lentiviral RNA genome, since Cas9 protein functions to bind gRNA. To test this, we split the non-integrating all-in-one vector (encoding both GFP-targeting gRNA and YFP donor DNA) into separate gRNA and donor DNA vectors, and Cas9 protein was added to each vector during lentiviral preparation. A GFP+ cell line was transduced with (1) Cas9 protein delivery all-in-one vector encoding both gRNA and donor DNA, (2) gRNA-encoding Cas9 protein delivery vector and donor DNA vector, and (3) gRNA vector and donor DNA-encoding Cas9 protein delivery vector. We observed more efficient GFP-to-YFP gene conversion with the all-in-one vector (YFP 23% and GFP 34%) and gRNA-encoding Cas9 protein delivery vector (YFP 14% and GFP 42%), compared to the donor DNA-encoding Cas9 protein delivery vector (YFP 7% and GFP 70%) and an untransduced control (YFP 3% and GFP86%). These data demonstrate that Cas9 protein is dominantly delivered by binding to gRNA encoded in the lentiviral genome. We then inserted multiple gRNA sequences (n=2-9) without additional promoters, permitting Cas9 protein binding without additional transcription. The addition of multiple gRNAs resulted in a strong increase in DNA breakage (GFP 12-35%) and a slight increase in GFP-to-YFP gene conversion (YFP 5-20%), compared to the all-in-one vector without additional gRNA (YFP 16% and GFP 44%) and an untransduced control (YFP 0% and GFP 88%). Greater amounts of Cas9 protein were detected in a multiple gRNA vector by Western blotting. These data demonstrate that adding gRNA sequences enhances Cas9 protein delivery and thus DNA breakage. As a therapeutic model for SCD, we designed several gRNAs targeting the β-globin gene with the SCD mutation (βs-globin) and demonstrated that the optimal gRNA for enhancing lentiviral Cas9 protein delivery is different from that for DNA targeting for genome editing. A non-integrating gRNA vector encoding an optimal gRNA for both DNA targeting and Cas9 protein delivery resulted in robust DNA breakage (56%) of βs-globin gene in human immortalized erythroid cells with the SCD mutation, allowing for efficient gene correction (21-46% at the protein level) of βs-globin gene with addition of a non-integrating donor DNA vector encoding a normal β-globin gene, as well as with Cas9 protein delivery non-integrating all-in-one vectors (Figure). In summary, we developed a Cas9 protein delivery system with lentiviral vectors, allowing for efficient one-time gene conversion with a non-integrating lentiviral vector encoding both gRNA and donor DNA. Our findings improve the prospects for safe delivery and efficient genome editing in SCD.



913. Cell Type Specific Co-Factors are Required for IFITM3 Antiviral Activity in Human Hematopoietic Stem and Progenitor Cells

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Innate immune factors may restrict hematopoietic stem cell (HSC) genetic engineering and contribute to broad individual variability in gene therapy outcomes. We have recently shown that the interferon-induced transmembrane protein 3 (IFITM3) constitutively blocks VSV-g mediated lentiviral vector (LV) entry into HSC and can be efficiently counteracted by the non-immunosuppressive Cyclosporine H (CsH) that transiently targets IFITM3 for lysosomal degradation in HSC. Several post-translational modifications play a crucial role in IFITM3 antiviral function and turnover but the molecular players involved remain elusive, and how CsH counteract IFITM3 is still unknown. We show here that cell-type specific co-factors are required for IFITM3 antiviral effects and the capacity of CsH to degrade it as IFITM3 over-expression recapitulates LV restriction and CsH

sensitivity in THP-1 but not in K562 cells. We excluded involvement of CypA, calcineurin and the known CsH target, FPR1 through targeted knockout/knockdown experiments. In search of other candidates, we compared the transcriptomes of CsH-sensitive HSC and THP-1 with that of the insensitive K562 at steady-state and upon IFN-stimulation. 450 genes were differentially regulated (adjusted p-value<0.01) in cyclosporin-responsive vs. non-responsive conditions. Cholesterol biosynthesis, antiviral responses as well as protein degradation and processing were among the top enriched pathways in cyclosporin responsive conditions. To address the impact of CsH at the protein level, we performed global proteomics in primary human HSC exposed or not to the drug. Out of 3136 proteins identified, CsH significantly downregulated 100 and upregulated 140 proteins (T-test FDR<0.05). Pathway enrichment analysis revealed that proteins upregulated by CsH are mainly involved in metabolic processes such as fatty acid and glucose metabolism as well as protein breakdown. To further narrow down on the players involved in IFITM3 antiviral effects and CsH sensitivity, we immunoprecipitated IFITM3 from primary human HSC, THP-1 and K562 exposed or not to CsH. 136 IFITM3 interactors were identified among the three cell types by Mass-Spec with some factors displaced from and others recruited to IFITM3 by CsH. Phagosome activity (KEGG, p= 0.00008911) and endocytosis (KEGG, p= 0.0004013) were among the top enriched pathways in agreement with known antiviral activities of IFITM3. We are in the process of screening the most relevant interactors in terms of their impact on IFITM3 antiviral activity and sensitivity to CsH-mediated degradation. Overall, our work will help uncover the mechanisms governing IFITM3 antiviral effects as well as unravel potential novel targets for improved gene therapy and antiviral approaches.

914. Safety Evaluation of Intracerebral Injection of Lentiviral *ABCD1* for X-ALD Gene Therapy

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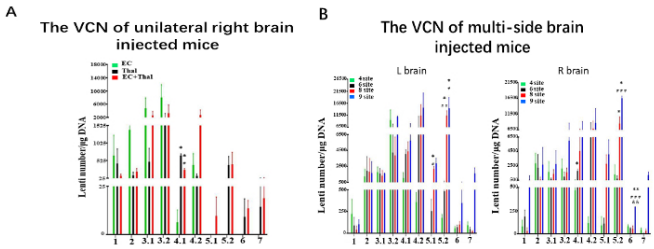
Background: X-linked adrenoleukodystrophy (X-ALD) is a genetic disorder due to *ABCD1* gene deficiency. *ABCD1* gene belongs to the ATP-binding cassette (ABC) transporter superfamily, which is located on Xq28 and codes for ALDP, a peroxisomal membrane protein. The malfunction of *ABCD1* leads to accumulation of very long chain fatty acids (VLCFA) in plasma and all body cells especially in central nervous system, which causes severe neurological dysfunction and death. The treatment for X-ALD is limited due to its irreversible disease progression. Gene therapy to correct the genetic defect has been one of the most promising treatments for X-ALD. ***Results:*** In this study, we aim to target X-ALD deficiency via direct intracerebral injection of lentiviral *ABCD1* (LV-*ABCD1*) gene in the central nervous system (CNS) in order to ameliorate the major neurological symptoms. Bone marrow cells from X-ALD patients and control neuroblastoma cells were transduced with an EF1αpromoter driven *ABCD1* lentiviral vector TYF-*ABCD1* (MOI 20 and 10, respectively), and vector integration copy number (VCN per μg genomic DNA) was determined by qPCR. High TYF-*ABCD1* transduction rate was detected in PT#11 (VCN=150.34), PT#13 (VCN=68.81) and neuroblastoma (VCN=82.64) cells. Intracellular immunostaining and Western blot analyses demonstrated that as compared to control, lentiviral modified X-ALD patients' cells showed high expression of ABCD1. For*in vivo* experiments, we injected TYF-*ABCD1* vector and a control green fluorescence reporter vector into the brain of C57BL/6J mice to access the safety and feasibility of the direct gene therapy approach. The biodistribution, toxicity and safety of the intracerebral lentiviral injection were analyzed on 7-week old male mice. The vector titer of TYF-*ABCD1* was above 10^9TU/ml. Vector distribution and VCN were examined in the brain, spinal cord, blood, urine, feces and various tissues. The *in vivo* expression of ABCD1 was quantitatively determined by direct immunofluorescence and confocal microscopy analysis. The results confirmed supraphysiological expression of ABCD1 in the injection sites. The mice that received TYF-*ABCD1* showed normal activities and continued to gain body weight. Tissue biopsies were examined after LV injection. In unilateral right side injected mice, VCN ranged from 6.37 to 7998.71 on the right brain (Fig.1A), and 6.57 to 205.57 on the left brain, indicating vector distribution from one side to the other. In multi-site cerebral injection groups (4, 6, 8, 9 sites, Fig.1B), the VCN ranged from 24.78 to 16573.3 after bilateral injections, indicating that TYF-*ABCD1* transduced most brain regions and spread to the cerebellum (Fig.1B). Confocal microscopy images illustrated supraphysiological ABCD1 expression surrounding the injected sites. For safety evaluation, we examined neuronal loss, astrogliosis and microglia activation and did not detect any abnormal changes in the injected brains. The overexpression of ABCD1 protein in the brain did not result in neural inflammation and immunopathological toxicity. ***Conclusion***: Our study has extensively demonstrated the feasibility and safety of intracerebral TYF-*ABCD1* injection. Ongoing preclinical study in *ABCD1* knockout mice and clinical trials will be necessary to illustrate the therapeutic potential of CNS lentiviral gene therapy to target neurological disease in X-ALD patients.



915. Profiling of Encapsidated Producer Cell RNA in Lentiviral Vectors

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Retroviruses package specifically host tRNA that are useful for reverse transcription of their genomic RNA, however, other classes of host cellular RNAs can also be encapsidated in the virion. Specifically, noncoding RNAs (ncRNAs) such as the 7SL RNA, a signal recognition particle RNA, can be preferentially packaged. Although ncRNAs can amount up to 50% of the virion RNA mass and the roles of ncRNAs are not yet fully understood, investigations of these packaged ncRNAs in retroviruses, including the human immunodeficiency virus 1 (HIV-1) is limited. To profile the encapsidated producer cell RNAs in lentiviral vectors (LV), we sequenced RNA extracted from three different lentiviral vector batches on the Illumina MiSeq platform. After quality filtering and Illumina adapter trimming, sequences were mapped to both the human genome (GRCh38) and vector sequences by means of the Burrows-Wheeler Alignment (BWA) tool. The correctly aligned reads were extracted based on the SAM flag information and then annotated to define the nearest RefSeq gene, and its biotype, at its map location. Controls included an in-silico dataset of simulated reads and a batch containing capsids devoid of the vector gRNA. An initial analysis of the sequences (7,717,758, 6,079,694 and 7,195,601 raw reads for the three lentiviral vectors, respectively) verified that our data fall in line with the HIV-1 parental virus, showing an abundance of mainly producer cell ncRNAs, within the capsids. As expected, the majority of reads aligned to hg38, where the 7SL RNA was one of the recurrent ncRNAs in the three LV. The reads obtained were used to perform a more in-depth analysis based on the clustering of annotated ncRNAs. The identified clusters were divided into homogeneous clusters, with contributions from only one sample, and heterogeneous clusters, with contributions coming from different samples to identify inter-sample variations in terms of packaged producer cell line ncRNAs. Gene ontology analysis on the cluster reads mapped to protein coding RNAs and a preferential packaging for RNAs related to ATP-binding, alternative splicing and metabolic activity processes. Additionally, these findings have been independently evaluated with highly sensitive intermediate CCS (Circular Consensus Reads) using the PacBio platform (IsoSeq) sequencing and SMRT Link software suite (v5.1.0.26412). These results will contribute towards the full characterization of LV packaged RNAs to assess and quality control LV-based gene therapy vectors.

** Senior authors to this work.*

AAV Vectors - Virology and Vectorology IV

916. A Novel Approach for Rapid and Transient Depletion of Pre-Existing Neutralizing Antibodies Against AAV Vectors

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Pre-existing humoral immunity to recombinant adeno-associated viral (AAV) vectors poses a significant challenge to patient recruitment in gene therapy clinical trials. Further, neutralizing antibodies (NAbs) are a significant hurdle to vector re-dosing, which might be essential for patients receiving an ineffective first dosage or to ensure sustained therapeutic gene expression in pediatric patients through adulthood. Strategies to evade pre-existing anti-AAV antibodies can include engineered capsids with distinct antigenicity or plasmapheresis, which can be utilized in combination. In addition, immunosuppression to prevent formation of NAbs during vector administration is being explored actively by several groups. Here, we propose a novel orthogonal approach to transiently deplete antibodies by enabling accelerated antibody clearance. As proof of principle, we utilize the IgG specific protease IdeZ (IgG-degrading enzyme of *Streptococcus equi ssp. equi zooepidemicus*) for targeted degradation of pre-existing antibodies. This cysteine protease, which cleaves IgG at the hinge region, removes the Fc region essential for binding Fc receptors and consequently, the long circulation half-life of antibodies. We demonstrate that IdeZ is cross-species compatible and efficiently cleaves IgG in mouse, rabbit, dog, primate and human sera. We synthesized recombinant IdeZ, which when dosed intravenously, efficiently cleared human IVIG administered to mice in less than 24 hours. Consistent with ongoing clinical trials, IdeZ was able to cleave human IVIG *in vivo* at clinically relevant doses without any side effects. IdeZ pre-treatment prevented human IVIG-mediated neutralization of distinct AAV clades (AAV3, AAV5 and AAV8) *in vitro*. Importantly, IdeZ rescued AAV8 liver transduction in human IVIG treated mice. Further, IdeZ also rescued AAV9 transduction in the liver and heart of mice passively immunized with 20 individual human serum samples containing anti-AAV antibodies. While results from vector redosing and non-human primate studies are forthcoming, we postulate based on the aforementioned results that IdeZ mediated antibody depletion represents a readily adaptable approach for clinical translation. Furthermore, extrapolation of the antibody clearance approach outlined in this study provides a foundation for exploring other pharmacological agents such as anti-Fc receptor monoclonal antibodies or soluble Fc receptor proteins for eliminating pre-existing antibodies to enable AAV gene therapy in the clinic.

917. *In Situ* Detection of Adeno-Associated Virus Genomes with DNA SABER-FISH

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Recombinant adeno-associated viruses (AAVs) are a promising modality for the treatment of genetic disorders with multiple recent successes in human patients. Despite these advances, a number of simple yet therapeutically relevant concepts regarding AAVs remain incompletely understood, in part due to the lack of readily adoptable methods to track viral particles or genomes. Here, we describe a novel application of DNA signal amplification by exchange reaction fluorescence *in situ* hybridization (SABER-FISH) that enabled the visualization and quantification of individual AAV genomes in the mouse retina. Using a serotype 8 AAV encoding a cytomegalovirus (CMV) promoter-driven GFP (AAV8-CMV-GFP), we performed subretinal injections in neonatal mice and found that intracellular AAV genomes were detectable within 3 hours of vector delivery. Upon maturation of the retina, we quantified AAV8-CMV-GFP genomes in individual rod photoreceptors, cone photoreceptors, and retinal pigment epithelium (RPE) cells and observed a correlation between the number of genomes and the level of transgene activity. Although GFP expression from AAV8-CMV-GFP was confined to photoreceptors and the RPE, we identified multiple cell types in the eye which also contained AAV genomes but did not express the vector, including microglia. Additionally, using 5-ethynyl-2'-deoxyuridine (EdU) to label dividing cells, we found that AAV8-CMV-GFP infected non-mitotic and mitotic cells in the developing retina at similar frequencies but was selectively not expressed in the latter, suggesting a mechanism of AAV or CMV promoter silencing dependent on cell cycle. In summary, DNA SABER-FISH can be used to visualize AAV genomes *in situ*, allowing for studies of viral processing and the tracking of AAV-infected cells following gene therapy administration.

918. Long-Read Characterization of Adeno-Associated Virus Packaged DNA by Direct Nanopore Sequencing Reveals Genome Fusions and Recombination between Genomes and Producer Plasmids

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Rapid and unbiased monitoring of transgene integrity and DNA contaminants in adeno-associated virus (AAV) gene therapy vectors is of major interest, because of clinical applications with increasing therapeutic doses. We here report direct nanopore next generation sequencing (NGS) of AAV single-stranded DNA (ssDNA) using a transposase-based protocol. Direct sequencing is enabled through transposase action on transient hairpins, which we prove by read-start and read-end analysis on circular bacteriophage M13 ssDNA. The technique enables an unprecedented deep view into packaged AAV DNA due to long reads and direct sequencing, in addition to its quick and straightforward sample preparation, which sets it apart from other NGS methods. Unlike short read techniques, our presented method achieves up to full genome read length and revealed single-nucleotide variants across the transgene cassette. Furthermore, assessments on the transgene methylation status were possible. Long reads also span packaged genome-genome and genome-backbone fusions and readily covered the often notoriously difficult to sequence inverted terminal repeats (ITR). Regarding genome-genome fusions, we find that these stem from rolling circle replication rather than rolling hairpin replication, and the former seems to be the dominant replication mode in our recombinant setting. A comparison with qPCR data showed that a qualitative statement on the share of contaminants is feasible and that a quantitative assessment is likely possible. Furthermore, the 3'-packaging signal is accessible with our method, and we confirm previous reports of the AAV p5 promotor as a secondary packaging signal, next to the ITRs. Additionally, single read investigation showed that a large proportion of reads of contaminating sequences from the producer plasmids, but not from human genomic sequences, terminate in an ITR, which got inserted by nonhomologous recombination. These combined findings highlight direct nanopore sequencing as a versatile tool for vector quality control and ongoing basic research on AAV biology to achieve homogenous therapeutics with the possibility to expand the scope of the technique to other ssDNA viruses and bacteriophages.

919. Non-Stringent Requirement in AAV D-Element Leads to Erroneous Encapsulation of Non-Vector Sequences

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Adeno-associated virus (AAV)-based gene therapy vectors have demonstrated promise for the treatment of a wide variety of diseases. One of the major concerns regarding current AAV-based treatments is vector-related impurities. These impurities are found in even highly purified preparations and cannot be separated by processing technology because they are packaged into the mature viral capsids and closely resemble the vectors themselves. One contaminant commonly observed is the plasmid backbone sequence. To fully dissect the cause of such erroneous encapsidation, we systematically analyzed the molecular basis of D element in the AAV vectors since D-element plays a pivotal role in the life cycle of AAV, such as genome rescue, replication, and integration. In this study we have generated a highly diverse random D-sequence library for analysis of characteristics of the D-sequence in recombinant AAV vector production. Unique to our model system, there is no wild type AAV D-element present. Core sequences of the terminal resolution sites (trs) site were replaced with randomized 8, 18, and 24 oligonucleotides. A highly diverse vector library harboring randomized oligonucleotides in ITR-D sequence were generated and vectors were produced. The recovered D sequences show very low stringency compared to wild type D sequences. To confirm this observation, vector plasmids with pseudo "D" sequence for typical plasmid backbone without any wild type D sequences were generated and used for vector production. Surprisingly, vectors can be produced with pseudo "D" sequences, with a yield of approximately 10% of regular vector containing the normal D sequence. In conclusion, the lax requirement of wild type D sequence is the cause of plasmid

backbone contamination in vector preparations in the current system. A production system without plasmid bone contamination will be presented and discussed.

920. In-Depth Parallel Profiling of Tissue and Cell-Type Tropism of AAV Variants by Single-Cell RNA Sequencing

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Adeno-associated viruses (AAVs) are popular gene delivery vehicles and there is a continuing high demand for AAV variants with improved transduction efficiency and specificity. Directed evolution and/or rational design have been used extensively to engineer the capsid of naturally occurring AAVs in order to better customize their properties for research and clinical work. While these engineering approaches are scalable and have generated useful variants, the subsequent transduction profiling of these variants remains either low throughput or lacks resolution across the many relevant cell and tissue types. Single-cell RNA sequencing (scRNA-seq) via droplet-based methods allows in-depth profiling of gene expression of several thousand individual cells. We established a tissue processing and data analysis pipeline that leverages the capabilities of scRNA-seq to achieve simultaneous characterization of AAV variants across multiplexed tissue cell types. To verify our approach, we retro-orbitally co-injected C57Bl/6 mice with PHP.eB (Chan et al., Nat. Neurosci., 2017) and a neuron-biased PHP.eB-evolved variant (Flytzanis*, Goeden* et al., ASGCT, 2019), each packaging a construct expressing different fluorophores. After two weeks of expression we harvested the brain and used one hemisphere for characterization by traditional immunohistochemistry and one hemisphere for characterization by scRNA-seq. Single-cell libraries were prepared with the Chromium Single Cell Kit by 10x Genomics and analyzed with multiplexed Illumina sequencing. As a proof of concept, we compared the two characterization methods by analyzing the infection rate of neurons (NeuN), astrocytes (S100b) or oligodendrocytes (Olig2). For immunohistochemistry, a cell was classified as infected based on expression of fluorophores while in scRNA-seq transduced cells were identified based on the presence of defining viral transcripts. Louvain community detection method (Blondel et al., J. Stat. Mech., 2008) followed by analysis of significantly differentially expressed genes was used to identify cell types in the scRNA-seq data set. Given the differences in RNA and protein abundance and detection thresholds between imaging and sequencing, the two characterization methods detect different absolute numbers of infection rates; however, the cell type transduction biases are consistent among the three different cell types we tested. After verifying our method, we further explored the data set beyond major cell types and discovered previously unnoticed sub-cell type enrichments in, for example, cortical inhibitory neurons. These findings are being confirmed by mapping mRNA expression using *in situ* hybridization chain reaction. Besides sub-cell type tropism characterization, we are analyzing the transcriptome of infected and non-infected cells in search of mechanistic insights into AAV transduction that could facilitate rational design of recombinant AAVs with disease-relevant cell-type specificity. Our approach will aid the gene therapy field to both characterize more thoroughly existing recombinant AAVs and guide engineering of novel AAV variants.

921. Novel AAV Vector for Delivery of Transgenes to the Photoreceptors and RPE via Intravitreal Injection Enables Rescue of Multiple Models of Retinal Disease

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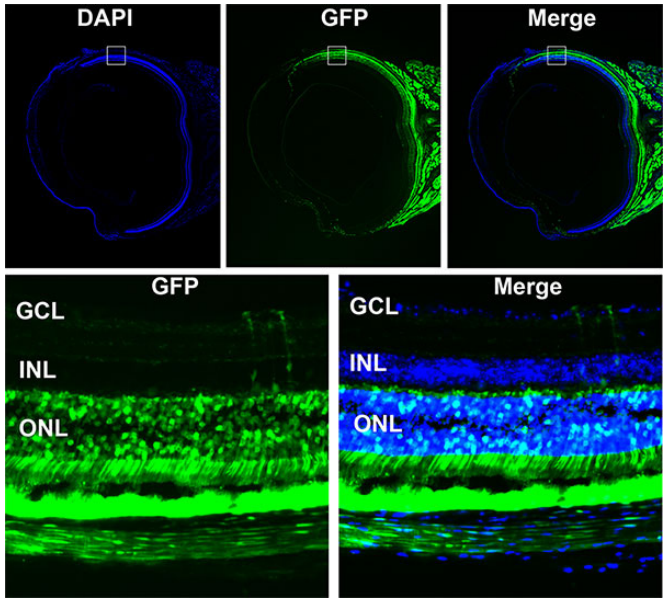
Purpose: The majority of genetic retinal disorders involve the photoreceptors or the RPE. AAV-mediated gene delivery to these cells generally requires a subretinal injection- a surgical approach with significant risks. In contrast, intravitreal injections are an office procedure with an excellent safety profile. Recombinant AAV vectors do not generally penetrate the retina and infect photoreceptors or RPE when injected into the vitreous. The purpose of our study was to develop an AAV vector that can infect cells in the outer retina following intravitreal injection. **Methods:** We have developed a novel cell penetrating peptide (Nuc1) that can deliver recombinant proteins into cells of the outer retina following intravitreal injection. Interestingly, this peptide does not need to be chemically linked to its cargo. We discovered that in addition to the delivery of proteins or antibodies into the outer retina, Nuc1 could facilitate the penetration of very large molecules including AAV vectors deeper into the inner retina. To expand these observations, we incorporated the Nuc1 sequence directly into the AAV coat proteins. Using this backbone, we generated GFP-expressing AAV vectors and injected them intravitreally in mice. In addition, we generated AAV vectors expressing NRF2, R-Ras, Aflibercept and Decorin. Each of these vectors were injected into the vitreous of an appropriate murine model of retinal disease. **Results:** Mice injected intravitreally with novel AAV expressing GFP exhibited pan retinal expression of GFP in the photoreceptors, RPE, bipolar cells and retinal ganglion cells. NRF2 expressing virus injected into the vitreous significantly reduced oxidative stress in wild type and NRF2 -/- mice following injection with MNU. R-Ras and Aflibercept -expressing AAV significantly reduced laser induced choroidal neovascularization (CNV). Decorin-expressing AAV significantly reduced CNV and fibrosis in the retina. **Conclusions:** We have generated a novel AAV vector that can deliver transgenes to the outer retina including photoreceptors and RPE following intravitreal injection. We have demonstrated efficacy of this vector in several animal models of retinal degeneration.



922. Brain Transduction Profiles of Novel Human-Derived Clade B and C Variants in Mice and Non-Human Primates

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Recombinant adeno-associated viruses (rAAVs) are the ideal vectors for human gene therapy because of their excellent safety profiles and therapeutic transgene delivery efficacies. Currently, the AAV serotype 9 (AAV9) capsid remains the gold standard for targeting the central nervous system (CNS). Efforts to discover novel AAVs with improved transduction efficiencies, unique neuronal cell-type specificities, and reduced host immunities continue to be major focuses for the gene therapy field. Using single molecule, real-time (SMRT) sequencing of proviral capsid libraries isolated from >800 human surgical specimens, we discovered a collection of novel clade B and C variants. Among these AAV capsid variants, approximately 28.5% exhibit higher packaging yields than prototypical AAV2. We screened a selection of 176 variants for *in vivo* brain tropism in NHPs by pooled injection of multiple capsids that each package a unique barcoded transgene via magnetic resonance imaging (MRI)-guided intracerebroventricular (ICV) administration. Using this approach, we have identified >20 variants that can confer brain transduction levels that are comparable or better than AAV9 in specific brain structures (hippocampus, cerebellum, and cortex) . In addition, these variants also showed higher transcript abundances (transgene RNA) per vector genomic copy (transgene DNA) in different brain tissues than AAV9. These results indicate that the selected capsids are more efficient at intracellular trafficking, endosomal escape, and/or nuclear import. Top 7 candidate capsids from each clade were then packaged with the eGFP reporter transgene and tested individually in mice by intra-hippocampal injections followed by fluorescence microscopy. Interestingly, the clade B capsids conferred better transduction in the brain than that of clade C capsids, demonstrating inter-species tropism differences among the variants. Importantly, several clade B variants performed better than AAV9 in transducing the hippocampus. Evaluation of amino acid variation within the VP3 (capsid surface) region hint at functional differences between the variants. However, differences among VP1-unique and VP2 regions, which correlate with quantifiable tropism differences between capsids, remain the most intriguing finding. Overall, we have identified a novel population of clade B and C variants from natural isolates with high brain transduction profiles. This work helps to expand the toolkit of vectorized AAVs and are potentially suitable for clinical utility. [a]Co-corresponding authors

Musculo-skeletal Diseases II

923. Abstract Withdrawn

924. A Universal Gene Correction Approach for FKRP Mutant Patient-Specific iPS Cells: Potential for Autologous Cell Therapy

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Mutations in the fukutin-related protein (*FKRP*) gene result in a broad spectrum of muscular dystrophy (MD) phenotypes, with most cases being classified as congenital MD type 1 (MDC1C) and limb-girdle MD type 2I (LGMD2I), but also including the rare and severe Walker-Warburg Syndrome (WWS). The hallmark of FKRP-associated diseases is the hypoglycosylation of α-dystroglycan (α-DG), which leads to disruption in the interaction of α-DG with extracellular matrix (ECM) proteins, in particular laminin-α2. This results in loss of anchoring of

the contractile apparatus to the ECM, culminating in cycles of muscle fiber damage, and ultimately, muscle degeneration. In this study, we developed a universal gene editing approach to correct FKRP mutations in iPS cell lines from three patients displaying broad clinical severity: LGMD2I (c.826C>A), CMD (c.217C>T, c.826C>A) and WWS (c.558 dup C, c1418T>G). Among these, only the most severe, the WWS iPS cell line, showed complete absence of α-DG functional glycosylation, which is recognized by the IIH6 antibody. LGMD2I and CMD iPS cell lines stained positive for IIH6, as previously shown in primary samples (Am. J. Pathol. 164:727-737, 2004). Our gene editing approach replaces the entire open reading frame, which is within exon 4. We used a pair of CRISPR-Cas9 ribonucleoprotein complexes to induce double-strand breaks at 5' and 3' ends, which deleted the FKRP coding region. We then used homology directed repair from an exogenous donor vector to successfully knock-in the WT FKRP coding region along with a selection cassette. Following gene editing, we selected iPS cells for antibiotic resistance, and corrected WWS iPS cells were subsequently purified by fluorescence activated cell *sorting* (FACS) for IIH6 positivity. We validated FKRP correction in WWS, CMD, and LGMD2I iPS cells by knock-in specific PCR and sequencing. To confirm the specificity of CRISPR-Cas9 gene editing in the corrected iPS cell lines, we performed PCR amplification and sequencing of the regions encompassing five potential off-target sites for each of the two guide RNAs used for editing. Upon analyzing the sequencing chromatograms using the Inference of CRISPR Edits (ICE) tool, we found no detectable off-target activity in any of the tested sites in all three corrected iPS cell lines. Using conditional expression of PAX7, we next generated gene edited WWS myogenic progenitors, and subsequently differentiated these iPS cells into myotubes expressing myosin-heavy chain (MHC). By IIH6 immunoblotting, we observed rescue of α-DG functional glycosylation in corrected WWS iPS cell-derived myotubes. Importantly, this was accompanied by the restoration of laminin binding capacity, as assessed by the laminin overlay assay. We then transplanted corrected WWS myogenic progenitors into FKRP^{P448L}-NSG mice, and our preliminary results revealed *in vivo* restoration of α-DG functional glycosylation, as shown by the presence of IIH6+ human-derived myofibers. Taken together, these findings provide proof-of-principle for the future therapeutic application of iPS cell-based autologous cell transplantation that could permanently restore gene function in FKRP mutant patients.

925. Effective Pseudo-Exon Skipping of a *COL6A1* Intronic Mutation in Cultured Muscle Interstitial Fibroblasts from a Novel Humanized Mouse Model

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Collagen VI-related dystrophies (COL6-RDs) are a group of frequently severe, congenital-onset muscular dystrophies for which there currently is no effective treatment. Our group has recently identified a new recurrent unexpectedly common *de novo* deep-intronic mutation (c.930+189C>T) associated with a severe COL6-RD phenotype, in the collagen 6 alpha 1 (*COL6A1*) gene. The intronic mutation creates a new donor splice site and in conjunction with a dormant splice acceptor drives the insertion in about 50% of transcripts of an in-frame pseudo-exon that exerts a dominant-negative effect on the collagen VI extracellular matrix assembly. Using morpholino antisense oligonucleotides (PMOs) targeted at potential splice enhancer sites within the pseudo-exon, we have previously shown that the pseudo-exon was skippable, in patient cultured fibroblasts (Bolduc et al., JCI Insight 2019). We have now created a humanized mouse model by knocking in the relevant *COL6A1* genomic region that contains the appropriate intronic context including the mutation, with the goal of testing the pseudoexon skipping approach in preclinical studies. We found that, comparable to human fibroblasts, cultured skeletal muscle interstitial fibroblasts isolated from the KI mouse express the mutant pseudoexon and respond efficiently to the PMOs that were designed for human cells (>90% skipping with 5μM treatment). We synthesized our lead antisense sequence on various chemical scaffolds (phosphorothioate 2'methoxyethyl, and vivo-PMO) to compare their *in vivo* activity. Data collection for systemic injections is ongoing. The *Col6a1/COL6A1* human KI mouse represents an invaluable tool to test human-ready antisense oligonucleotides and assess their potential to target the muscle interstitial fibroblasts, producers of collagen VI.

926. Hematopoietic Stem Cell Gene Therapy Corrects Neuromuscular Manifestations in Preclinical Study of Pompe Mice

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Pompe disease is a genetic disorder caused by deficiency of the enzyme acid alpha-glucosidase (GAA) resulting in accumulation of glycogen in both muscle and central nervous system (CNS). The classic infantile disease presents as a rapid progressive myopathy and if untreated patients typically die within the first year of life. Enzyme replacement therapy (ERT) prolongs life expectancy, but most patients still succumb in early life. High antibody titers, in part, could explain diminished therapeutic responses in ERT treated patients. In less severe late onset Pompe disease patients, ERT results have been inconsistent and often ERT does not halt disease progression. Hence, there is a clear unmet medical need to develop treatment options for these patients that could provide a life-long cure. We hypothesized that overexpression of GAA fusion proteins in the hematopoietic system could target both muscle and CNS compartments and potentially provide a cure. A promoter was selected that has been used previously in hematopoietic stem and progenitor cell (HSPC) gene therapy clinical trials and that provides robust expression. Subsequently, ten lentiviral vectors were constructed with therapeutic transgenes containing fusion tags to enhance secretion and uptake in muscle and CNS through glycosylation independent lysosomal targeting (GILT). Gene-modified HPSCs were transplanted

in irradiation and busulfan conditioned Pompe mice, and followed for 4 months after transplantation. GAA enzyme activity levels were increased in vector containing peripheral blood mononuclear cells, bone marrow, thymus, and spleen. GAA enzyme activity levels could also be detected in plasma and correlated well with GAA protein content. Heart, diaphragm, as well as skeletal muscle had detectable GAA enzyme activities, except for brain. Hex4, a glucotetrasaccharide which is elevated in urine of both patients and mice, was markedly reduced after treatment. Glycogen content was significantly reduced in heart, diaphragm, skeletal muscle, and virtually absent in brain and spinal cord. Biochemical findings were confirmed by histological stainings, and pathology was evidently mitigated. Heart mass was reduced in the GILT-tag treated Pompe mice. Average vector copy number in bone marrow was below 5 and there was no effect on the hematopoietic compartment or glucose metabolism in the treated mice. No significant differences in therapeutic efficacy were observed between irradiation or busulfan conditioning. Certain vectors containing double tags resulted in reduced responses with higher remaining glycogen content and the single GILT tag was the most effective. The conclusion is that using GILT technology to deliver the therapeutic protein by the hematopoietic system to the required tissues mitigated disease pathology in both CNS and muscle. This approach could be a viable option to translate into clinical application and potentially cure Pompe patients.

927. Abstract Withdrawn

928. Follistatin Overexpression in *Sleeping Beauty*-Transposed, *In Vitro* Differentiated Human Plasmablasts: Enhancement of Muscle Mass and Strength in NSG Mice after Adoptive Transfer

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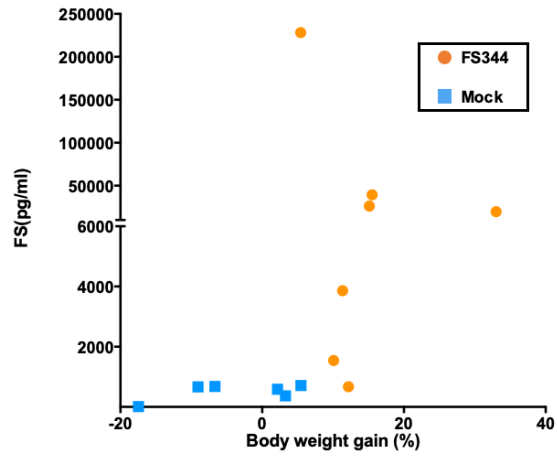
Background: The downregulation of excessive muscle cell regeneration by myostatin can be inhibited by follistatin (FS). Treatment with FS can increase muscle growth and has potential as a supportive therapy for musculoskeletal disorders. Several animal studies support this suggestion; in particular, FS encoded by adeno-associated virus (AAV) can dramatically improve muscle development in mouse models of Duchenne and fascioscapulohumeral muscular dystrophies (DMD, FSHD). We have utilized a proprietary product platform whereby primary human B cells are genetically engineered using a *Sleeping Beauty* (SB) transposon construct encoding a therapeutic candidate protein. The B cells are subsequently differentiated *in vitro* into plasmablasts and adoptively transferred into the mammalian host, where they further differentiate into plasma cells that are capable of durable, high-level production of the desired protein. In humans, long lived plasma cells can persist for decades. The product platform thus presents the opportunity for the long-term systemic delivery of therapeutic biomolecules. Additionally, this gene therapeutic approach utilizes autologous B cells, so antigenicity is not anticipated, and multiple longitudinal doses are possible. In this study we generated primary human B cells engineered to express FS344, an FS splice variant in which a propeptide is cleaved to produce FS315, which is free to circulate in the serum and minimizes localization to gonadal tissues with possible off-target effects. Methods: To facilitate B cell engraftment, mice were given autologous human $CD4^+$ T helper cells seven days prior to B cell treatment. One group of NSG mice (n = 7) was treated with the SB-FS344 B cells, while a control group was treated with mock transposed B cells (n=6). Blood was sampled serially to detect both human IgG (hIgG) and FS by ELISA. Body weights were measured to determine if FS expression effected increase in size. Mice were sacrificed at two months after B cell treatment. Muscles were harvested for weight measurement, contractile force, and histological analyses. Results: High-level hIgG expression (>100 µg/mL) indicated successful B cell adoptive transfer in both treatment and control groups, and FS expression in the experimental group reached approximately 30-fold higher than the background level observed in control mice. FS expression in the treatment group correlated with total body weight gain of about 5-10% over the controls (see figure), and the effect of the overexpressed FS was recapitulated with increased muscle weight and contractile force. Conclusion: FS344 delivered by SB-engineered human B cells enhanced muscle growth and strength in NSG mice. This approach can also be used in mouse models of muscle wasting, whereby prolonged delivery of FS44 may ameliorate disease. These results would lend support to the clinical candidacy of FS344 expressing human plasmablasts in the treatment of human muscle disorders.



929. Combined CNS and Systemic Directed Gene Therapy in a Mouse Model of Pompe Disease with Advanced Disease at Treatment

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Pompe disease is a lysosomal storage disease caused by mutations in the acid-alpha-glucosidase (GAA) gene that lead to glycogen

accumulation in the heart (i.e. cardiomyopathy), muscles, and motor neurons (i.e. neuromuscular disease). The only currently approved therapy, enzyme replacement therapy, has suboptimal efficiency to correct muscle pathology, and cannot cross the blood-brain-barrier, meaning long-term survivors of classic infantile Pompe disease typically experience progressive neurologic deterioration. Adeno-associated virus (AAV)-mediated gene therapy has the potential to target all the tissues involved in the disease pathophysiology, namely muscle, heart, and the central nervous system. We hypothesized that a dual-route of administration approach (i.e. intravenous and into the cerebrospinal fluid) using the same vector would correct both peripheral and neurological manifestations of the disease. We developed an optimized gene therapy vector using a pan-tropic AAV capsid carrying a human GAA transgene engineered to improve both secretion and uptake of the enzyme. This optimized gene therapy efficiently corrected heart, muscle, and CNS pathology after intravenous administration of 2.5 x 10^{13} GC/kg in young Pompe mice, while a 10-fold lower dose was efficacious in heart and muscle only. Because a significant proportion of patients that will be eligible for gene therapy may already have advanced pathology, we elected to treat post-symptomatic Pompe mice (7 months of age) and follow them for at least 6 months post-treatment (in life phase ongoing). Mice received a low or high dose of vector via intravenous, intracerebroventricular, or dual-route of administration. Behavioral and functional endpoints for these mice are being periodically assessed using standard methods until vehicle-treated mice reach humane endpoint. The study is still ongoing and preliminary efficacy results will be presented.

New Techniques in Gene Therapy for Neurological Disorders

930. Monosynaptic Tracing Induced by a Novel AAV Show Appropriate Circuit Integration of Human Dopamine Neurons (in a Rat Model of Parkinson's Disease)

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Summary: Brain repair using human embryonic stem cell (hESC) transplantation in Parkinson's disease has shown great promise as a future treatment option. However, little is known about what directs maturation and circuit integration of transplanted cells. Here, we have used retrograde infectivity of novel AAV capsids in combination with mono-synaptic rabies tracing to investigate the maturation and circuit integration of hESC-derived dopaminergic neurons into a Parkinson's disease rat model. With our AAV delivered methodology, we were able to accurately map and evaluate the connectivity of the host rat neurons onto human transplanted dopaminergic cells. We found that nigral transplantation of identical hESC-derived neurons receive distinctly different afferent input depending on the graft innervation pattern, suggesting a previously unknown directed circuit integration. **Introduction:** Cell transplantation in Parkinson's disease has been intermittently pursued in the clinic over the last four decades. The cell sources have continuously evolved as has the understanding of what governs a successful restoration of the impaired circuitry. Currently, the most broadly used cell source of choice is human embryonic stem cells (hESCs), which are differentiated into dopaminergic (DA) neurons. Yet, it is not fully understood how the transplanted cells integrate into the host circuits, information that is key in order to evaluate and optimize the therapeutic potential of the transplantation therapy in Parkinson's disease. Using a novel assembly and screening method (Davidsson et al., 2019), we previously identified several new AAV capsids with unique properties, where increased retrograde transport was one of the key features. The AAV-MNM008 capsid was identified due to its high efficiency for retrograde transport from the striatum to endogenous DA neurons and also to hESC-derived DA neurons grafted to rat the striatum. In this study, we thus utilized Cre-restricted, AAV-induced mono-synaptic rabies tracing to assess the circuit integration of hESC-derived DA transplants. **Key Results:** We show efficient retrograde transport of our newly developed capsid, AAV-MNM008, to grafted hESCs thought projections in the prefrontal cortex and striatum. Using retrograde transport in combination with monosynaptic rabies tracing, we found the grafts to innervate DA target areas in many areas of the forebrain, neocortical areas as well as thalamic areas. Importantly, we found that the afferent inputs to the grafted neurons are different depending on the cellular DA subtype (A9- or A10-like). **Conclusion:** We have used a novel host-to-graft tracing method to show circuit integration of DA neuron transplants into the brains of a Parkinson's disease rat model. We thoroughly mapped graft connectivity throughout the brain and conclude that most of the vital circuitry is reestablished to transplanted DA neurons. These encouraging findings suggest that the host system is plastic and adaptive after a nigral DA transplant and that the host afferents may be able to differentiate between DA neuron subtypes in the transplants.

Reference: Davidsson M. et al. (2019) A systematic capsid evolution approach performed in vivo for the design of AAV vectors with tailored properties and tropism. Proc Natl Acad Sci U S A, 116 (52)

931. Selective Intein Degradation for Safe AAV Mediated Large Gene Delivery

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Intein-mediated protein trans-splicing is being evaluated to reconstitute large proteins via adeno-associated viral (AAV) vectors, thus overcoming their limited cargo capacity. In this system, a large coding sequence is split into two or more parts each flanked by split-inteins which are independently cloned in AAV vectors. Upon AAV co-transduction and protein synthesis, inteins self-ligate and -excise resulting in the seamless reconstitution of the large protein. We have shown the efficacy of the system in the retina of animal models, however, excised inteins which are a by-product of trans-splicing raise

safety concerns being non-mammalian products. To overcome this limitation, we included in the trans-splicing system a newly designed short destabilizing domain (DD) derived from dihydrofolate reductase that once embedded within the excised intein leads the fused protein to rapid ubiquitination and subsequent proteasomal degradation. The small molecule trimethoprim binds and stabilizes DD thus preventing proteasomal degradation of the fusion protein. DD inclusion in AAV intein vectors reduces the amount of intein following trans-splicing *in vitro* without impairing the protein trans-splicing mechanism of either reporter or therapeutic proteins which are reconstituted at similar amounts to those obtained from AAV intein vectors without DD. By reducing the amounts of intein after trans-splicing, DD inclusion should mitigate the safety concerns around the use of AAV intein vectors for delivery of large genes to the retina. Experiments addressing the efficacy of the platform *in vivo* are ongoing.

932. Allele-Specific AAV-Based Silencing of Mutant Ataxin-3 Alleviates Neuropathology and Motor Deficits in Spinocerebellar Ataxia Type 3

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Introduction: Spinocerebellar ataxia type 3 or Machado-Joseph disease (MJD) is the most common dominantly inherited ataxia worldwide. It is associated with the expansion of a (CAG)n tract in the coding region of the causative gene MJD1/ATXN3, which translates into an expanded polyglutamine tract conferring toxic properties to the ataxin-3 protein, and inducing severe clinical features. Gene silencing targeting both mutant and non-mutant ataxin-3 alleles holds great promise for its treatment. Nevertheless, it is unknown whether neuronal cells in the human brain will tolerate long-term silencing of wild-type ataxin-3. Therefore, we aimed at developing an AAV-based miRNA gene therapy that would promote allele-specific silencing of mutant ataxin-3 and alleviation of MJD upon intracranial or intravenous injection. **Methods:** Specific gene silencing miRNAs targeting SNPs in linkage disequilibrium with the disease-causing expansion were firstly designed and tested in modified neuronal cell lines. An AAV9 vector encoding the most effective artificial microRNA (AAV9-mirATAX3) was then generated and validated in a lentiviral-based mouse model of MJD upon intracranial injection. Next, severely-impaired transgenic mice were intravenously-injected with AAV9-mirATAX3 at postnatal day one (PN1), submitted to behavioral tests at three different time points, to magnetic resonance imaging/spectroscopy (MRI/MRS) at PN75, and sacrificed at PN95. **Results**: The silencing potential of the mirATX3 sequence demonstrated superior specificity in vitro compared to the silencing sequence previously reported. AAV9-mirATAX3's treatment reduced the number of protein aggregates and neuropathology in both animal models and led to significant improvements in behavioral tests in the transgenic model. Moreover, MRI/MRS data indicated that mirATXN3 treatment ameliorates the levels of a specific set of neurometabolites, which can be used as therapeutic biomarkers. The intravenous injection of adult animals further demonstrated that AAV9-mirATAX3 has also the ability to transduce the CNS of adult MJD transgenic mice. **Conclusion:** This study provides compelling evidence that AAV9-mirATAX3 is able to silence mutant ataxin-3 in different SCA3 animal models, through different routes of administration, which may have an important impact on the treatment of MJD.

933. CRISPR/Cas9-Mediated Excision of ALS-Causing Hexanucleotide Repeat Expansion in C9ORF72

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Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the brain and spinal cord. Although mutations in several genes have been implicated in ALS, a clear understanding of the mechanisms that drive ALS progression is still lacking and thus has hindered efforts to find a therapy that could stop or reverse ALS progression. In 2011, a hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{30+}$ in the intronic region of C9ORF72, was discovered to be a major cause of ALS, contributing to about 40% of familial and 10% of total ALS cases. Up to 50% of patients with this expansion also develop Frontotemporal dementia (FTD). Several hypotheses have emerged to explain the mechanism behind this HRE, including haploinsufficiency, RNA binding proteins sequestration in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide production. Both C9ORF72 ALS and FTD are aggressive diseases with no treatments to significantly slow disease progression or extend life expectancy. While understanding the disease mechanisms of these repeats is valuable, gene therapy suggests this understanding may not be essential to treat or cure C9ORF72 FTD/ALS. In order to excise the HRE from the C9ORF72 genomic locus and restore the gene to a normal or healthy state, we are using a CRISPR/Cas9 gene editing approach, in which we designed gRNAs to target the HRE. We delivered Cas9 and gRNAs via AAV9 vectors. Here we demonstrate that we can edit C9ORf72 by excising the HRE in primary cortical neurons and in different mouse models containing the C9ORF72 expanded HRE (ranging from 100-1000 repeats) through various delivery routes. We demonstrate that editing C9ORF72 HRE results in reduction of RNA foci and toxic dipeptides, both hallmarks of C9-ALS/FTD, making this an attractive therapeutic approach.

934. Gene Editing of the Ube3a Antisense-Transcript in an Angelman Syndrome Mouse Model

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Introduction: Angelman syndrome (AS) is a severe, rare neurodevelopmental disorder. Characteristic symptoms include delayed development, intellectual disability, severe speech impairment, problems with movement and balance (i.e. ataxia), and early-onset recurrent seizures (i.e. epilepsy). No curative therapy is currently available for AS. AS develops due to the lack of Ubiquitin-protein ligase E3A (UBE3A; also known as E6AP ubiquitin-protein ligase) expression in neurons. UBE3A expression in neurons is mono-allelic from the maternally inherited allele; the paternally inherited UBE3A allele is silent. Individuals affected by AS have a large deletion or loss-of-function mutations within the UBE3A gene on the maternally inherited allele; this results in a complete loss of UBE3A expression in neurons. One approach to reinstate UBE3A expression in neurons is to unsilence the paternal UBE3A gene that is fully functional but not transcriptionally active. Previous studies have demonstrated that genetically reinstating UBE3A expression in the young Ube3a-ko mouse brain significantly improved symptoms. The silencing of paternal UBE3A expression involves an antisense transcript (ATS) that suppresses the extension of UBE3A mRNA past the transcriptional start site. Here, we sought to interfere with the extension of the ATS into the UBE3A-coding region to enable the full-length extension of UBE3A mRNA and protein expression. **Results:** In a proof-of-concept study, we utilized CRISPR/Cas9 gene editing and achieved Indel formation in the ATS sequence in up to 20% of mouse neurons *in vivo*. In a reporter mouse model that carries a wild-type maternal Ube3a allele and a paternal, silenced Ube3-YFP fusion gene, we demonstrated that Indel formation resulted in unsilencing of Ube3a-YFP and subsequent protein expression. Expression of Ube3a from the maternal allele remained unaffected. In the same study, we did not detect Indel formation (compared to wild-type brain control) or neuronal YFP expression with vectors comprising either non-targeting sgRNA+Cas9 or targeting sgRNA+dCas9 (nuclease-deficient Cas9). To investigate a potential therapeutic benefit of paternal Ube3a unsilencing, we administered neonatal AS model mice with an adeno-associated virus (AAV) encoding the CRISPR/Cas9 gene-editing system. The AS model mice were Ube3a-knockouts on the maternal allele but carried a normal, silenced paternal Ube3a allele. We observed Ube3a protein expression in the neurons of treated AS-model mice but not in vehicle-treated control mice. This result is indicative of paternal Ube3a unsilencing. Protein expression persisted for at least three months. When we subjected the treated AS-model mice to a battery of neurobehavioral tests, we observed significant performance improvements in the rotarod and marble-burying tests. We also observed a strong trend of improvement in open-field activity and the elevated-maze test. **Conclusions:** We conclude that reactivating Ube3a by gene editing in a limited neuronal population can ameliorate the symptoms of AS in a mouse model. This approach may spur the development of a similar translational therapeutic approach in the future.

935. Treatment of a Mouse Model of ALS by CRISPR Base Editing

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Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the progressive loss of motor neurons in the spinal cord and brain, which results in paralysis and ultimately death. While most cases of ALS are sporadic, dominant mutations in the Cu-Zn superoxide dismutase 1 (SOD1) gene are responsible for ~20% of inherited or familial forms of the disease. To this end, we previously demonstrated that CRISPR-Cas9 gene editing technology could be used to disrupt the expression of a mutant SOD1 gene in motor neurons, resulting in delayed disease onset and improved survival in a mouse model of the disorder. However, potential clinical applications of Cas9 nucleases are limited in part by their reliance on DNA breaks, which could lead to deleterious large deletions and chromosomal rearrangements, and non-homologous end-joining, a stochastic DNA repair pathway that could give rise to undesirable and potentially mutagenic outcomes. More recently, CRISPR base editors, a gene-editing modality that can be used to introduce targeted C > T or A > G alterations in DNA, have emerged as an alternative to CRISPR-Cas9 nucleases that could overcome some of their limitations. We sought to harness CRISPR base editing to permanently disable the expression of the mutant SOD1 gene *in vivo* and treat ALS. To accomplish this, we designed cytosine base editors (CBEs) to introduce targeted C > T base substitutions that could create a premature stop codon in the SOD1 coding sequence, thereby silencing its expression. According to qRT-PCR and western blot analyses, we found that the most efficient CBE reduced SOD1 expression by ~50% in HEK293T cells. Deep sequencing of the SOD1 gene further revealed that ~19% of the analyzed reads contained the target C > T substitution. We next sought to determine whether base editing could reduce SOD1 in an animal model of the disease following *in vivo* delivery using an adeno-associated virus (AAV) vector. Though AAV are promising therapeutic gene delivery vehicles, they possess a limited carrying capacity that restricts their ability to deliver a full-length base editor. To overcome this limitation, we created a split-intein CBE compatible with dual AAV particle delivery by fusing N- and C-intein fragments from the DnaB protein of *Rhodothermus marinus* to a CBE that we split into two halves. Following co-delivery, these two intein-containing halves participate in a protein trans-splicing reaction that reassembles the full-length CBE. We packaged the N- and C-terminal split-intein CBE into AAV9 and injected 56- to 60-day-old G93A-SOD1 mice with 8 x 10^{10} particles each of the N- and C-terminal vector encoding either the sgRNA targeting the SOD1 gene or the mouse Rosa26 locus into the lumbar CSF. Immunofluorescent analysis revealed widespread CBE expression throughout the spinal cord by four weeks post-injection. In particular, we determined that ~80% of the cells in the anterior horn expressing the reactive astrocyte marker GFAP were positive for the CBE. Importantly, compared to control animals, we found that mice treated by base editing had an ~11% increase in mean survival and an ~39% increase in disease duration. Behavioral analysis further revealed that treated mice had improved motor function by rotarod and lost

weight ~43% slower following disease onset. Moreover, compared to control mice, we found that base-edited animals had ~40% fewer SOD1 reactive inclusions in the ventral white matter, ~28% less inclusions in the anterior grey column, smaller and less hypertrophic astrocytes, and the target C > T edit in the mutant SOD1 gene. This study thus demonstrates that a split-intein CBE can be used to lower mutant SOD1 in the spinal cord and improve therapeutic outcomes in a mouse model of SOD1-linked ALS.

936. Direct Vagus Nerve Injection of AAV9 as a Treatment Approach for Autonomic Dysfunction in Giant Axonal Neuropathy

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Giant axonal neuropathy (GAN) is a rare pediatric disorder characterized by progressive sensory and motor neuropathy that presents as early as 3 years of age and with mortality typically by the third decade of life. GAN is caused by autosomal recessive loss-of-function mutations in the *GAN* gene. While GAN is primarily described as a progressive peripheral neuropathy, pathology is apparent throughout the autonomic nervous system (ANS) and patients frequently present with enteric and autonomic dysfunction. An NIH-sponsored Phase I study (NCT02362438) is underway to test the safety of lumbar-injected, intrathecal (IT) delivered scAAV9/JeT-GAN to treat the most severe aspects of GAN, namely the motor and sensory neuropathy. The distribution of IT-delivered AAV9 to peripheral ganglia is limited, so we explored injecting AAV9 directly into the vagus nerve to target genes to ANS neurons in adult rats. In agreement with previous studies, there was efficient targeting of vagal neurons of the nodose ganglia, dorsal motor nucleus, visceromotor neurons and fibers of the nucleus ambiguous, and viscerosensory neurons and fibers of the nucleus tractus solitaries. Further, we assessed the feasibility to target genes to ANS neurons in subjects already treated with an IT injection of AAV9. Rats were immunized with AAV9 by an IT injection of AAV9/GAN and then received a direct vagus nerve injection with AAV9/GFP 4 or 14 weeks after the IT injection. AAV9-immunized rats had efficient GFP transduction in vagal nerve fibers and neurons, albeit to a reduced level as compared to naïve rats. Histological analysis revealed an excellent safety profile without evidence of neuroinflammation or significant chronic inflammatory infiltrates. We then tested this approach in a GAN rat model that we have previously developed. Adult GAN rats received a single IT or a combination IT and vagus nerve injection of AAV9/GAN or vehicle and were monitored for up to 20 months post-injection. Importantly, the follow-up monitoring indicated that the injections of AAV9/GAN were well-tolerated. Rats were implanted with telemetry devices to record blood pressure, heart rate and body temperature under basal conditions and following ANS stimulation. Analysis of ANS function and post-mortem assessments of ANS pathology and vector biodistribution are ongoing. If successful, a simultaneous dual-route administration could be proposed for future patients. To our knowledge, this approach has not been utilized in large animals or humans, so we performed a pilot safety and feasibility study in dogs. Adult hounds received a direct vagus nerve injection of increasing volumes of vehicle and were allowed to recover for 2 weeks. Vocalization, weight, eating and fecal output were monitored pre and post-surgery. Overall, the injections were well-tolerated at all doses and minor changes to vocalization and frequency of fecal output in animals returned to baseline by the end of the study. Post-mortem analysis showed that vagal nerve fibers and neurons were microscopically normal. Future studies will include the assessment of direct vagal nerve injection of AAV9/GAN in naïve or pre-immunized non-human primates. If successful, the intention would be to translate this approach into clinical testing for patients with GAN and could include patients that already participated in the AAV9 IT clinical trial. Results from these studies are immediately applicable to GAN but may extend to treating a wider variety of neurological diseases.

Gene Therapies for Hemophilia and Immune Disorders

937. Modeling, Optimization and Comparative Efficacy of HSC- and T-cell Based Editing Strategies for Treating Hyper IgM Syndrome

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HIGM1 is caused by mutations of *CD40L*, whose absence in CD4 T cells impairs their helper signaling for B cell activation and Ig class-switching. Since its unregulated expression leads to lymphoproliferations/lymphomas, we aimed to correct *CD40L* while preserving physiologic regulation. Corrected autologous T cells could provide prompt therapeutic benefit to patients by resolving pre-existing infections and, if not lasting, bridge them towards a definitive cure by hematopoietic stem cell (HSC) transplant. To investigate this strategy, we infused wild-type (WT) T cells into HIGM1 mice conditioned or not with different lymphodepleting regimens and achieved long-term, stable T cell engraftment and confirmed partial rescue of antigen-specific IgG response and germinal center formation in splenic follicles after TNP/KLH vaccination. Remarkably, infusion of T cells from mice previously exposed to the antigen, better modeling the harvest of autologous cells from patients, was effective even in absence of conditioning. Moreover, these levels of immunologic reconstitution were sufficient to control a common HIGM1 pulmonary infection by opportunistic Pneumocystis, further highlighting the therapeutic

value of this approach. We thus designed a gene editing strategy, based on CRISPR/Cas9 electroporation and AAV6 transduction, to insert a corrective cDNA in the first *CD40L* intron of human T cells and correct most disease-causing mutations without risk of expression upon off target integration. By exploiting a culture protocol that preserves long-term surviving T stem memory cells, we reproducibly obtained ~35% editing in both healthy donor and patients derived T cells. CD40L expression and physiologic regulation were restored on edited T cells, reaching 60% of WT expression level. By performing T-B cells co-culture, we confirmed restoration of CD4 T cells ability to provide normal contact dependent helper function to B cells, as assessed by in vitro proliferation, class switching and IgG secretion assays. To increase the yield of edited T cells before transplant, we coupled corrective cDNA with a clinical-compatible selector gene and confirmed that enriched T cells preserved their engraftment capacity in NSG mice. Surprisingly, presence of the selector gene in the transcript allows higher CD40L expression, now comparable to healthy controls. Since surface expression of reporter was observed also in resting cells, we exploited an optimized, truncated version of EGFR gene for allowing in vivo tracking of edited cells and, in case of adverse events, their depletion by a clinical-grade monoclonal antibody. We then investigated applying the same correction strategy to HSC to possibly provide an even broader and prolonged therapeutic benefit to patients who are amenable to HSC transplant. By using a recently optimized protocol we obtained stable ~30% *CD40L* editing in HSC after xenotransplant. Finally, we modeled this strategy in HIGM1 mice by transplanting escalating doses of WT HSC together with mutant ones and found that a threshold of 25% functional cells is sufficient to partially restore serologic immunity against different antigens. Remarkably, however, level of phenotypic rescue obtained by the two strategies was similar, thus positioning T cell based strategy ahead for clinical testing, given the easier application and lower safety concerns raised by T cell manipulation.

938. Production of Genetically Engineered T and NK Cells is Maintained in Humans by Common Long Term Lymphoid Progenitors 15 Years after Loss of Transplanted Hematopoietic Stem Cells

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Our mathematical modelling of integration site (IS) data in clinical gene therapy (GT) supports the existence of a population of long-term lymphoid progenitors (LtLP) capable of surviving independently from hematopoietic stem cells (HSC). To date, no experimental setting has been available to validate such statistical prediction neither in the mouse nor in humans. We here report the first formal evidence *in vivo* in humans that *de novo* T and NK cells production was maintained by common genetically engineered LtLP for 15 years after loss of transplanted HSC in 5 SCID-X1 patients treated with gammaretroviral vector HSC-GT. In these patients, starting from 6 months after GT the vector copy number (VCN) of myeloid lineages and of B cells has declined rapidly and has remained constantly below detection limits ever since, while T and NK cells remained vector positive for up to 19 years (average VCN of 2.1 and 2.3 respectively). Such data would be consistent with permanent loss of engineered HSC and survival of vector-marked long-living circulating mature lymphocytes. Strikingly and unexpectedly, by a comprehensive immunophenotyping of T cells overtime we instead detected vector-positive short-living CD45RA+CD62L+CD95- naïve T cells (Tn) in the circulation of these patients. We obtained functional validation of the identity of such Tn cells by IFN-y production assays and we confirmed positive thymic activity by measurement of TREC content. High throughput sequencing of 1,193 T-cell receptor (TCR) rearrangements in FACS-sorted T-cell subtypes confirmed that engineered Tn cells had normal TCR diversity and that new rearrangements were detectable overtime. Similarly, Vbeta repertoire measured by spectratyping displayed normal profiles in all patients. These results suggested that a *de novo* T-cell production by a putative LtLP population is maintained in these patients in absence of gene-corrected HSC. Using Tn data as surrogate markers of LtLP clonal dynamics and composition, we collected and analysed 12,756 unique IS from 5 populations including Tn cells in a window of 10.1 to 14.9 years after loss of transplanted HSC. Clonal diversity was stable in all T subtypes and IS sharing across subpopulations and timepoints was high and consistent with active *in vivo* output by LtLP and with differentiation of Tn into memory/effector T cells. Analysing nature and recapture probability of IS we observed that IS in LtLP are significantly enriched in genes involved in lymphocyte survival/activation and we could estimate that T-cell production is sustained by 2,092-6,056 individual engineered LtLP clones. By analysing 651 IS collected from CD3-CD56+ NK cells we observed that up to 41.2% of them were shared with independently analysed Tn suggesting that the LtLP active in these patients have a dual T/NK restricted potential. Lastly, we tracked 52 clones bearing IS in MECOM, CCND2 and LMO2 including the IS originally associated with leukemia in one patient, showing absence of any sustained clonal expansions 19 years after GT, the longest follow up available in clinical HSC-GT. In conclusion, our data provide the first formal evidence *in vivo* in human that a *de novo* production of genetically engineered T and NK cells can be physiologically maintained by a population of LtLP surviving up to 15 years after loss of transplanted HSC (*manuscript in preparation*). Identification and exploitation of such human LtLP population would open new possibilities for the development of next generation GT and cancer immunotherapy approaches.

939. CRISPR/Cas9 Targeted *MAGT1* Gene Addition into XMEN Patient Hematopoietic Stem Cells and Peripheral Blood T Cells

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Mutations in *MAGT1* gene encoding for the magnesium transporter 1 cause a recently described 'X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection, and neoplasia' (XMEN). The resulting glycosylation defect impairs the expression of key immune regulatory proteins like CD28, CD70, and NKG2D, receptor critical for the cytotoxic function of natural killer (NK) and $CD8^+$ T cells. Impaired antiviral and anti-tumor cytotoxicity leads to chronic EBV infections and development of lymphoproliferative disorders. There is currently no specific therapy available, and potentially curative hematopoietic stem cell (HSC) transplant is associated with a high risk of fatal hemorrhagic complications. We assessed the feasibility of targeting a gene addition approach with CRISPR/Cas9 technology and rAAV6 delivery of the MAGT1 cDNA for correction of XMEN patient $CD34^+$ HSCs for gene therapy, and of XMEN patient T cells for treatment of chronic viral infections. Mobilized peripheral blood $CD34^+$ HSCs from XMEN patient (IRB-approved protocol) were gene-edited (GE) with Cas9 mRNA/sgRNA and AAV donor. Using a cocktail of agents to enhance targeted integration (TI) and to maintain HSC 'fitness', we achieved highly efficient gene correction (50-60% TI) and excellent cell viability (> 95%) post-electroporation (EP). Functionally, GE XMEN $CD34^+$ HSCs showed correction of glycosylation defect by restoration of NKG2D expression and NK cytotoxic activity of *in vitro*-differentiated NK cells (20-40%) compared to naïve XMEN cells (5-10%) which was comparable to healthy donor (HD) controls (30-40%). When GE XMEN $CD34^+$ cells were differentiated *in vitro* into T cells using the Artificial Thymic Organoid (ATO) system, the GE differentiated T cells had similar levels of NKG2D expression as normal T cells. Next, we performed transplantation studies (Institutional Animal Care and Use Committee approved protocol). We observed robust engraftment (mean 10%, up to 40%) at 16 weeks after transplantation of XMEN (GE, naïve) and HD $CD34^+$ HSCs into newborn immunodeficient mice. Transplanted mice showed highly corrected NKG2D-expressing $CD8^+$ T (~75%) and NK (~35%) cells in peripheral blood, spleen and thymus. Molecular analysis confirmed high TI (50-60%) in mice bone marrow human $CD45^+$ cells indicating successful engraftment and persistence of GE HSCs following transplant. In parallel, we applied the same approach to XMEN T cells. Interestingly, we observed increasing percentages of GE cells following *in vitro* culture (from 40% to 60% after 5 weeks), highlighting an important survival advantage of corrected cells. This indicates that relatively low levels of correction will likely be necessary for clinical benefit. In summary, we demonstrate a rapid application of a robust gene correction approach to a newly described primary immunodeficiency disease, where improved GE process achieved efficient TI of *MAGT1* cDNA in human XMEN patient $CD34^+$ HSCs, sustained engraftment and multi-lineage differentiation capabilities. High-throughput sequencing for potential off-target insertions/deletions are underway.

940. AAV Integration Analysis after Long Term Follow Up in Hemophilia a Dogs Reveals the Genetic Consequences of AAV-Mediated Gene Correction

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One of the safety concerns for AAV gene therapy is the potential for integration events that could be genotoxic and lead to tumorigenesis. While recombinant AAV primarily remains as an episome, integration events have been detected in mouse models and hepatocellular carcinoma has been observed after neonatal delivery of AAV vectors. The long term follow-up of 9 hemophilia A (HA) dogs for as long as 10 years after AAV delivery of canine factor VIII (cFVIII) provides the unique opportunity to assess the potential for long term consequences of AAV integration. The cFVIII gene was delivered to HA dogs using AAV8 or AAV9 using two delivery approaches: (1) two chain delivery that co-delivers the cFVIII heavy chain in one AAV vector and the cFVIII light chain in a second AAV vector or (2) B-domain deleted cFVIII in a single AAV8 vector (Sabatino 2011). FVIII expression was between 1.9% and 11.3% of normal levels. While FVIII expression was stable for 7 dogs, 2 dogs had a gradual increase in FVIII expression that began 3 years after vector administration and resulted in levels that were about four times the initial levels. Clinically, there was no liver toxicity and no tumors were detected in any of the dogs. One hypothesis for the increase in FVIII expression in the 2 dogs is AAV integration followed by clonal expansion. Furthermore, studies of AAV integration in this large animal model followed for 6-10 years may provide important insights into the AAV genome after gene therapy. Vector copy number (VCN) analysis was performed on liver samples (5-29 per dog, n=8 dogs) by Q-PCR. DNA copy numbers were between 0.0 and 7.8 copies per diploid genome. Integration target site analysis was performed on liver samples (n=3/dog) from 6 AAV-treated HA dogs alongside liver samples from naïve HA dogs (n=2). The sites were isolated using ligation-mediated PCR, Illumina paired-end sequencing followed by analysis using the custom software pipeline, AAVenger. In the 20

liver DNA samples, more than 2,000 unique AAV integration events were identified throughout the canine genome. Clonal abundances were estimated by counting the unique genome breaks associated with integration positions, which showed that the maximum clonal abundance ranged from 1 to 138. Expanded cell clones were observed with integration near genes previously associated with growth control and transformation in humans with the most abundant clones located in DLEU2L, PEBP4 and EGR3. Integration events associated with clonal expansion were detected in EGR3, EGR2, CCND1, LTO1 and ZNF365 in multiple dogs. Validation of integration sites in the most abundant clones was performed using targeted PCR to isolate junction fragments followed by Sanger or Illumina sequencing. The analysis of integrated AAV vectors revealed complex rearrangements of the vector genome that were partially deleted and/or rearranged as well as one intact transgene coding sequence. In addition, further analysis of the integration events indicates that the AAV vector integrated within itself with high frequency. These studies demonstrate the long term efficacy and safety of AAV administration but also highlight the complex nature of AAV vector genomes that are present long term.

941. First-In-Human Gene Therapy Study of AAVhu37 Capsid Vector Technology in Severe Hemophilia A: Safety and FVIII Activity Results

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Introduction: Gene therapy for hemophilia A has the potential to reduce the treatment burden for patients and care providers. It may eliminate the need for regular factor VIII (FVIII) prophylaxis by facilitating the long-term expression of endogenous FVIII at levels sufficient to normalize coagulation. BAY 2599023 consists of an adeno-associated virus (AAV) vector with capsid serotype hu37 (AAVhu37), and a genome that directs expression of B-domain-deleted (BDD) human FVIII under the control of a liver-specific promoter/enhancer combination optimized for liver transgenic expression, used for the first time in humans. Preclinical studies have indicated a good safety profile and the potential to achieve FVIII expression at therapeutic levels over an extended period. This analysis reports the safety and FVIII activity achieved with BAY 2599023 in the first two cohorts of this first-in-human study. **Methods:** This phase 1/2, open-label, dose finding study (NCT03588299) included male patients with severe hemophilia A who each received a single intravenous infusion of AAVhu37. Patients were aged ≥18 years with no history of FVIII inhibitor development, no detectable immunity to the AAVhu37 capsid and >150 exposure days to FVIII products. Primary endpoints were adverse events (AEs), serious AEs (SAEs) and AEs/SAEs of special interest (S/AESIs). Secondary endpoint was change in FVIII activity from baseline. Informed patient consent and ethics committee approval were obtained. **Results:** Two, 2-patient cohorts were enrolled sequentially. Following 52 weeks of safety observation of the first cohort (0.5×10^{13} GC/kg), no SAEs, study-drug-related AEs or S/AESIs were reported. Liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) remained <1.5× upper limit of normal. Corticosteroids were not used in either patient. Clear evidence of FVIII expression was observed in both the first and the second patient, with stable FVIII coagulant activity of 3-8% and 13-21% respectively. Since June 4, 2019, two additional patients were enrolled sequentially into the second dose cohort, with a dose of 1.0×10^{13} GC/kg. As of January 1, 2020, both second-cohort patients have completed at least 24 weeks' follow-up. At the time of this report, both patients are off prophylaxis: patient 3 with stable FVIII coagulant activity of 25-41% and patient 4 with stable FVIII activity of 6-9%. In Cohort 2, one AESI, mild elevation in ALT/AST liver enzymes, was recorded in patient 3. There were no additional clinical symptoms reported for this patient, nor loss of FVIII activity levels. A short course of corticosteroid treatment was initiated with a rapid return to normal ranges in both AST and ALT. Long-term safety and FVIII activity will be monitored continuously in all treated patients. **Conclusion:** In this first-in-human study, four patients were treated with BAY 2599023 at doses of 0.5×10^{13} GC/kg and 1.0×10^{13} GC/kg. There was measurable expression of endogenous FVIII in all patients and early indications of hemostatic efficacy. The data generated demonstrate successful translation from preclinical to clinical development and proof-of-mechanism for BAY 2599023.

942. Engineered Antigen-Expressing CAAR-T Cells Targeting Autoreactive B Cells to ADAMTS13 in the Context of Thrombotic Thrombocytopenic Purpura

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Acquired thrombotic thrombocytopenic purpura (aTTP) is life-threatening thrombotic microangiopathy characterized by abnormal bleeding and clotting due to dysregulated platelet activation. aTTP results from an autoantibody response directed against a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS13), a plasma protease that cleaves von Willebrand Factor (vWF). vWF is synthesized as multimers, which serve to activate platelets to stem bleeding in the context of endothelial injury. Under normal physiologic conditions, in the absence of endothelial injury, the length of vWF multimers is regulated through cleavage by ADAMTS13 and this process prevents pathologic platelet activation and clotting. Pathologic autoantibodies against ADAMTS13 results in severely reduced activity of ADAMTS13. The persistence of large vWF multimers in the circulation results in the formation of platelet thrombi in the microvasculature leading to the clinical and pathological features of TTP. Current therapies aimed at eliminating pathogenic antibodies and B cells do not provide durable remission in up to 30% of patients. To develop a curative treatment, we developed chimeric auto-antigen receptors (CAARs) to target ADAMTS13-specific B cells responsible for the production of the autoantibodies that cause aTTP. We generated receptors employing different combinations of ADAMTS13 domains as extracellular CAAR domains linked to intracellular signaling domains derived from T cell or NK cell receptors. ADAMTS13-based CAAR-T cells specifically killed target cells expressing human autoantibodies directed at the cysteine-rich spacer domain (CS) of ADAMTS13, a

region known to contain enzyme-inhibitory epitopes. The potency of the CAAR-T cells was comparable to that of a CD19-directed CAR-T. In the presence of anti-CS expressing target cells, we observed CD107 degranulation and cytokine production. ADAMTS13 CAAR-T cells also proliferated in the presence of anti-CS expressing targets. These results suggest that ADAMTS13-based CAAR-T cells may be an approach to curing aTTP and support further testing in *in vivo* models.

943. AAV Mediated Expression of Activated Factor V (FVa) for Hemophilia

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Background: Gene therapy using Adeno-Associated Virus (AAV) vectors has been successfully applied in hemophilia patients following single drug administration; however, patients with inhibitors are excluded from these trials. During coagulation, activated factor V (FVa) functions downstream as a co-factor of FXa to amplify thrombin generation; therefore, we hypothesize that continuous expression of FVa by gene therapy can correct both Factor IX and FVIII deficiency regardless of clotting factor inhibitor. **Methods:** To make FVa constructs for human FVa expression and function, we have cloned an AAV cassette by deleting human FV B domain and linking the FV heavy chain with the light chain using a furin cleavage motif. AAV8 vectors encoding human FVa driven by the TTR promoter (AAV8/TTR-hFVa) were administrated into mice with hemophilia A and B with or without FVIII inhibitors by intravenous injection. Hemostasis monitoring, including FVIII-aPTT, tail transection, the saphenous vein bleeding assay (SVBA) and rotational thromboelastometry (ROTEM), were performed. To evaluate the risk of thrombosis after hFVa gene therapy, thrombin/antithrombin III (TAT) and fibrinolysis were analyzed by ROTEM . **Result:** After transfection of plasmid FVa driven by the CBA promoter into 293 cells, the heavy chain (110kd) was detected with the antibody GMA-044, which specifically recognizes the hFV HC, This result indicates that FVa protein can be properly processed and formed by the furin cleavage intracellularly. Next, we administered $1x10^{12}$ particles of AAV8/TTR-hFVa in hemophilia B mice via the tail vein. The complete phenotypic correction was achieved when compared to wt mice over 28 weeks with a normal activated partial thromboplastin time (aPTT). This study suggests that utilization of AAV vectors to deliver FVa is safe and supports hemostasis. Next, we administered AAV8/TTR-hFVa in hemophilia A mice. Again, a long term hemostasis correction, represented by aPTT, was achieved. The mice treated with FVa gene therapy were subjected to tail transection, and significantly less blood loss was observed when compared to untreated mice (42/44 AAV8/TTR-hFVa treated mice survived with blood loss of 8.7±7.2mg/kg body weight vs 8/13 survived with blood loss of 34.5±12.9mg/kg body weight in untreated hemophilia mice), which was also confirmed by SVBA in a different pilot study. It is no surprise that AAV8/TTR-hFVa treatment also resulted in hemostasis correction represented by shortening of aPTT time and less bleeding by tail transection assay in mice with pre-existing anti-FVIII inhibitor, similar efficiency to that in mice without FVIII inhibitors. In comparison to WT and untreated hemophilia mice, gene therapy with AAV8/TTR-hFVa was not associated with elevations in TAT levels, and fibrinolysis on ROTEM was normal. These results imply that continuous expression of FVa via AAV gene delivery would not lead to clot formation. **Conclusion**: FVa-based AAV gene therapy showed the promise for hemostasis correction in hemophilia regardless of clotting factor inhibitor development and no risk for thrombosis. Additionally, this treatment has the potential to achieve the goal of "one drug for multiple clotting factor deficiencies."

AAV Vectors Preclinical and Proof-of-Concept Studies in Systemic Diseases

944. PR001 Gene Therapy Increased GCase Activity and Ameliorated *GBA1*-Associated Disease Phenotypes

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Mutations in the *GBA1* gene are believed to be the most common etiology of lysosomal storage diseases. *GBA1* mutations cause Gaucher disease (GD), an autosomal recessive inherited disorder, with severe mutations leading to neurological manifestations (neuronopathic Gaucher disease, or nGD). Additionally, *GBA1* mutations are the most common known genetic cause of Parkinson's disease (PD), with a prevalence of 7-10% of the patient population. Deficiency in the *GBA1* encoded enzyme glucocerebrosidase (GCase), a key lysosomal enzyme required for the normal metabolism of glycolipids, leads to the accumulation of the glycolipid substrates, resulting in lysosomal dysfunction that ultimately leads to inflammation and other pathological changes. With our gene therapy product candidate, PR001, we aim to increase GCase activity in nGD and PD patients with *GBA1* mutations (PD-GBA) in order to ameliorate the lysosomal dysfunction and slow or stop disease progression. We evaluated PR001 in *in vivo* and *in vitro* studies. We used two established mouse models of GCase deficiency that display phenotypic characteristics consistent with nGD and PD-GBA. Intracerebroventricular (ICV) injection of PR001 increased GCase activity, decreased glycolipid substrate accumulation, improved motor abnormalities, and reduced neuropathological changes. These therapeutic benefits were persistent, with lasting effects observed at 6 months post-treatment. Select safety endpoints from these studies, as well as toxicology studies in nonhuman primates, demonstrated that PR001 was well-tolerated at all doses tested. Supportive *in vitro* studies showed that PR001 increased GCase activity and decreased α-Synuclein levels in a cell line and primary rodent neurons. Overall, these findings support the clinical development of PR001 for nGD and PD-GBA. We are initiating Phase 1/2 clinical trials to investigate safety, tolerability, biomarkers, and clinical measures of efficacy in nGD and PDGBA patients.

945. Developing AAV-Mediated Clinically Translatable Gene Therapy for Maple Syrup Urine Disease (MSUD) Caused by *BCKDHA* Mutations in a Bovine Model

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Maple syrup urine disease (MSUD) is a rare genetic disorder affecting degradation of the branched-chain amino acids (BCAA) leucine, isoleucine, and valine and their ketoacid derivatives. MSUD is caused by biallelic mutations in one of three genes that encode subunits of the branched-chain ketoacid dehydrogenase complex (BCKDC), namely *BCKDHA*, *BCKDHB*, and *DBT*. In normal humans, the liver and skeletal muscle exhibit relatively high BCKDC activity. Severe ('classic') MSUD is fatal without treatment. Dietary BCAA restriction is the mainstay of treatment but is difficult to implement. It has imperfect efficacy, and affords no protection against episodic and life-threatening encephalopathic crises. Liver transplantation is an effective alternative to dietary therapy, but entails surgery risks and long-term immunosuppression. MSUD affects approximately 1 per 185,000 births worldwide and is screened for in most U.S. states and developed countries. The birth incidence is much higher (~1 per 500) among Old Order Mennonites of North America due to a common *BCKDHA* founder variant (c.1312 T>A; p.Tyr438Asn) that abrogates BCAA oxidation, rendering patients biochemically unstable within days of life. No suitable *Bckdha*$^{-/-}$ rodent model exists, but a naturally occurring *BCKDHA* loss-of-function mutation (c.248C>T) was identified in cattle herds. Homozygous newborn calves have a phenotype similar to the human disease. In this study, we set out to leverage the bovine model to develop a gene replacement therapy for *BCKDHA* variants of MSUD. Surprisingly, overexpressing BCKDHA in a *BCKDHA*$^{-/-}$ HEK293T cell line only slightly restored BCKDC activity. We also found that BCKDHB in these cells was diminished as previously reported, and was not restored upon overexpressing BCKDHA during the course of transient transfection experiment. Therefore, a dual-transgene strategy was used to express BCKDHA and BCKDHB from a single AAV vector (opti-*BCKDHA/B*). The co-expression stabilized both proteins and fully restored BCKDC activity to normal level in the *BCKDHA*$^{-/-}$ HEK293 cell line. We systemically delivered AAV9 vectors into neonatal and young adult WT mice at escalating dose regimens that can efficient target the liver and muscle to determine safety and gene transduction efficiency. The general health condition and body weight remained unaffected by the injection of vectors up to 1.3E14 GC/kg, indicating that the vector treatment was well tolerated. Large-scale AAV vector production and generation of *BCKDHA* c.248C>T homozygous calves are under way, and converge on the goal of dosing newborn MSUD calves with rAAV9-opti-*BCKDHA/B* antenatally or within the first hours of life. This pre-clinical proof-of-concept study employs a highly relevant, large animal disease model; it should provide valuable information about the safety and efficacy of gene therapy that can be quickly and reliably translated to clinical development. Furthermore, the opti-*BCKDHA/B* AAV vector can potentially treat both *BCKDHA* and *BCKDHB* variants of MSUD that together represent more than 70% of all MSUD patients. [a]Co-corresponding authors.

946. A Novel NAGA Variant Designed to be Non-Immunogenic in Humans and Provide Broad Cross-Correction in Fabry Disease

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Fabry disease is an X-linked hereditary metabolic disorder due to a mutation in the alpha-galactosidase A (GLA) gene. Deficiency of GLA enzyme leads to a constant accumulation of Gb3 and its deacylated derivative globotriaosylsphingosine (lysoGb3) in cells and plasma, resulting in cell abnormalities and organ dysfunction affecting heart, kidney and brain. Current enzyme replacement therapy (ERT) has limited therapeutic effects, because of poor cross-correction, and the induction of immunogenicity, and is very costly. We explore the use of modified α-N-acetylgalactosaminidase (NAGA) as a non-immunogenic and more effective alternative for GLA. Wildtype NAGA shows high structural resemblance to GLA and by modifying 2 amino acids the modified NAGA elicits GLA-like activity. The modified NAGA protein was previously reported to degrade Gb3 in cultured Fabry fibroblasts and prevents Gb3 accumulation in liver, kidney and heart of Fabry mice. Six AAV vectors expressing modified NAGA were generated with distinct design and two different promoters, i.e. liver specific or constitutive. *In vitro* all AAV5-modified NAGA vectors showed GLA activity upon transduction of liver cells. Modified NAGA produced in human cells can be taken up by Fabry fibroblasts, providing evidence for cross-correction. Injection of the AAV5-modified NAGA vectors in C57Bl/6 mice resulted in 10 and 20-fold higher GLA activity in plasma and liver, compared to untreated mice. Furthermore, AAV5-modified NAGA administration to *GLA* knockout mice showed significant lowering of (Lyso)Gb3 in plasma and target organs. Most importantly, administration of AAV5-modified NAGA vectors to non-human primates showed significant elevation in modified NAGA levels in plasma. Administration of AAV to mice and non-human primates was well-tolerated and did not show increased liver biomarkers of hepatic toxicity. Thus, the use of AAV gene therapy expressing modified NAGA, which possesses GLA-like activity warrants further investigation as an attractive approach for Fabry disease.

947. AAV Gene Immunotherapy Reverses MS-Like Disease Against Multiple Epitopes and in Genetically Diverse Mice

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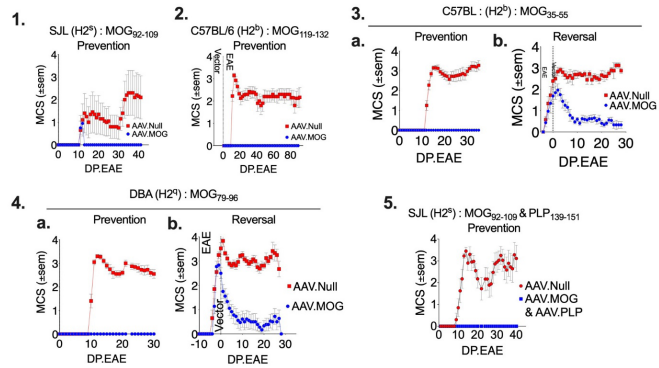
An autoimmune disease like Multiple Sclerosis (MS) is caused by a breakdown of tolerance against endogenous proteins. Regulatory T cells (Tregs) maintain homeostasis between immune activation and suppression; however, in MS the mechanisms dependent on thymically produced Treg cells appear insufficient to prevent the initial priming of encephalithogenic T cells. Using the experimental autoimmune encephalomyelitis (EAE) model of MS, we have previously shown that neuroinflammation and clinical symptoms can be prevented or completely ameliorated, even in preexisting disease, by extrathymically induced antigen (Ag)-specific Tregs. Historically, Ag-specific therapies have remained elusive due to genetic differences and the various encephalitogenic epitopes present. Thus, a treatment must be capable of overcoming such barriers in order to provide the most effective therapy for patients suffering from MS. To address this, we have developed a powerful gene-immunotherapy capable of dynamically adjusting to the unique Ag-specific requirements needed to restore tolerance through de novo-induced Treg. Here, we demonstrate the effectiveness of an Adeno-Associated Virus (AAV) gene-immunotherapy (AAV.MOG) to prevent and/or ameliorate pre-existing EAE disease induced with multiple antigenic epitopes of myelin oligodendrocyte glycoprotein (MOG_{35-55}, MOG_{79-96}, MOG_{92-106}, or MOG_{1-125}). Moreover, we show the same hepatocyte directed MOG expressing AAV vector is therapeutically effective in genetically diverse mice (C57BL*H-2^b; DBA/1*H-2^q; SJL*H-2^s). To demonstrate prevention of disease, mice were given AAV.MOG (or AAV.Null) vector 2-weeks before EAE was induced. In all scenarios, mice receiving the AAV.MOG gene-immunotherapy showed virtually no signs of disease onset nor cellular infiltration or demyelination within the CNS (hallmarks of EAE) (Fig. 1, 2, 3a, 4a, 5). In striking contrast, controls developed severe demyelinating EAE. Next, to show the therapeutic effectiveness of the immunotherapy to ameliorate/reverse active pre-existing disease, EAE was induced using one or more immunogenic epitopes, prior to administering treatment at a predetermined disease severity. Remarkably, after an initial increase in disease severity, mice receiving AAV.MOG had significant reductions in neurological impairment (clinical disease score) ($p<0.05$), cellular infiltration, and demyelination as compared to controls (Fig. 3b, 4b). Lastly, to further demonstrate the strength and effectiveness of this gene-immunotherapy on disease, we administered a cocktail of vectors encoding two different neuroproteins (AAV.MOG & AAV.PLP). At 14 days post injection, disease was induced using both dominate immunogenic EAE inducing epitopes (MOG_{92-106} & $PLP_{139-151}$). Amazingly, the mice receiving the immunotherapy failed to develop disease, whereas the control mice quickly developed a severe relapsing-remitting EAE disease. These results, in combination with data submitted in additional abstracts, establish a novel gene-immunotherapy platform for treating MS that is universally applicable and superior to the traditional non-specific immunosuppression therapies currently available.



948. Preclinical Gene Replacement Therapy with a New scAAV9/*SUMF1* Viral Vector for the Treatment of Multiple Sulfatase Deficiency

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Multiple Sulfatase Deficiency (MSD) is a rare autosomal recessive disorder characterized by the deficient enzymatic activity of all known sulfatase. MSD patients frequently carried two loss of function mutations in the *SUMF1* gene, encoding a sulfatase modifying factor which activates 17 different sulfatases. MSD patients shown common features of mucopolysaccharidosis, and metachromatic leukodystrophy, including neurologic impairments, developmental delay, visceromegaly and skeletal abnormalities. The severity of the disease varies depending on the residual SUMF1 activity, being the neonatal forms the most severe. There are currently no approved therapies for MSD patients. Gene therapy is a promising approach to provide a long term therapeutic benefit. We designed a preclinical study in *Sumf1* deficient (KO) mice to test the capacity of a newly developed self-complementary AAV9/*SUMF1* vector for their use in a gene replacement therapy. Sumf1 KO mice are characterized by a short life span with a median survival of 10 days, craniofacial and skeletal deformities, tremors and hind limb clasping. As a proof of concept, we injected scAAV9/*SUMF1* in Sumf1 KO mice at post-natal day 1 (PND1) via intracerebroventricular (ICV). The ICV treatment was able to rescue *Sumf1* deficiency resulting in improved survival and extended life span (median survival of 300 days) without signs of neurological impairments after one-year post-treatment. We also tested the efficacy of the scAAV9/*SUMF1* in symptomatic mice at PND7 by intrathecal delivery (IT) and a combined treatment targeting CNS and systemic organs by intravenous injection (IV). Both IT and combined IT/IV treatment were able to rescue the mouse phenotype to a similar extent as the ICV treatment, improving their survival (70 % survival at 200 days), growth and behavioral outcomes. No significant signs of toxicity derived from the viral vector were seen in treated mice at one-year post-treatment. Biochemical studies to assess sulfatase activity and biomarkers are still ongoing. Our preclinical results indicated that the use of scAAV9/*SUMF1* in a gene replacement therapy could certainly provide a benefit for MSD patients.

949. Efficacy and Safety of CSF- Delivered AVXS-401 in Mice and Non-Human Primates for the Treatment of Friedreich's Ataxia

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Friedreich's Ataxia (FA) is the most common autosomal recessive ataxia, typically presenting during childhood in 1 in 50,000 individuals. It is characterized by progressive sensory and spinocerebellar tract neurodegeneration, hypertrophic cardiomyopathy and increased incidence of diabetes, resulting in progressive loss of coordination and muscle strength with subsequent motor incapacitation. The mean time to loss of independent gait is typically 8 years from onset. FA patients require mobility aids, such as a walker, or are wheelchair-bound by their teens to early 20s. The majority of deaths occur between 16-45 years of age due to congestive heart failure and arrhythmia. All cases of FA arise from gene repression due to increased tri-nucleotide (GAA) repeat expansion in the first intron of the FXN gene leading to reduced expression of frataxin. Frataxin is an essential mitochondrial protein involved in the biosynthesis of iron-sulfur clusters and the regulation of mitochondrial iron transport, electron chain transfer, respiration and energy metabolism. Published work showed that FA-like symptoms in two conditional mutant murine models, cardiac and neurosensory, were rescued via exogenous re-expression of FXN in the CNS and the heart by intracranial, intraperitoneal, or intravenous delivery of transgene therapy. For clinical translation, we developed AVXS-401, a self-complementary adeno-associated virus (AAV9) based gene replacement therapy to provide sustained expression of FXN in key tissues relevant to FA. Toxicity studies in wildtype mice proved AVXS-401 is safe and well-tolerated. Dose ranging efficacy studies following a one-time, pre-symptomatic intracerebroventricular (ICV) administration of AVXS-401 in conditional FXN-deficient mice in the CNS (Pvalb) demonstrates amelioration of behavioral phenotypes, rescue of Purkinje neurons and cerebellar gliosis at low doses. ICV delivery of AVXS-401 in cardiac mutants (MCK) results in full recovery of cardiac functions as measured by magnetic resonance imaging (MRI), prevention of histopathological evidence of cardiomyopathy and >300% increase in median survival at efficacious doses. Scale up of therapeutic doses to non-human primates (NHP) showed that AVXS-401 is safe and well tolerated with no aberrant behavior, clinical or anatomical pathology attributable to frataxin expression. Importantly, AVXS-401 provides durable mRNA transcription in the CNS and heart of NHP at 6 months post-injection with frataxin expression detectable above endogenous levels. Dose escalation studies by intrathecal (IT) administration in NHP show a dose correlation between mice and NHP by ddPCR quantification of vector genomes. Together these pre-clinical data show that AVXS-401 is suitable for first-in-human studies.

950. Bicistronic AAV Gene Therapy for Tay-Sachs Disease

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GM2 gangliosidosis (Tay-Sachs, Sandhoff disease) are fatal monogenetic diseases that result from a deficiency of hexosaminidase (Hex), which is an enzyme of the ganglioside degradation pathway. These diseases are clinically indistinguishable in humans and are caused by mutations in the alpha and beta subunits, respectively. Symptoms present in children as lack or loss of developmental milestones (sitting, crawling, etc.) and progress to inability to swallow, seizures and eventual semi-vegetative state. Tay-Sachs also occurs in sheep (TSD sheep), and this model is useful in testing of novel therapies. Adeno associated viral (AAV) gene therapy has shown efficacy in animal models, but has the limitation of intracranial delivery using two viral vectors to deliver both therapeutic genes. Here we describe the use of a single, bicistronic AAV9 vector construct to co-express both Hex subunits simultaneously. TSD sheep were injected with 2E13 vg/kg intravenously (n=5) or 3E14 vg total via the cerebrospinal fluid (n=7). Studies are ongoing with treated animals currently surviving over 1.5 years, while untreated TSD sheep reach humane endpoint at ~ 9 month of age. Both IV and CSF AAV treated TSD sheep exhibit marked attenuation of neurologic disease as well as normal cognition as measured by maze testing. MRI, EEG, CSF and other fluid biomarker analyses were performed. Four of the IV treated animals (16-18 months old) were euthanized due to musculoskeletal complications. The CSF treated animals are ongoing with only mild symptoms, with the oldest two animals reaching 2 years of age. These data show promise for a minimally invasive treatment for Tay-Sachs and Sandhoff disease using this new vector construct.

Tolerance and Vaccine Approaches

951. Creating AAV Specific CAR T-Cells and CAR Regulatory T- Cells to Model and Mitigate AAV Immune Responses

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Effector Tcell responses to AAV capsid have been observed to limit transgene expression in clinical trials of intravenous (IV) rAAV gene therapy, such as in hemophilia, an immune response that

was not accurately predicted by animal models. Clinical trials with intramuscular (IM) delivery, with and without immune suppression, also reported significant immune infiltration, but minimal clearance of transgene expression. This was attributed to an immune modulatory role of T-regulatory cells identified within the injected muscle. In order to better understand the capsid immune response, and modulate it, we have created T-cells and T-regulatory cells specific for AAV capsid using chimeric antigen receptor (CAR) technology. We created CAR T-cells specific for AAV capsid using an ScFv from a previously published anti-AAV antibody, and a 3rd generation CAR construct. In order to create T-regulatory cells we added a 2A sequence to the CAR construct and a FoxP3 transcription factor. The AAV-CAR-T-cells robustly expressed IL-2 and IFN-γ when co-cultured with AAV-infected cells, suggesting antigen specificity against AAV-transfected cells. In a cytotoxicity assay, AAV-CAR-T-cells killed 80% of cells infected with AAV3b and AAV1, but not mock infected controls. Coculture with AAV-CAR-T-regs significantly reduced this effect. To model the clinical cellular immune response against AAV capsid, we injected AAV-CAR-T-cells into AAV-injected animals. Mice received 5×10^{10} vector genome of AAV1-Alpha-1 antitrypsin (AAT) IM followed three weeks later by either 2.5×10^{6} AAV-CAR-T-cells or saline IV. We observed a robust reduction in AAT levels in animals that received AAV-CAR-T-cells but not in saline treated animals. T-cells infiltrated injected muscle only in the animals receiving AAV-CAR-T-cells. To examine the immune modulatory ability of AAV-CAR-T-regs *in-vivo*, we employed the previously described immune response against AAV-Rh32.33 capsid. Mice received an intramuscular injection of 5×10^{10} vector genome of AAV-Rh32.33 expressing AAT, and one week later a systemic injection of 5×10^{6} of AAV-CAR-T-regs, non-specific T-regs, or saline. In mice receiving AAV-Rh32.33-AAT (followed by saline), AAT levels began to increase over the first two weeks, and then dropped below the limit of detection, indicating a native effector T-cell response. However, mice receiving $5x10^{6}$ AAV-CAR-T-regs continued to show increasing expression over 5 weeks, indicating successful suppression of the effector T cell response. Non-specific T-regs showed only partial suppression, allowing for 25% of the expression level of the AAV-CAR-T-reg group. These results confirmed that AAV-CAR-T-regs can suppress the expected capsid mediated immune response against Rh32.33 capsid allowing for continuous expression of delivered transgene. This study demonstrates that anti-AAV-CAR-T-cells provide a powerful new tool to model the cellular immune response against AAV capsid in a mouse model and the ability of anti-AAV-CAR-regulatory T-cells to modulate the CD8-T-cell immune responses directed against capsid.

952. Combination of Rapamycin and Anti-IL-15 to Preserve Transgene Expression and Allow for Re-Administration in Hepatic AAV Gene Transfer

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Hepatic gene transfer of codon-optimized human factor VIII (FVIII) with an adeno-associated virus serotype 8 (AAV8-co*F8*) in hemophilia A (HA) animal models can induce tolerance and durably replace FVIII. However, in some HA animal models tolerance is not always observed. For instance, we identified a sub-strain of BALB/c-HA that reliably forms FVIII function-inhibiting antibodies (inhibitors) with this same approach. Hence, we sought to establish a regimen of transient immune suppression to prevent this response. We previously showed combining antigen, growth factor Flt3L, and the mTOR inhibitor rapamycin can induce tolerance to proteins via effector T cell deletion and regulatory T cell induction. Initial experiments sought to test the protocol during FVIII gene therapy in BALB/c-HA mice (1-2E11 vg/mouse vector dose, which sustains FVIII expression of ~10-20% normal in B6/129-HA mice, a strain that does not develop inhibitors after hepatic gene transfer). While inhibitor formation was merely delayed by several weeks in most of the immune-modulated BALB/c-HA mice compared to non-immune-suppressed controls, 25% never developed inhibitors and retained FVIII activity. A subsequent optimization experiment extended the protocol duration to ten weeks, and tested rapamycin alone or combined with Flt3L. Although no inhibitors formed, FVIII activity was mostly lost at later time points. Other experiments aimed to sustain FVIII activity levels through various alternative immune-modulators in combination with rapamycin, including IL-2/anti-IL-2 antibody complexes, anti-B-cell activating factor, and anti-IL-15 (n=8-10/group). While several regimens were partially successful, 8 weeks of rapamycin/anti-IL-15 combination achieved nearly undiminished FVIII levels throughout the 16-week experiment (mean=14.3%). In comparison, FVIII levels in mice receiving only rapamycin steadily declined after it was stopped until near total loss. IL-15 is a cytokine critical to memory CD8+ T cells. Thus, we are now addressing the hypothesis that rapamycin-treated mice had a memory CD8+ T cell response that was blocked by anti-IL-15, thereby preserving therapeutic FVIII expression after rapamycin treatment was stopped. Interestingly, in rapamycin-treated mice, we did not detect anti-AAV8 antibodies seen in vector-only mice (~10 μg/mL). This suggested AAV re-administration might be possible; thus, 17 weeks after the initial gene transfer, we administered an identical dose of AAV8 vector expressing human FIX (n=7-10/group). Three weeks later, average FIX levels of 4.5 μg/mL and 2.7 μg/mL were measured for mice originally treated with rapamycin and rapamycin/anti-IL-15, respectively, versus 7.2 μg/mL in naïve controls, indicating re-administration was successful. Without immune modulation with the first vector dose, mice failed to express FIX. All re-administered mice were immune competent and developed high levels of anti-AAV8 antibodies after the second gene transfer. Anti-IL-15 failed to suppress antibody formation in the absence of rapamycin. In summary, transient rapamycin treatment with hepatic AAV gene transfer prevents antibody formation against transgene product and vector. However, a yet to be defined subsequent immune response is prevented by monoclonal antibody therapy against IL-15.

953. Robust Antigen-Specific Immune Tolerance Can be Achieved Through Platelet-Targeted Gene Therapy Even in a Primed Model via Peripheral Clonal Deletion and Treg Cell Induction

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Our previous studies have demonstrated that platelet-specific gene transfer under control of the αIIb promoter (2b) results in effective immune tolerance induction not only to the coagulation factor VIII and IX, but also to the non-coagulant protein ovalbumin (OVA) in a model without pre-existing immunity (the unprimed model). Modulating existing immune responses and reestablishing tolerance is more challenging than preventing a primary immune response. In the present study, we explored how the reactivity of immune responses affects the efficacy of transgene expression and the potential immune tolerance mechanisms in platelet-specific gene transfer in the primed model. Here we used OVA as a surrogate protein for this study. Recipient wild-type (WT) CD45.1 mice were primed with OVA full-length protein. Platelet-OVA expression was introduced by 2bOVA lentivirus transduction of Sca-1$^+$ cells from either CD45.2/WT or CD45.2/OTII donors followed by transplantation into OVA-primed WT/CD45.1 recipients preconditioned with 6.6 Gy total body irradiation. We found that pre-existing high-reactive immune responses devastate neoprotein OVA expression in recipients in both the WT/WT and OTII/WT models. Using the WT/WT mouse model, 6 of 7 2bOVA-transduced recipients had sustained platelet-OVA expression with levels ranging from 4.53-8.84 μg/10^8 platelets. Anti-OVA total IgG titers declined with time in 2bOVA-transduced OVA-primed recipients even when rechallenged with OVA. In contrast, recall memory immune responses were elicited in 2bGFP and untransduced transplanted controls. Furthermore, full-thickness tail skin grafts from CAG-OVA transgenic mice were successfully engrafted onto 2bOVA-transduced recipients and survived throughout the remaining lifetimes of the animals (greater than 8 months). In contrast, skin grafts were completely rejected in control of 2bGFP-transduced and untransduced transplanted recipients within 60 days. To ensure that the immune system was not inactive in 2bOVA-transduced recipients, animals were immunized intravenously with unrelated antigen human recombinant FVIII (rhF8) 200 U/kg/week for 4 weeks, an immunization protocol known to induce anti-FVIII immune responses even in wild-type animals. All 2bOVA-transduced recipients developed anti-FVIII inhibitory antibodies after rhF8 immunization with no significant differences between the 2bOVA group and the 2bGFP and untransduced transplanted control groups. Using the OVA-specific TCR transgenic model (OTII/WT), we showed that OVA-specific CD4 T cells were deleted and regulatory T (Treg) cells were expanded in peripheral lymphoid organs, but not in thymus in 2bOVA-transduced OVA-primed recipients. The levels of platelet-OVA expression negatively correlates with the percentages of OVA-specific CD4 T cells, but positively correlates with Treg cells. Immune tolerance was also achieved in the OTII/WT model even though donor-derived CD4 T cells are OVA-specific. However, our studies showed that high-reactive immune responses can influence the efficacy of platelet-targeted gene therapy, indicating that there is a counterpace process between immune reaction and immune tolerance after platelet-targeted gene therapy. Taken together, our studies demonstrate that platelet-targeted gene therapy can induce antigen-specific immune tolerance even when the immune response has been mounted, suggesting that platelet-targeted gene therapy is a promising approach to induce immune tolerance for treatment of diseases with undesired immune responses or even with pre-existing immune responses, such as hemophilia A with inhibitors or autoimmune diseases.

954. Immunization with a Synthetic DNA Vaccine Encoding the OspA Antigen of *Borrelia burgdorferi* Provides Durable Immunity Against Lyme Disease

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Lyme Disease (LD) is highly prevalent in Europe and North America and its incidence is rapidly increasing. The etiological agents are bacteria spirochetes of *Borrelia* species. In North America, *B. burgdorferi* (ST1) is by far the most prevalent *Borrelia* species responsible for 99% of all LD cases. These bacteria are transmitted to humans through Ixodes tick bites. The disease presents as a local skin lesion (erythema migrans), and is occasionally accompanied by fatigue, fever, headache, arthralgia and/or myalgia. If left untreated or is refractory to antibiotic treatment, serious persisting manifestations can develop affecting the nervous system, joints, and/or the heart. In recent years there has been a steep increase in LD cases that has prompted renewed interests in the development of a vaccine. Historically, vaccines for Lyme disease have mostly relied on recombinant-based immunogens with limited immune response durability. The current study describes the development and the evaluation of a novel synthetically engineered DNA vaccine, pLD1 encoding the outer surface protein A (OspA) of *Borrelia burgdorferi*. We show that immunization of C3H/HeN mice with pLD1 elicits robust humoral and cellular immune responses, and a single dose confers complete protection to a live *Borrelia burgdorferi* bacterial challenge. Furthermore, we assessed intradermal (ID) delivery of pLD1 in Hartley guinea pigs. We demonstrated that a robust and durable humoral immune response was induced and maintained to at least 78 weeks. We also provide support of the efficacy of our DNA vaccine in this model by demonstrating in an *in vitro* correlate of protection assay, that antibodies targeting known protective epitopes on OspA protein are prominently generated. Overall, the present study provides the basis for the advancement of pDL1 as an innovative vaccine candidate which elicits a strong and durable immune response targeting the OspA antigen of Lyme disease.

955. Phase I-IIA Study of GLS-5300 Vaccine for Mers-Cov: Preliminary Results Demonstrate B and T Cell Immune Response Elicited in Lower-Dose 2- and 3-Vaccination Intradermally Administered Regimens

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Background: Novel coronaviruses (CoV) caused 3 global outbreaks over the past 2 decades: SARS-CoV (2002), MERS-CoV (2012), and 2019-nCoV in Wuhan, China. Each caused pneumonia with mortality of 10%, 35% and 2%, respectively (2019-nCoV estimated). GLS-5300 DNA vaccine targeting MERS-CoV Spike (S) was first to enter clinical trial, was safe and immunogenic (Lancet ID; 2019). In Phase I, a 3 dose series at Day0, 4 and 12 weeks of GLS-5300 at either 0.67, 2 or 6mg was given IM followed by electroporation (EP, IM+EP) with CELLECTRA-5P device. GLS-5300 induced antibodies (Abs) in 94%, Tcell response in 76%, and neutralizing Abs in 50% of participants. No dose response was observed. GLS-5300 response was similar to those recovered from natural MERS-CoV infection. The absence of dose response and prior experience showing benefits of ID+EP vs IM+EP (JID; 2019) led us to design this trial of lower ID dosing with an arm for a 2-dose regimen. We report results from MERS-002, the ongoing Phase I/IIa study of GLS-5300. **Methods:** MERS-002 is an open label, dose ranging, phase I/IIa study of GLS-5300. Participants were enrolled at 2 Korean sites into 3 groups receiving GLS-5300 ID+EP with the CELLECTRA-3P device: Group 1 received three 0.3mg doses at Day0 and weeks 4 and 12; Group 2 received three 0.6mg doses at Day0 and weeks 4 and 12; Group 3 received two 0.6mg doses at Day0 and week 8. Safety and tolerability of GLS-5300 was evaluated at each visit. Samples were collected at baseline, before each dose, and at both 2 and 4 weeks post dose 2 and post dose 3. Study data through 4 weeks after the primary series for a subset of immunoassays were included here. **Findings:** GLS-5300 given ID+EP was well-tolerated with no vaccine-associated SAEs. Preliminary results were available for: full length S (flS) ELISA, EMC2012-Vero neutralization (MERS-neut) and MERS-CoV S IFNg ELISPOT. GLS-5300 at 0.6mg induced MERS-CoV-specific Abs by flS ELISA and MERS-neut in 74% and 48%, respectively, after 1 dose. After the 2 or 3 dose vaccine series at 0.6mg per dose, flS ELISA response was seen in 100% and 92% of participants, respectively. MERS-neut response was 92% in both 2 and 3 dose 0.6mg groups. Antibody responses and rates were higher during and after primary series in 0.6mg group regardless of regimen than 0.3mg per dose. GLS-5300 induced Tcell responses via MERS-CoV IFNg ELISPOT in 60% and 84% receiving 0.6mg after the 2 or 3 dose series, respectively. Compared to 0.67mg of GLS-5300 given IM+EP in the first trial, 0.6mg of GLS-5300 given ID+EP in MERS-002, binding Abs appeared sooner and neutralizing Abs were observed in a higher fraction of participants (92% vs 50%) while Tcell reactivity was similar between vaccination schema. **Conclusions:** GLS-5300 was well tolerated with no vaccine-associated SAEs. Like prior studies, DNA vaccines given by ID+EP had fewer injection-related AEs relative to IM+EP. In MERS-002, 0.6mg of GLS-5300 in a 2-dose regimen spanning 8 weeks had similar reactivity and rate to the longer 3-dose regimen. GLS-5300 was safe and immunogenic when given IM+EP and, similarly, when given ID+EP in both 2- and 3-dose regimens in this ongoing MERS-002 Phase I/IIa trial. A Phase II clinical evaluation of the use of GLS-5300 to prevent MERS-CoV infection in endemic regions is planned.

956. Profiling the CD8 Response Following Liver Directed AAV Gene Therapy

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Autoimmune diseases are amongst the most common afflictions from which Americans suffer. According to the American Autoimmune Related Diseases Association (AARDA), there are currently ~50 million people suffering from an autoimmune disease in the United States. That is more than suffers from cancer (9 million) and heart disease (22 million), combined. Further, autoimmune diseases are one of the top 10 leading causes of death amongst women up to the age of 64. While the etiology of many autoimmune diseases remains unclear, the loss of immune tolerance prior to disease onset is a common feature. In general, tolerance is defined as the failure of the body to mount an immune response to an antigen. In the case of autoimmune diseases, the loss of tolerance may be resulting in the failure of specific regulatory cells within the body to suppress self-reactive effector cells. It has been postulated that the restoration of immune tolerance in patients suffering from an autoimmune disease would provide the most effective treatment. The use of liver directed, AAV gene therapy has proven to be an effective treatment capable of re-establishing immune tolerance in an animal model of the autoimmune disorder, Multiple Sclerosis. In this model, it has been established that liver directed AAV gene therapy results in the production of antigen (Ag)-specific $CD4^+$ $Foxp3^+$ Tregs. Traditionally, any immune tolerance induced following liver directed AAV therapy has been attributed to the production of Ag-specific T cells. More recently, we have established that the use of liver directed AAV gene therapy also results in a significant increase in multiple $CD8^+$ T cell populations (Fig 1). Upon further investigation, it was determined that some of these T cell populations are in fact regulatory in nature. Also, we have shown that the presence of $CD8^+$ regulatory cells is significantly increased in animals suffering from an autoimmune disorder following treatment with liver directed AAV as compared to controls. This data suggest that liver directed AAV gene therapy results in the production of multiple regulatory cell types and that the immune tolerance seen may not be due solely to the production of Ag-specific CD^+Foxp3^+ Tregs. Understanding the role that $CD8^+$ regulatory cells play in induction and maintenance of immune tolerance will lead to the development better treatments of multiple autoimmune diseases.

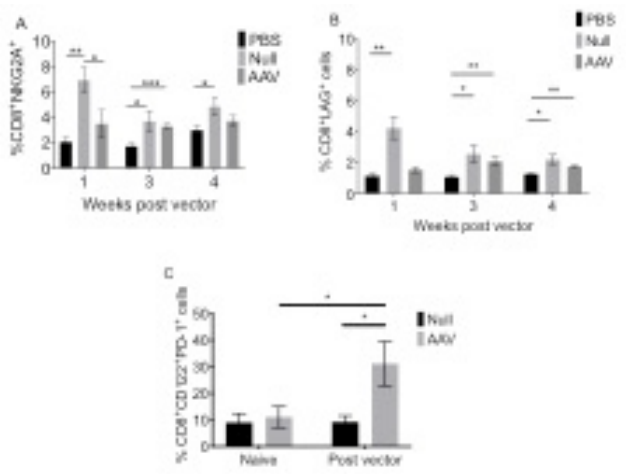

Figure 1: Increase in CD8+ regulatory cells following administration of liver directed AAV gene therapy. A. Percent of CD8+NKG2A+ cells found in the blood of mice 1,3,4 weeks post vector administration. B. Percent of $CD8^+LAG^+$ cells found in the blood of mice 1,3,4 weeks post vector administration. C. Percent of $CD8^+CD122^+PD\text{-}1^+$ cells found in blood pre- and post vector.

957. Engineering of an Enhanced DNA-Based Immunotherapeutic Against *Neisseria gonorrhoeae*

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The Center for Disease Control and Prevention categorizes *Neisseria gonorrhoeae* as an urgent health threat due to rising infection rates and increased levels multi-drug resistant strains. Alternative approaches to combat this increasingly resistant pathogen must be developed. Previous studies have shown that administration of recombinant monoclonal antibody (mAb) 2C7, which targets a highly conserved epitope within the surface-exposed lipooligosaccharide (LOS) antigen, can reduce bacterial burden and facilitate clearance of infection via activation of the classical complement pathway. Furthermore, engineering of the Fc region of 2C7 (E430G mutant) lead to increased C1q engagement and rapid clearance of *N. gonorrhoeae* infection following a low, single intravenous dose of 1μg. To expand the potential use of this therapy to address the widespread need, we applied the synthetic DNA (SynDNA) platform that allows for the *in vivo* production of DNA-encoded monoclonal antibody (dMAbs). This is a validated, safe and cost-effective alternative to traditional mAb therapy. We used a dual plasmid system to generate optimized DNA constructs encoding four forms of 2C7 that were previously shown to differentially activate complement *in vivo*: 1) wild type 2C7 (WT; complement-activating); 2) 2C7-E430G (complement-enhancing variant); 3) 2C7-E345K (complement-enhancing variant) and 4) 2C7-D270A/K322A (complement-abrogating variant). All 2C7 variants were successfully expressed *in vitro* and *in vivo,* with a single administration resulting in serum levels which far exceeded the predicted mAb 2C7 therapeutic dose. These *in vivo*-produced 2C7 dMAbs were able to bind the LOS antigen and demonstrated potent activity in an *in vitro* serum bactericidal assay. Importantly, their *in vivo* efficacies were subsequently demonstrated using an established prophylactic model of *N. gonorrhoeae* infection. As expected, all groups that received complement-activating 2C7 dMAb variants (2C7_WT, 2C7-E430G and 2C7-E345K) demonstrated rapid and comparable clearance of primary infection relative to control groups. However, the 2C7_E430G variant showed superior, dose-sparing protection following a delayed re-challenge and in subsequent passive transfer studies. These data verify protective capacity of mAb 2C7 and support the continued development of 2C7-based immunotherapies using the versatile synthetic DNA platform.

Genome Editing in Inborn Errors of Metabolism

958. AAV-Mediated Homology-Independent Targeted Integration in Newborn Hepatocytes Corrects Mucopolysaccharidosis Type VI

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Mucopolysaccharidosis type VI (MPS VI) is a severe lysosomal storage disorder caused by deficiency of arylsulfatase B (*ARSB*) that results in widespread accumulation and urinary excretion of glycosaminoglycans (GAGs). A liver gene therapy clinical trial with an adeno-associated viral (AAV) vector encoding ARSB is currently ongoing in MPS VI subjects, 4 years old or older (NTC03173521). This age threshold has been defined to avoid the loss of transgene expression caused by AAV episomal genome dilution occurring during liver growth, as observed in newborn AAV-treated animals. To overcome this limitation, we propose an AAV-based genome editing approach that uses Cas9-mediated homology-independent targeted integration (HITI) to insert a correct copy of the *ARSB* coding sequence in the albumin locus of murine neonatal hepatocytes. As the most abundant protein secreted by hepatocytes, albumin is a safe harbor for integration that can achieve high and stable levels of transgene expression. In order to assess the feasibility and efficiency of this approach, we first used the reporter gene Discosoma red (DsRed). We have designed a 2-AAV vector system. One vector encodes for SpCas9 under control of the liver-specific hybrid liver promoter. The other vector encodes for an albumin-specific or a scramble (negative control) gRNA expression cassette, as well as a donor DNA comprised of: i. a STOP codon (to block the translation of albumin), ii. a kozak sequence (to start translation of the gene of interest), iii. the DsRed gene and iv. the bovine growth hormone polyA.

This donor DNA is flanked with the same target sites recognized by the albumin-specific gRNA. One month after neonatal injection of 4*10^13gc/kg of each vector we observed 3,3% efficiency of targeted integration and 10% of INDELs in gRNA-treated hepatocytes, while no integration or INDELs were observed in scramble-treated livers. We then performed in-depth characterization of HITI precision by next-generation sequencing, and observed 40-60% of precise integration without INDELs in the 5' and 3' junctions of integration. We also performed off-target integration characterization which showed no integration of our donor DNA in other loci due to Cas9-mediated off-target cleavage. To determine whether the observed HITI efficiency was enough to achieve therapeutically relevant correction of the MPSVI phenotype, we replaced DsRed in our donor DNA with the coding sequence of *ARSB*. Newborn MPSVI mice treated with 6*10^13gc/Kg of Cas9 and donor DNA showed stable expression of ARSB up to 6 months after injection, with serum levels reaching 8-20% of normal mice. Importantly, significant reduction of urinary GAGs was observed in MPS VI treated mice compared to untreated controls. These results lay the groundwork for neonatal treatment of MPSVI. Additionally, the strategy we have developed can easily be adapted for other metabolic diseases that demand early treatment to achieve major phenotype correction.

959. Selective Expansion of Gene-Targeted Hepatocytes In Vivo Leads to Therapeutic Levels of Transgene Expression

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Many gene therapy approaches are limited by the inherent inefficiency of gene targeting. In proliferative cell types such as hepatocytes, this inefficiency can be overcome by selection for cells with successful gene targeting events. Previously, we described a system for expanding gene-targeted hepatocytes by blocking expression of cytochrome P450 reductase (Cypor). Cypor is required for the metabolism of the drug acetaminophen to its hepatotoxic intermediate, NAPQI. Therefore, Cypor-deficient hepatocytes are protected from acetaminophen-induced liver toxicity and will have a selective advantage over wild type hepatocytes when treated with high doses of acetaminophen. We have previously shown that selection of Cypor-deficient hepatocytes with acetaminophen in vivo can increase the proportion of targeted cells from less than 1% to over 30%. This effect is robustly reproducible, and can be achieved using either a CRISPR knockout or a shRNA-mediated knockdown of Cypor. We have also reported that Cypor-deficient hepatocytes are stable in the absence of continued selective pressure from acetaminophen. Here, we report data on the use of acetaminophen selection to achieve therapeutic levels of transgene expression using a lentiviral vector. We have created lentiviruses expressing the selectable Cypor gRNA or shRNA along with either human factor IX (hFIX), the therapeutic transgene for hemophilia B, or phenylalanine hydroxylase (PAH), the therapeutic transgene for phenylketonuria. Through repeated cycles of acetaminophen selection following a single lentivirus administration, therapeutic transgene expression can be increased by over 100-fold. We have successfully used this method to increase plasma hFIX concentration from subtherapeutic levels to upwards of 10,000 ng/mL, well above the therapeutic threshold of 250 ng/mL. We are now applying the same method to use the PAH-expressing vectors to treat a mouse model of phenylketonuria. One concern for the clinical applicability of this system is that a deficiency in Cypor will result in a metabolic pathology. Cypor is an obligate cofactor for all Cytochrome P450 (Cyp) enzymes and is involved in many metabolic processes. Selection of Cypor-deficient hepatocytes by acetaminophen is limited to pericentral zone 3 hepatocytes as only zone 3 hepatocytes express the Cyp enzymes that metabolize Acetaminophen to NAPQI, using Cypor as a cofactor. As zone 3 hepatocytes express different Cyp enzymes from hepatocytes in other zones, it would be desirable to maintain some zone 3 hepatocytes that are able to conduct Cytochrome P450-related metabolism. To address these concerns, we have shown that we can halt selection to retain a sizable proportion of zone 3 hepatocytes that express Cypor while still increasing therapeutic transgene expression by 100-fold. This indicates that we can achieve significant levels of selection while still retaining the ability of the liver to conduct zone 3 metabolic activity. Overall, these results suggest that acetaminophen selection of Cypor-deficient hepatocytes can effectively be used to increase transgene expression above a therapeutic threshold with minimal impact on the normal metabolic activities of the liver.

960. *In Vivo* Genome Editing at the *Albumin* Locus Corrects Two New Mouse Models of Methylmalonic Acidemia (MMA) Caused by Methylmalonyl-CoA Mutase Deficiency (Mmut)

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Methylmalonic acidemia (MMA) is a severe metabolic disorder with treatment options limited to restriction of dietary protein intake, and symptomatic management. Medical management notwithstanding, MMA can result in multi-systemic impairment and death. The majority of patients with MMA harbor mutations in a single gene, which encodes the mitochondrial enzyme methylmalonyl-CoA mutase (*MMUT*), making it an excellent candidate for gene therapy. Adeno-associated viral vectors (AAV) designed to increase *Mmut* expression in the liver can rescue MMA mice from neonatal lethality, increase enzyme activity in hepatic tissue, and restore growth to near normal levels. However, translation to humans with MMA will be limited by the loss of vector genomes over time. Genome editing using the CRISPR/Cas9 system represents an alternative approach for the *in vivo* therapy of genetic disorders such as MMA. Targeting the insertion of a *MMUT* cDNA into a highly expressed locus in the liver could provide the therapeutic benefit of canonical gene therapy, while mitigating the risks associated with off-target integration, and enable permanent correction. We identified two targets in the safe harbor locus albumin (*Alb*), designed and tested guides in cell lines, and then prepared AAV serotype 8 vectors expressing the *Alb* gRNAs and SaCas9 under expression of a liver-specific promoter. To assay therapeutic editing, the Cas9 vector

was co-delivered with a second AAV8 vector containing a codon-optimized *MMUT* rescue cassette. Two newly generated knock-in mouse models of MMUT MMA were used to assay the efficacy of *in vivo* editing. Mmut p.Gly715Val homozygotes manifest a milder MMA phenotype, are growth impaired, and display elevated levels of plasma methylmalonic acid. Mmut p.Arg106Cys mice display a more severe form of the disorder, resulting in less than 10% of mice surviving to two months of age without intervention. Survival, weight, plasma methylmalonic acid levels, and RNA in situ hybridization were studied to assess the therapeutic effects of *MMUT* insertion into the *Alb* locus in MMA and control littermates. In the more severe Mmut p.Arg106Cys model, targeting the *MMUT* rescue cassette to the first codon of *Albumin* afforded substantial rescue from mortality ($p<0.001$), correction of growth retardation, and significant reduction in levels of plasma methylmalonic acid ($p<0.0001$, n=8 treated mutants vs controls). In Mmut p.Gly715Val mice, *Alb* editing in intron 1 with an *MMUT* donor preceded by an IRES sequence similarly resulted in growth correction and improvement in metabolites ($p<0.001$ for biomarker reduction, n=8 treated mutants vs controls). RNA *in situ* hybridization to detect *MMUT* transgene expression showed widespread expression, with up to 50% of the hepatocytes expressing *MMUT* mRNA after editing with either vector, and was accompanied by robust MMUT protein expression. A semi-quantitative PCR assay to detect integration junctions further supports that the expression of MMUT derived from integration events. These results are consistent with observations from different MMA mouse models that demonstrate hepatocytes corrected after genome editing have a selective growth advantage. Targeting albumin using CRISPR/Cas9 delivered by AAV can be readily applied to other inborn errors of metabolism, particularly those for which correction of diseased hepatocytes results in robust clinical and/or metabolic improvement.

961. Genomic Instability and Tumorigenesis are Long-Term Effects of Therapeutic CRISPR/Cas9 Genome Editing in Hereditary Tyrosinemia Type I

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The immense therapeutic potential of CRISPR/Cas9 has been demonstrated in various animal models, however, little is known about the long-term consequences of CRISPR/Cas9 gene editing *in vivo*. Recently, our lab developed a novel concept called Metabolic Pathway Reprogramming (MPR) which utilizes CRISPR/Cas9 to re-route a metabolic pathway instead of having to correct the disease-causing gene. The efficacy of this approach was shown in a murine model for hereditary tyrosinemia type 1 (HT-I). Hereditary tyrosinemia type I (HT-I) leads to accumulation of toxic metabolites due to the deficiency in the fifth and last step of the tyrosine catabolism - fumarylacetoacetate hydrolase (FAH). If untreated, this condition can cause severe clinical consequences, such as metabolic decompensation and development of liver cancer. Therefore, patients are treated with nitisinone, a small molecule drug inhibiting the second step of the tyrosine catabolism, hydroxyphenylpyruvate dioxygenase (HPD), thereby preventing metabolic decompensation and tumorigenesis. We have previously shown in the murine model for HT-1 (*Fah*$^{-/-}$ mouse) that a one-time treatment with CRISPR/Cas9 deleting the *HPD* gene is an attractive genetic alternative to a life-long therapy on nitisinone. In present study, we evaluate the long-term (12 months) effects of gene editing in the *Fah*$^{-/-}$ mouse. We demonstrate an efficient elimination of HPD (>99.9%) and a complete correction of the deleterious tyrosinemic phenotype using CRISPR delivered by Adenovirus and Adenovirus Associated Vectors (AAV). Unexpectedly, we detected a markedly increased tumor incidence in the genome edited (10/17 mice) compared to the control (5/21 mice) group. Normal liver tissue and all examined tumors in the gene edited group were deleted for *HPD* hence, tumorigenesis was not driven by tyrosine catabolites. Whole genome sequencing identified multiple integrations of both gene therapy vectors across the murine genome, copy number variations and large deletions across all tumor samples. Interestingly, the tumor suppressor Dicer1 was recurrently lost across all tumor samples, as confirmed by qPCR analysis. In summary, our study is the first report of a severe adverse event of gene editing. Our findings reveal potential limitations of CRISPR/Cas9 approaches in cancer-prone disorders like HT-1. Even though genome engineering has tremendous therapeutic potential, this study concludes that it can also promote genomic instability and tumorigenesis, highlighting the need for further evaluation of long-term effects of viral vector mediated gene therapy.

962. Crispr/Cas9-Mediated Genome Editing Z-Allele Mutation in Hepatocytes of Alpha-1 Antitrypsin Pizz Ferrets Using Adenovirus-Associated Virus

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Alpha-1 antitrypsin (AAT) deficiency (AATD) is caused by mutations in the *SERPINA1* gene and is one of most common hereditary monogenic disorders. The homozygous Z-alleles (PiZZ) is the most prevalent variant that accounts for 96% of patients with AATD. This leads to misfolding and aggregation of AAT protein within hepatocytes, where the protein is produced and secreted, as well as an insufficient circulating AAT. Together, these defects lead to chronic liver disease and COPD by mechanisms of gain-of-toxic function in the liver and loss-of anti-protease function in the lung, respectively. Owing to unique advantages over rodents in size, lifespan, and a high degree of similarity to humans with regard to lung biology, the domestic ferret (*Mustela putorius furo*) has served as an excellent model for lung diseases, including COPD and cystic fibrosis (CF). *SERPINA1* Z-allele (AAT-PiZZ) and *ROSA26*-CAG-^{Lox}tdTomato^{LoxStop}-EGFP (ROSA26-TG) Cre-reporter ferrets have been recently created using a CRISPR/Cas9-mediated genome editing approach in ferret zygotes. Notably, the AAT-PiZZ ferret is the first endogenous AATD model

with a genetic Z-allele mutation, and it develops both liver and lung disease phenotypes similar to that observed in AATD patients. Unlike the mouse, which has six *Serpina1* genes, ferrets and humans have a single *SERPINA1* gene. While transgenic mice overexpressing human AAT-PiZZ in the liver develop hepatic disease, this model has many copies of the PiZZ allele. Thus, the AAT-PiZZ ferret offers an advantage as a gene-editing model for hepatocytes. Toward this goal, we found that a simultaneous introduction of single-guide RNAs (sgRNAs) to two different endogenous loci create double-editing events that can be enriched using a surrogate reporter, in this case a Cre reporter activated by delivery of a LoxP gRNA. We have also performed AAV serotype optimization for ferret liver gene transfer and these studies demonstrate that rAAV9-Cre robustly transduces ferret hepatocytes *in vivo*, converting the ROSA26-TG reporter in >95% of hepatocytes. We hypothesize that *in vivo* correction of the AAT-PiZZ allele will provide a selective advantage for survival of gene-edited hepatocytes. To approach this question, we are using double transgenic ROSA-TG:AAT-PiZZ ferrets and hepatic AAV-mediated gene transfer of Cas9 and two gRNAs targeting LoxP sites and *SERPINA1* Z-allele, together with a short WT AAT DNA donor sequence. The conversion of the Cre reporter from Tomato to EGFP will be used as a surrogate marker of edited cells and assessment of positive selection for gene editing hepatocytes at the AAT-PiZZ allele. These studies will lay the foundation for testing reversal of hepatic pathology in the AAT-PiZZ ferret model using AAV gene editing.

963. Treatment of Juvenile Mice with Methylmalonic Acidemia (MMA) by Targeted Integration of *MMUT* into *Albumin* Using a Promoterless AAV Vector

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MMA is a rare and heterogenous inborn error of metabolism most commonly caused by a deficiency of methylmalonyl-CoA mutase (MMUT). Patients suffer from frequent episodes of metabolic instability, severe morbidity, and early mortality. Elective liver transplantation has emerged as a treatment option for severely affected patients and can eliminate the potentially lethal metabolic instability associated with this disorder. Gene therapy has been explored in MMA mouse models as an alternative therapy to liver transplantation. Conventional adeno-associated viral (AAV) mediated gene delivery was highly effective in the treatment of neonatal mice with severe MMA. However, as has been seen in many other mouse models, hepatocellular carcinoma was observed in aged MMA mice, as well as control littermates, that were treated with AAV in the neonatal period. To minimize the potential of vector-related insertional mutagenesis and preserve MMUT expression after therapeutic gene delivery, we designed a promoterless AAV vector that utilizes homologous recombination to achieve site-specific gene addition of human *MUT* into the mouse albumin (*Alb*) locus, Alb-2A-MMUT (GeneRide™). Previously, we reported that after a latency period of several months, MMA mice treated as neonates displayed continuous enhancement of MMUT expression accompanied by weight gain, reduction of disease-associated plasma biomarkers, increased 1-C-13 propionate oxidation capacity, increased Alb-MMUT integration events, and increases in a treatment associated plasma biomarker (Alb-2A), which correlated strongly with Alb-MMUT integration and was predictive of protein expression in the liver. Aged MMA mice, treated as neonates with very high doses of vector, have not developed HCC (30 mice followed 13 to 26 months after treatment). We have now investigated the efficacy of the treatment of juvenile animals with MMA. A time-course study has revealed rapid reduction of disease-associated plasma biomarkers, a greater persistence of viral episomes compared to neonatal treatment, and similar Alb-MMUT integration rates as seen in the treated neonates at the same point post-treatment (at 2 months post-treatment an average of 0.041±0.025 for 4 juvenile treated animals vs. 0.096±0.034 for 12 neonatally treated animals; P=0.39). As with neonatal treatment, plasma Alb-2A levels correlated with Alb-MMUT integration rates and hepatic MMUT protein expression in juvenile MMA mice, further emphasizing the use of this circulating protein biomarker as a means to track correction and expansion of corrected cells longitudinally. It is likely that the increased hepatic turnover associated with the underlying disease creates an environment where therapeutic integration events into Albumin can be enriched in the absence of physiologic liver growth, which, if translatable to humans, would expand the therapeutic window for genome editing into Albumin as a treatment for MMA.

964. *Ex Vivo* Editing of Human Hematopoietic Stem Cells for Erythroid-Specific Expression of Therapeutic Proteins

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Genome editing technologies can correct the underlying disease-causing mutation of most genetic disorders; however, insufficient expression from the edited loci and the need of countless mutation-specific editing strategies represent major obstacles to clinical translation. Previous studies identified the human albumin gene as an ideal locus to express integrated transgenes at high levels by the liver. Unfortunately, this approach is limited by pre-existing immunity, as it relies on *in vivo* delivery of nucleases and donor DNA, and by liver conditions affecting hepatic functions. Here, we developed an alternative platform for erythroid-specific expression of proteins via *ex vivo* targeted integration of therapeutic transgenes in hematopoietic stem/progenitor cells (HSPCs). Specifically, by inserting transgenes under the control of the endogenous α-globin promoter, we aim to redirect a fraction of the striking globin synthesis capacity of erythroid cells (~7.2gr/day) for therapeutic protein expression without interfering with physiological erythropoiesis. We reasoned that the combination of robust transcription and the abundance of transgene-expressing cells should maximize protein production, reducing the number of integration events required to reach therapeutic levels. To develop this platform we used CRISPR/Cas9 to identify non-coding regions in α-globin genes where the integration of a promoterless GFP

cassette resulted in reporter expression in erythroid cell lines (K562 and HUDEP2) and HSPCs. We further showed efficient expression of different therapeutic transgenes by the same locus, including transmembrane proteins, secreted clotting factors and soluble lysosomal enzymes. Remarkably, all transgenes were expressed at high level (~100 fold more than hPGK promoter) and were upregulated during erythroid differentiation (~100 fold). Next, we focused on targeted integration of two therapeutic enzymes in HSPCs: human Factor IX (FIX) for Hemophilia B and lysosomal acid lipase (LAL) for Wolman disease. We demonstrated that erythroblasts derived from edited HSPCs were able to secrete both therapeutic proteins, which retained their enzymatic activity. In addition, erythroid-secreted LAL was able to cross-correct Wolman fibroblasts and to reduce cholesterol and lipid accumulation in these cells. To evaluate if modified HSPCs maintained their homing, engraftment and multilineage potential, we transplanted immunodeficient mice and monitored human cells for 16 weeks. All mice showed successful engraftment in bone marrow, spleen and blood. Notably, we observed modified cells in different hematopoietic lineages, demonstrating that HPSCs reconstituted the entire hematopoietic system. Finally, we assessed the safety of our CRISPR strategy. First, we confirmed that erythroblasts derived from edited HSPCs displayed erythroid markers and retained globin chains and hemoglobins expression. Then, we verified that selected CRISPR/gRNAs are specific for α-globin gene, with undetectable off-target events (1% InDel threshold) as assessed by PCR analyses of *in silico* predicted off-targets and unbiased genome wide screening (IDLV capture). Overall, we established a safe and novel *ex vivo* nuclease-based platform to achieve robust erythroid-specific expression of proteins for different therapeutic applications, including haemophilia and inherited metabolic disorders.

Vector and Cell Engineering, Production or Manufacturing IV

965. Genome-Wide CRISPR Screen in HEK293 Identifies Putative Cellular Restriction Factors for AAV Manufacturing

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The ability to produce sufficient vector quantity to meet demand is a practical yet highly relevant challenge for gene therapy. Traditional mammalian-cell-based rAAV production platforms utilize triple plasmid transfection of HEK293 cells. Moreover, despite high transfection efficiency of all three plasmids, previous observations revealed only a subset of transfected cells actually contained assembled AAV capsids. We thus hypothesized that host factors in HEK293 can repress vector production, and removal of such factors could exponentially increase vector yields from mammalian culture systems. To screen for such factors, we prepared genome-wide CRISPR knockout libraries of HEK293 cells which were then triple-transfected by PEI-MAX, immunostained with anti-capsid antibody to identify producer cells, and FACS sorted on this stain at 12h, 18h, and 24h post-transfection. Of the most significantly enriched genes in the positive populations, three are subunits of the same epigenetic regulatory complex, and a fourth gene regulates pH within endomembrane compartments. We tested these four genes for a gain in rAAV production by generating knockout (KO) cell lines deficient for these four hits individually and in all permutations of double KO. Single KO cell lines produced 4 fold higher titer compared to WT cells, and initial tests in double KO cell lines produced up to 9 fold higher titers in crude lysates, as measured by qPCR on DNase resistant particles. The KO lines have no apparent growth defects, and harvested vector transduces cultured cells at efficiencies indistinguishable from vector harvested from WT HEK293 cells. Our studies present a viable approach to significantly increasing vector quantities using standard culture, transfection, and harvest conditions, and could therefore be implemented in any production setting. Ongoing studies aim to elucidate the mechanisms responsible for these increased titers, to inform further improvements upon mammalian cell rAAV production systems.

966. CRISPR-Based High-Throughput Screening Reveals Host Factor SKA2 and ITPRIP Promoting AAV Manufacturing

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An increasing number of clinical trials have established that gene therapy is a promising strategy for the treatment of genetic disorders and that the recombinant adeno-associated virus (rAAV) is an efficient vehicle for therapeutic gene delivery. However, the doses required as well as the patient numbers involved in many clinical targets is increasingly making AAV manufacturing a barrier for the clinical development and commercialization of AAV gene therapies. To address this problem, we have employed CRISPR-Cas9 technology with Synergistic Activation Mediator (SAM) guide libraries for genome-wide screening of host factors, which revealed SKA2 and ITPRIP as effective enhancers for rAAV production. First, doxycycline-induced expression of SKA2 or ITPRIP in 293T cells increased rAAV titer by 2.2-fold and 3.3-fold, respectively. Second, transfection-based rAAV production upregulates mRNA transcription of endogenous SKA2 and ITPRIP, which implies that expression of these molecules could be facilitated by rAAV. Third, expression of SKA2 or ITPRIP had no effect on the transfection efficiency, cell viability, or transcription of AAV CAP genes, which suggests that the replication of AAV transgenes or capsid packaging could instead be affected. Finally, we found that ITPRIP modulates cell cycle in a manner that facilitates rAAV production. This highlights the importance of cell engineering in AAV production as well as the importance of host factors facilitated by AAV for its own replication. Taken together, our results indicate that the host factors,

revealed by our CRISPR-based high-throughput screening system, have a significant effect on AAV production potentially by modulating cell cycle in the triple-plasmid transfection system.

967. CRISPR Screening Identifies Gene Knockouts That Improve Lentiviral Vector Titers in HEK293 Cells

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Lentiviral vectors have emerged as important tools for treating genetic and acquired human diseases. As clinical studies have progressed there has been a growing demand for large amounts of purified vectors. However, the manufacturing of large, high titer viral stocks remains challenging. To address this issue, we developed a CRISPR library screening method to identify gene knockouts that increase lentiviral productivity. Briefly, the gRNA sequences from the Brunello knockout library were cloned into a modified vector backbone containing an intact 3' LTR sequence, allowing for integrated vector genomes to be rescued. HEK293 cells were transduced with this modified library, such that transduced cells on average contained one potential gene knockout. These cell populations were then expanded, and integrated vector genomes were rescued by transfection. New virions were harvested, and the process of sequential transduction and rescue-transfection was iterated. Through this workflow, gRNAs that knock out genes that suppress vector production were enriched and these enriched gRNAs were identified with accessible NGS methods. Further validation of three top hits was carried out by generating clonal HEK293 knockout cell lines using RNP transfection. Two of the three hits tested affecting an E3 ubiquitin ligase and a protease inhibitor, yielded increases in viral titer of 5 and 2.8-fold, respectively, when compared to cell clones transfected with a control RNP. Similar approaches may be used to identify additional hits that could be combined to yield cell lines with improved titers.

968. Novel Strategy for Generating rAAV Vectors Using Pseudorabies Virus

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Robust and long-term gene expression in a variety of different cell types has made the use of recombinant adeno-associated viral (rAAV) vectors very successful in pre-clinical and clinical studies. However, producing large-scale quantities of rAAV necessary for systemic administration and large clinical trials has been an ongoing challenge in the field. In this study, we evaluated the use of the porcine pathogen, Pseudorabies Virus (PRV), to facilitate rAAV production. Since this virus is non-infectious to humans, it is safer than the established Herpes Simplex type I system, in which contaminating replication-competent virus is a serious safety concern. Furthermore, by using a molecular clone of rPRV cloned in a bacterial artificial chromosome (BAC), we are able to manipulate the viral genome using bacterial genetics quickly and efficiently. This method accelerated virus production by 1) obviating the need for extensive plaque purification, and 2) eliminating the risk of parental virus contamination. We generated two different replication-deficient rPRVs: one encoding the AAV *rep* and *cap* genes, and the other encoding our gene of interest (GOI) flanked by AAV2 inverted terminal repeat (ITR) sequences. Initial transfection and infection experiments with these viruses resulted in yields as high as 1e5 rAAV gc/cell. We are currently optimizing rAAV production by co-infecting a variety of different cell lines.

969. Exploring Optimal *REP* Gene Expression Levels for the Generation of a Stable AAV Packaging Cell Line

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Over one hundred ongoing clinical trials using recombinant adeno-associated virus (AAV) vectors are showcasing their popularity and potential to treat various genetic disorders. A great obstacle for the commercial application of AAV vectors is the complexity and cost associated with large-scale production even to support clinical trials. Generation of a safe AAV packaging or producer cell line for large-scale production has proven to be challenging, mainly due to the toxicity caused by the AAV Rep proteins. The native AAV *REP* gene encodes for four Rep proteins (Rep-78, -68, -52, and 40) which have both specific individual and overlapping functions. To date, the importance of these proteins for high titer AAV vector production has not been fully understood. Our previous results have shown that only one large Rep protein (-78 or -68) combined with only one small Rep protein (-52 or -40) is sufficient for high titer AAV production. For generation of an AAV packaging cell line, we have tested the minimal AAV *REP* level of expression to obtain high titer of vectors by transient transfection in HEK 293-SF cells using plasmids containing cDNAs for individual Rep proteins. Using the CMV minimal core promoter, without the CMV enhancer, provides adequate *REP* expression levels resulting in AAV titers comparable to the standard triple-plasmid transfection method with AAV2 REP-CAP genes. This demonstrates that low amounts of Rep proteins are required for AAV vector production. Considering the toxic effect of the Rep proteins on producing cells, Rep68 and Rep40 were chosen together with the use of an inducible gene expression system as the most sensible option for stable packaging cell line generation. A low copy number of *REP* genes was integrated into HEK 293-SF cells using lentivirus and the production of Rep proteins was demonstrated in cell pools after induction. Optimization of AAV production using clones of cells isolated from the pools and expressing Rep68 and Rep40 after induction is currently being investigated.

970. rAAV for Tumor Therapy Using Transcriptional and Translational Control of Suicide Gene Expression Purified by a Newly Developed Affinity Chromatography Based on the PKD Domains of AAVR

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Gene therapy for cancer with recombinant viruses can be targeted to tumor cells via surface marker binding, cell type specific promoters, and tuning mRNA stability via miRNA overcoming the lack of specificity of conventional non-invasive therapies. We integrated these options in recombinant adeno-associated virus (rAAV) for suicide gene delivery in a virus-directed enzyme prodrug therapy (VDEPT) context. rAAV2 vectors with thymidine-kinase (TK) under the control of survivin (SUR), cyclooxygenase-2 (COX-2) or the C-X-C motif chemokine receptor 4 (CXCR-4) promoter were constructed and tumor specificity was compared to the strong cytomegalovirus immediate early promoter (CMV) driven gene expression. For an additional layer of tumor-specificity the let-7a microRNA (miRNA) target sequence was introduced into CMV- and SUR promoter driven expression cassettes. In the presence of the prodrug ganciclovir (GCV) a cytotoxicity assay demonstrated dramatic repression of CMV-promoter driven gene expression upon miRNA de-targeting, leading to the highest tumor-specificity among all investigated transgene expression cassettes. Contrary to expectations, the CMV promoter may be a good candidate for tumor specific gene expression when combined with miRNA de-targeting strategies. In addition to increased tumor specificity, successful application of rAAVs in cancer gene therapy also relies on efficient, cost-effective and scalable purification strategies. We established an affinity chromatography based on selected single Polycystic Kidney Disease (PKD) domains of the natural AAV receptor (AAVR), which mediates transduction of many serotypes. Affinity chromatography based on a relevant biological interaction yields functional particles of sufficient purity in one step and simplifies downstream processing of rAAV.

971. Lac Repressor Inducible Control of AAV Capsid Protein Ratios and Decoupling VP1, VP2 from VP3 in the Sf9 insect cell/Baculovirus Expression Vector (BEV) Platform

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The Sf9/baculovirus expression vector system (BEV) is an efficient, cost-effective and scalable method to produce AAV for large patient populations. Based on wild type (wt) AAV, efficacious vectors are thought to require the expression of VP1, VP2 and VP3 capsid proteins at a 1:1:10 ratio. Most if not all commercial BEV cloning kits are restricted to engineering foreign genes into a single location at the polyhedrin (polh) or Tn7 locus of the BEV. The VP1, VP2 and VP3 capsid genes share a common gene sequence and they would be unstable if expressed in tandem under independent promoters. Coinfection with multiple recombinant BEVs or the use of inducible Sf9 cell lines is a complex option not ideal for commercial scale. These facts force recombinant AAV specific BEV cloning strategies to emulate the wt AAV transcriptome with VP1, VP2 and VP3 sharing a common ORF region. To achieve the wt capsid protein ratios, the commercial BEV system uses noncanonical translational start codons to reduce the abundance of VP1 and VP2 relative to VP3 which has a normal ATG start codon. AAV is a mammalian virus and we have found that different capsid serotypes generated in the Sf9/BEV system can produce non-optimal or non-wt like VP1:VP2:VP3 ratios despite using the same noncanonical start codons for VP1 and VP2. In some AAV serotypes almost no VP1 and VP2 is produced resulting in reduced AAV infectivity. We have developed a new BEV technology for producing AAV capsid proteins that is not restricted to one locus for foreign gene insertion. We have applied our technology to decouple VP1, VP2 and VP3 expression such that these proteins are stably and independently expressed from separated locations in the BEV genome. We have also engineered the *E.coli* Lac Repressor system into our BEV such that we have inducible and predictable control of the expression of VP1 and VP2 relative to VP3. The goal of this work is to produce an AAV-based capsid which is most efficacious for the delivery of gene therapy agents. The capsid VP1:VP2:VP3 ratios found in wt AAV capsids found in nature are not necessarily the optimal ratios for a AAV VLP destined for the delivery of foreign therapeutic DNA constructs into human tissues. Freed from the AAV transcriptome biology and with the full potential of the BEV and the solidity of the Lac Repressor system, we now have the ability to predictably tune VP1:VP2:V3 ratios towards making an abundant and superior AAV therapeutic drug product in Sf9/BEV system.

Pharmacology/Toxicology Studies

972. Gene Transduction, Hematopoietic Stem Cells (HSCs) Engraftment, and Red Blood Cell (RBC) Physiology in Sickle Cell Disease (SCD) Gene Therapy

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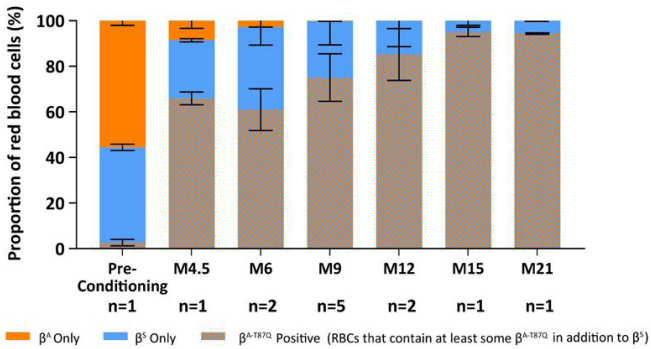
LentiGlobin for SCD drug product (DP) contains autologous CD34+ HSCs into which modified β-globin gene ($\beta^{A\text{-}T87Q}$) was delivered by lentiviral vector (LVV). This gene therapy is being evaluated in the ongoing phase 1/2 HGB-206 study (NCT02140554). Use of a refined manufacturing process led to a median (min-max) DP vector copy number (VCN) of 3.8 (2.8-5.6) copies/diploid genome and % transduced cells (% LVV+) of 80 (71-88) %. These DP characteristics led to robust HbA^{T87Q} production and better outcomes in the recently treated cohort. We performed exploratory assays in this subset of

patients to assess: 1) persistence of LVV+ cells in bone marrow (BM) and peripheral blood (PB), 2) effect of VCN on $\beta^{A\text{-}T87Q}$ and sickle β-globin (β^S) in PB mononuclear cell (PBMC)-derived erythroid colonies, 3) % of RBCs expressing $\beta^{A\text{-}T87Q}$, and 4) impact of $\beta^{A\text{-}T87Q}$ on RBC sickling. Data from ongoing analyses in earlier study patients will be presented. Individual colonies derived from pre-infusion DP and, post-infusion, from PBMCs and CD34+ BM HSCs were tested for LVV presence by qPCR and for β^S expression by ultra-performance liquid chromatography. $\beta^{A\text{-}T87Q}$ in RBCs was assessed by single cell western (scW) using an anti-β^S antibody and an anti-$\beta^A/\beta^{A\text{-}T87Q}$ antibody. RBC sickling (% sickled RBCs over time) was measured by imaging flow cytometry on 2% O_2-exposed RBCs. Data are median (min-max). Engraftment of transduced cells was stable: percentages of LVV+ colonies from PBMCs at 9 months and BM at 12 months post-infusion in 5 patients were 79.2 (67.0-88.4) % and 81.5 (60.6-88.1) %, respectively. In colonies from PBMCs and BM post-infusion vs DP, frequency of high-VCN progenitors was reduced. VCN and HbS were inversely correlated per analyses of PBMC-derived erythroid colonies (n=1 patient): β^S was 62 (33-87) % of total β-globin in colonies with VCN=1 and 23 (0-55) % in colonies with VCN ≥ 3. To understand how 80% of LVV+ cells in the DP relates to $\beta^{A\text{-}T87Q}$ expression in RBCs, we measured the % of $\beta^{A\text{-}T87Q}$-positive RBCs by scW (Fig 1). At last visit in 8 patients with ≥ 9 months of follow-up, 83.0 (65.5-95.8) % of RBCs contained $\beta^{A\text{-}T87Q}$, with > 90% $\beta^{A\text{-}T87Q}$-positive RBCs in 4 patients. Samples from untreated patients with SCD, sickle cell trait individuals and 7 HGB-206 patients with ≥ 6 months of follow-up were used to assess the % of sickled RBCs. The % sickled RBCs from LentiGlobin-treated patients was similar to trait individuals and significantly lower than for untreated patients with SCD. These data show consistent engraftment and persistence of LVV+ cells post-treatment. Lower frequency of high-VCN progenitors from post-infusion PB or BM vs DP suggests they may not contribute to the gene-modified cells in the long term. LentiGlobin treatment also results in nearly pancellular $\beta^{A\text{-}T87Q}$ expression and decrease in β^S, affecting SCD pathophysiology as shown by reduced RBC sickling. Together, these early data begin to describe the complex interplay between DP characteristics and the physiology of erythroid progenitors after LentiGlobin gene therapy.

Figure 1. Single RBC western assay from HGB-206 patients



Mean is depicted - if N=1, data show technical replicates; *Pre-conditioning sample does not contain any $\beta^{A\text{-}T87Q}$, signal is due to error rate of multiples

973. InGeTox - A Modular Vector Safety Prediction System

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An Individualized Genotoxicity Testing (InGeTox) system based on patient-derived induced pluripotent stem cells (iPSC) and their hepatocyte-like cells (HLC) derivatives was developed within the CRACK-IT Challenge for the prediction of vector safety in gene therapy by the integrated analysis of:

- Cell susceptibility to DNA damage by integrating vectors.
- Vector integration site (IS) identification and clonal contribution assessment.
- Cellular transcriptome for aberrant transcript detection and integration-induced altered gene expression.
- Vector associated methylome profiling.

The reference standards used were lentiviral vectors (LV), pHR with SIN LTR and pHV with full LTR, and AAV vectors with liver-specific ApoE or strong ubiquitous CB7 promoters. IS analysis was performed by shearing-based S-EPTS/LM-PCR and identified a total of 348,432 and 4,391 IS for the LV and AAV vectors, respectively. Overall, LV-derived IS numbers were higher in iPSC compared to HLC (214,285 vs 56,770 IS), although iPSC collected 30 days post-infection presented an average 2.8-fold reduction compared to the earlier timepoint (77,377 IS). As expected, AAV samples presented a ~2-logs lower number of IS. AAV-ApoE yielded a 2.4-fold higher IS for both HLC and iPSC when compared to its homolog. The ongoing qPCR studies will enable the evaluation of vector integration frequencies. For AAV samples, (nr)LAM-PCR was performed to evaluate S-EPTS/LM-PCR performance and higher IS retrieval was observed for the shearing-based method in all samples (894 vs 298 IS/µg DNA). Highly polyclonal integration profiles were observed on LV-infected samples, even for iPSC at both timepoints, and no clonal outgrowth was detected. AAV-treated samples also exhibited a polyclonal integration profile, where the most abundant clone constituted 4.18%. Common integration site (CIS) analysis revealed top 10 CIS with orders ranging between 10-1170 in LV- and 2-22 in AAV-infected samples. Overall, iPSC CIS exhibited higher orders compared to HLC (on average, 197.9 vs 13.9) and iPSC receiving the pHR vector showed the higher CIS orders (on average, 312.1). No CIS overlap was observed in HLC infected with the different AAV although, for iPSC, 4/10 CIS were common to both vectors. Initial methylome analysis comparing the methylation profile of transduced and untransduced controls revealed no significant changes on a global level. However, significant differentially

methylated regions (DMR) were observed in in LV-infected (range 29-1077 DMR) and AAV-treated (range 220-314 DMR) samples. Of note, pHV-infected iPSC collected 30 days post-infection showed a hypo-methylated DMR within HMGA2, previously associated to clonal expansion in a clinical β-Thalassemia study. No DMR was located within genes linked to gene therapy severe adverse events. The integration of the ongoing single cell clone studies and transcriptome analysis with these datasets will enable to define the influence of the vectors' integration on host's gene expression and determine the genotoxic potential of gene therapy vectors.
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974. A Single Administration of AAV5-hFIX in Newborn, Juvenile and Adult Mice Leads to Stable hFIX Expression up to 18 Months after Dosing

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Recombinant adeno-associated viruses (rAAV) are replication-deficient, non-integrating viruses commonly used as vectors for gene therapies. Currently an AAV serotype 5 vector with a hFIX transgene expression cassette designed for liver-directed expression of human Factor IX is being studied in clinical trials for hemophilia B. A major concern when using a single treatment with AAV is the possibility of losing transgene over time due to growth and/or natural turnover of cells. Here, we investigated the duration of hFIX expression in plasma after treating wildtype mice at different stages of development, from neonates to adults, with AAV5-hFIX. Since hemophilia B is predominantly found in male patients, male C57Bl/6 mice received a single intravenous infusion of $2x10^{14}$ gc/kg AAV5-hFIX or vehicle. Neonates were dosed at 2 days old, weanlings at 3 weeks old, juveniles at 6 weeks of age and adult mice were dosed at 11 weeks or 6 months of age. All mice were sampled for plasma starting 4 weeks after dosing and then every three months. One cohort of mice (n=10 per group) was necropsied 4 weeks after dosing, while the other cohort (n=20 per group) was followed until 18 months of age, which is nearly the complete lifespan of a mouse. At necropsy the livers were weighed and collected for vector DNA analysis by QPCR. The plasma samples were used to determine the hFIX protein levels at each timepoint. All AAV5-hFIX injected animals showed hFIX protein expression from the first sampling timepoint at 4 weeks post dosing until sacrifice. The absolute levels of hFIX protein were lower in mice treated at a younger age, reflecting the lower total amount of vector genome copies injected in those animals due to the low body weight at dosing. The highest hFIX expression was seen 4 weeks after dosing and decreased to a stable level of hFIX protein within 3-6 months after dosing, after which the expression remained stable for the duration of the study. Remarkably, none of the animals in any group showed loss of hFIX expression after stabilization, even after more than a year of follow up and significant growth of the liver. A correlation was found between the amount of genome copies injected, the genome copies detected in the liver after necropsy and the hFIX protein expression in plasma at necropsy. This study shows that even in animals treated with AAV5-hFIX at a very young age, followed by significant growth, hFIX expression was sustained up to 18 months after dosing. This contradicts the expectation that vector DNA is lost during replication of the cells due to growth or natural turnover. Further studies to unravel the underlying mechanisms are ongoing.

975. *In Vitro* Transformation Assays for Non-Clinical Safety Assessment of CRISPR/Cas9 Genome-Edited Cells

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Cas9-mediated cleavage in undesired DNA regions (off-target editing) is one of the main safety concerns for the use of CRISPR/Cas9 genome editing in gene therapy. Although theoretically rare, off-target cleavages could lead to malignant transformation. To date, the gold standard for non-clinical tumorigenicity assessment is monitoring the potential of genome-edited cells to form tumors in NOD scid gamma (NSG) mice. However, *in vivo* tumorigenicity studies are lengthy, costly and their relevance as well as predictability is in dispute. Thus, assessing the transformation potential *in vitro* could provide additional cost-efficient, time-saving and relevant safety information. Here, we evaluated two *in vitro* transformation assays for their ability to detect the transformation potential of genome-edited cells, and compared to an *in vivo* tumorigenicity study. A known hallmark of malignant transformation is anchorage independent growth, which can be assessed by soft agar colony forming assay (SACF). Recently, an assay based on growth in low attachment plates (GILA), has been described and suggested as a quicker alternative to a classic soft agar. We compared the sensitivity and performances of GILA and a miniaturized digital SACF by using a CRISPR/Cas9 based approach to transform immortalized MCF10A cells and measuring ATP levels (GILA) or counting colonies using high content imaging (SACF). As potential candidates for a positive control, we chose genes whose deletion induced hyperproliferation in a genome wide CRISPR/Cas9 knock out screen. The screening revealed PTPN12 (Tyrosine-protein phosphatase non-receptor type 12), a tumor suppressor often inactivated in triple negative breast cancer, as strong inducer of hyperproliferation. We confirmed that PTPN12 deletion enables anchorage independent growth of MCF10A cells in GILA and SACF, and assessed the limit of detection for both assays by titrating sgRNAs targeting PTPN12 as well as spiking PTPN12 $^{(-/-)}$ in wild type cells. We found that SACF is more sensitive than GILA with a limit of detection of ~12.5% transformed cells. Moreover, we identified four weeks as optimal incubation time to count colonies for SACF whereas measuring ATP levels for GILA shows best results after two weeks. Next, we compared the sensitivity of SACF and GILA to *in vivo* tumorigenicity studies by injecting different ratios of PTPN12 $^{(-/-)}$ and wild type MCF10A cells subcutaneously or in the fat pad of NSG mice. No tumor formation was observed after three months. In

conclusion, mini SACF could be a valid, quick and cost-efficient assay for non-clinical tumorigenicity assessment of genome-edited cells while GILA could be used for a rapid pre-screening of potential targets.

976. Preclinical Assessment of an In Vivo Lentiviral Vector Cure for Hereditary Tyrosinemia Type 1

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Lentiviral vectors are durable gene therapy tools, but their application is largely limited to ex vivo administration due to perceived risk of genotoxicity from vector integration. Here we applied direct in vivo lentiviral gene therapy to a porcine model of a tumorigenic liver disease, hereditary tyrosinemia type 1 (HT1) to demonstrate the safety and efficacy of this approach. This integrating vector was well tolerated, and portal vein injection showed both improved liver targeting compared to systemic administration and higher efficacy than in ex vivo cell therapy application in the same indication. This treatment resulted in cure of the disease as evidenced clinically by the ability to thrive off the protective drug NTBC, as well as biochemically by normalization of tyrosine levels and liver function tests. Furthermore, treated animals showed neither significant residual fibrosis nor tumorigenicity. In fact, the vector demonstrated a benign genomic integration profile and restored wild type levels of expression for many cancer-associated genes that showed dysregulation during disease progression and ultimately the development of hepatocellular carcinoma. Therefore, application of lentiviral vectors in vivo is safe, prevents cancer formation in this model, and should be considered more widely for indications where integration offers advantages over other gene therapy platforms.

977. Physiologically-Based Pharmacokinetic Modeling for Adeno-Associated Virus Gene Therapy

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Adeno-associated viruses (AAV's) are non-pathogenic human parvoviruses capable of transducing non-dividing cells and effecting long-term gene expression. In recent years, AAV's have proven to be an exceedingly promising and popular vehicle with which to effect gene therapy. Despite the tremendous interest in this novel therapeutic modality, fundamental, quantitative understanding of the pharmacokinetics and pharmacodynamics of AAV's is largely underdeveloped. This lack of understanding constitutes a major unmet need as the body's responsive production of neutralizing antibodies following AAV exposure can render readministration impractical. This paradigm makes the need for dose-informing AAV pharmacology critical as we often have only one opportunity to administer the optimal dose via the optimal route. To address this gap in understanding, we have developed a physiologically-based pharmacokinetic (PBPK) model for the distribution of the transgene DNA of an AAV therapeutic molecule targeting CNS tissue. Figure 1 depicts the PBPK model structure which includes three compartments for the cerebrospinal fluid (CSF) and three correspondingly connected compartments for the spinal cord. Compartments representing the blood, brain and liver are also included. To parameterize this model, we have used data collated from several studies featuring the administration of AAV's to non-human primates (NHP's) via several routes of administration including: intravenous, lumbar puncture and intra-cisterna magna. AAV DNA transgene content was quantified in all matrices using quantitative polymerase chain reaction (qPCR). Data was obtained for all compartments in the model structure except for the CSF Thoracic compartment. Values for rate transfer constants for this model were fit using Monolix. Model fit is demonstrated, and key predictions are presented. In particular, the model predicts rapid equilibration of the CSF compartments. In addition to constituting an unprecedented modeling effort for AAV gene therapy, this PBPK model serves as a tool to simulate AAV biodistribution and concentrations at the site of action as a function of route of administration (intracisternal magna, lumbar puncture, intravenous). Furthermore, this model serves as the first step in realizing a broader quantitative pharmacology modeling platform for AAV's by connecting administration characteristics with biodistribution over time.

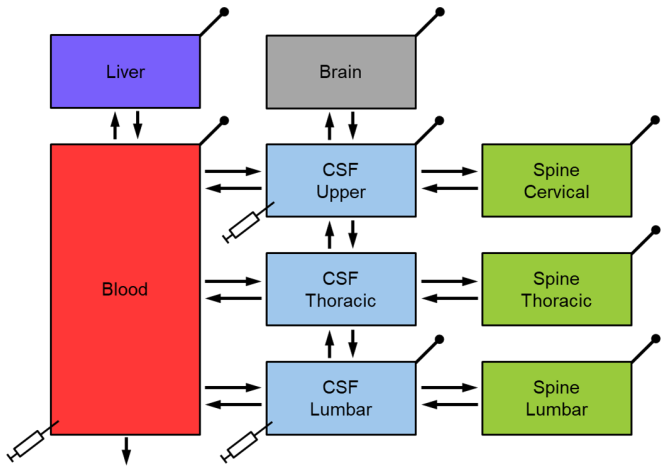

Figure 1: Physiologically-based pharmacokinetic (PBPK) model structure for adeno-associated viruses (AAV's). Compartments for which quantification of AAV transgene were obtained are indicated with lines ending in circles and compartments in which drug was dosed are indicated with syringe symbols.

978. XT-150 Human IL-10v Plasmid DNA: Results from 6-month GLP Safety Study and Efficacy After Intra-Articular Administration to Companion Dogs with OA

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Osteoarthritis (OA) is a disease of unknown etiology that manifests as significant impairment in joint function resulting from degeneration and destruction of tissues within the synovial capsule. Over 25 million sufferers in the US, and millions more world-wide, are poorly treated by current therapeutic options that target symptomatic relief. Importantly, this disease is also prevalent in the veterinary population and is especially common in dogs, cats, and horses. An important component of OA in all species is the production of proinflammatory cytokines within the joint capsule and the resultant alteration of cell functions within the joint. Interleukin-10 (IL-10) can reverse proinflammatory cytokine effects but the short half-life of IL-10 as a therapeutic protein is not practical for further development. A plasmid expressing a novel, long-acting IL-10 variant has been developed for targeted expression. Here we describe the results of a 6-month GLP toxicology study in Beagle dogs and an ongoing double-blind, placebo-controlled pilot study in companion dogs with OA examining an aqueous formulation of this plasmid-based therapy, XT-150. In the safety study, we demonstrate that XT-150, delivered intra-articularly to dogs, is well-tolerated and produces no toxicologic effects, with a no-observed-effect level (NOEL) at doses 10-fold above the anticipated therapeutic dose. In the ongoing pilot efficacy study, XT-150 has been shown to provide durable improvements in overall quality of life and pain measures in these animals. At the end of the double-blind period, 8 weeks after XT-150 intra-articular injection, both clinical veterinary ratings of pain and owner assessment of pain and quality of life measures were markedly different from those of vehicle-treated dogs. Because of the similar natural history and progression of OA in human and veterinary populations, these data are supportive of translation of IL-10-based therapies into the clinic in both human and veterinary settings.

RNA Virus Vectors

979. Development of the Novel FN3 Displayed Lentiviral Vectors for CD4-Targeted In Vivo Gene Delivery

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Cell type specific delivery is a critical and challenging step for *in vivo* gene therapy. Paramyxovirus entry glycoproteins, which can pseudotype lentiviral vectors, consist of separate attachment (H or G) and fusion (F) proteins. In this way they are amenable to re-targeting strategies through the independent engineering of the H or G components. To date, glycoproteins from measles virus (MeV) and Nipah virus (NiV) have been re-engineered by mutating their natural receptor binding sites and adding alternate targeting ligands based on single chain antibodies (scFvs) or Designed Ankyrin Repeat Proteins (DARPins). However, scFv displayed vectors often have an inherent structural stability problem which can lead to vector and producer cell aggregation and low titers. DARPins are a much more stable ligand, but the selection of molecules with appropriate binding characteristics from synthetic libraries is expensive and time consuming. To address these issues, we examined the potential of an alternative ligand based on human fibronectin domain III (FN3). FN3s offer many advantages including their smaller size and reduced complexity which facilitates the selection of ligands with appropriate specificities from libraries while displaying the high-binding affinity and specificity of antibodies. In addition, we reasoned that their smaller size and lack of disulfide bonds should facilitate incorporation into chimeric viral proteins. As a proof of principle study, we tested a number of different ligands and glycoprotein combinations to develop CD4-targeted lentiviral vectors. This included using MeV or NiV glycoproteins and incorporating CD4 binding DARPins (5) or FN3s (9) as the targeting ligands. As a control, we included a CD4 DARPin MeV vector previously described (Zhou et al. *J. Immunol.* 2015). The vectors were tested to confirm specific transduction of CD4+ T cells, including in cultures of non-stimulated PBMCs *in vitro.* The results showed successful incorporation of all ligands into both MeV and NiV vector particles, with FN3-NiV vectors in general giving 10-fold higher titers than the equivalent FN3-MeV vectors. A subset of the vectors were also administered to humanized mice, where we also observed specific human CD4+ T cell transduction in blood, lymph node, spleen, and bone marrow. The most optimal CD4 FN3 NiV vectors displayed compatible titers and *in vivo* transduction efficiencies as the control CD4 DARPin MeV vector. Further analysis of the spleen samples showed that FN3-NiV vectors were able to transduce resting and memory CD4 T cell subsets, which are important subsets for several therapeutic applications, including HIV latent cell targeting. These findings, therefore, demonstrate that FN3 is a useful ligand with great potential for targeted vector development.

980. Using Humanized W-41 NSG Mice to Test the Safety and Efficacy of Intraosseous Delivery of Platelet-Specific Factor VIII-Lentiviral Vectors to Human Hematopoietic Stem Cells as an *In Vivo* Gene Therapy for Hemophilia

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Hemophilia A is a disorder that causes a deficiency in functional factor VIII (FVIII) secreted in plasma. This congenital disease, which is cumbersome and expensive to treat, presents as a promising model for gene therapy. Our lab has optimized delivery of FVIII lentivirus driven by the platelet-specific GpIbα promoter (G-FVIII-LV) to hematopoietic stem cells (HSC) in the bone marrow via intraosseous (IO) injection.

This mode of therapy would be viable for patients who have developed inhibitors to FVIII, as the FVIII is stored within the α-granules of platelets, avoiding detection by the immune system while in circulation. We have previously shown that IO delivery of G-FVIII-LV partially corrected the bleeding phenotype in HemA mice for at least 5 months. In this study, our aim was to evaluate the safety and efficacy of this therapy in human HSCs, using humanized NSG mice as our *in vivo* model. We first confirmed that G-FVIII-LV and G-GFP-LV could successfully transduce human HSCs using human $CD34^+$ ($hCD34^+$) cells isolated from G-CSF mobilized donors. HSCs treated with LVs were subsequently either expanded or preferentially differentiated into megakaryocytes, which are platelet precursors. After 7 days of culture, $CD41a^+$ megakaryocytes showed higher expression of each transgene than did $CD41a^-$ cells. Genomic DNA of cells transduced with G-FVIII-LV at MOI of 0.5 was isolated and analyzed by MGS-PCR for integration site analysis. No clonal dominance was found in the 124 unique integration sites identified. We then tested these LVs *in vivo* in humanized NSG mice. NSG mice with the W-41 mutation in the *Kit* gene, which promotes higher engraftment efficiency, were preconditioned with busulfan (25mg/kg) 24 hours before the transplantation of $hCD34^+$ cells via RO injection. Eight weeks later, we observed over 80% engraftment of $hCD34^+$ cells in bone marrow. After treatment with clodronate liposomes, human platelets comprised over 21% of total platelets isolated from PBMC on average. We performed IO delivery of G-FVIII-LV and G-GFP-LV in transplanted mice and did not observe any apparent toxicity in mice after surgery due to treatment. Expression of each transgene was detected in the platelets of the respectively transduced animals; FVIII was measured by ELISA and GFP was measured by flow cytometry. Genomic DNA from the human HSCs of the transduced humanized mice was isolated, and these samples are currently being analyzed for retroviral integration sites to assess the safety of this therapy *in vivo*. We anticipate polyclonal integration of our virus since there is no selective advantage of G-FVIII-LV transduced HSCs. Evidence of FVIII expression in platelets over five months after IO injection from our previous studies suggest successful transduction of HSCs, but we are also currently assessing the efficiency of transduction in the multipotent long term-HSC population ($Lineage^-Sca1^+C\text{-}Kit^+CD150^+CD48^-$), specifically. Given its safety and efficacy in humanized mouse models, IO delivery of platelet-specific FVIII presents as a promising *in vivo* gene therapy for correcting the hemophilia phenotype.

981. Unwanted DNA Methylation and Transcriptomic Changes Can be Induced in CD34+ Cells by Vectors During the Ex-Vivo Gene-Modification Process

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Lentiviral (LV) or Adeno-Associated Vectors (AAV) are commonly used for the ex-vivo gene modification of human hematopoietic stem/progenitor cells (HSPC) for gene addition or genome editing. We have previously reported that unwanted genome-wide DNA over-methylation can be triggered in CD34+ HSPC following infection by some LV batches including Integration-Deficient Lentiviral Vectors (IDLV). In the present work, we compared DNA methylation and transcription in HSPCs 24 hours post-infection by LV, IDLV or AAV6. We confirmed previous observations as some of the LV and IDLV batches tested produced DNA hyper-methylation shortly after infection as measured by genome-wide DNA methylation arrays. The effect was caracterized by a reproducible differential methylation in the TSS regions of a subset of around 200 genes annotated in stem cell pathways. However, despite DNA methylation modifications at promoter regions, RNAseq revealed no differential gene expression at this time point (24 hours post-infection). In contrast, transduction in the same conditions with an AAV6 induced no DNA methylation changes but a strong differential expression of around 70 genes. Those genes were especially enriched for the p53 pathway indicating cell cycle alteration and apoptotic processes. Thus, some gene therapy vectors can have measurable effects on target cells independently of their insertional capacity. Experiments are ongoing to determine the functional impact of vector effects on the viability and engraftment of gene-modified cells.

982. Abstract Withdrawn

983. Development of an Epithelial Mesenchymal Transition Tracing Vector

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INTRODUCTION: Epithelial mesenchymal transition (EMT) is a process whereby epithelial cells lose adhesion and gain migratory properties of mesenchymal cells. EMT is involved in the repair of damaged epithelial tissue, and unchecked, can lead to the development of fibrotic tissue. EMT has been primarily observed in vitro, however detecting EMT in vivo is challenging, with current methods only able to observe a snapshot in time rather than cell changes over time. Here we have developed a simple cell lineage tracing technique that allows us to visually observe which epithelial and mesenchymal cells transdifferentiate in vivo. We designed a permanent GFP/mScarlet fluorescence switch that, when dual stained for specific cell markers, provides details on the current cell type, as well as whether the cell was previously of the epithelial or mesenchymal lineage. **METHODS:** A bicistronic vector was designed to contain 1) CRE recombinase under the control of the epithelial-specific E-Cadherin promoter, and 2) a floxed GFP/mScarlet sequence driven by the constitutive SFFV promoter. E-cadherin mediated Cre-recombinase expression results in a switch from GFP expression to permanent and exclusive mScarlet expression in cells. Vector functionality was tested on rat airway epithelial cells (E-Cadherin expressing), a NIH-3T3 fibroblast cell line (N-Cadherin expressing), and on E-cadherin and N-cadherin knockout HEK293T cells. **RESULTS:** GFP expression was observed in all three cell lines 48 hours after transduction. Epithelial and HEK293T cells expressed both GFP and mScarlet for more than one week before mScarlet was exclusively expressed. The NIH-3T3 cell line expressed GFP only, for the duration of the experiment. **CONCLUSION:** In vitro proof of concept validation studies

have demonstrated the ability to identify epithelial cells that have undergone EMT. Due to GFP having a half life of ~26 hours, we observed a period of dual expression where cells expressed both GFP and mScarlet. However, once mScarlet expression was observed, GFP expression diminished as GFP transcription ceased and GFP protein turnover occurred. This vector has the potential to determine the extent of EMT occurring in animal models of fibrosis, as well as elucidate the life-course of airway epithelial cells. Acknowledgments: Project funding provided to Dr Nathan Rout-Pitt by the Women's and Children's Hospital Research Foundation

984. Optimizing Lentiviral Gene Therapy Protocol Using Hematopoietic Stem and Progenitor Cells for Preclinical and Clinical Study

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Lentiviral gene therapy holds promise for treating many monogenic disorders. However, clinical application can be limited by suboptimal transduction of hematopoietic stem cells (HSCs) and quantity of available lentiviral vector (LV). We recently reported clinical study of lentiviral gene therapy for X-linked severe combined immunodeficiency using a protocol in which patient $CD34^+$ cells were incubated with two successive rounds of transduction. This protocol requires extra manipulation with more vector consumption and is very time consuming. Therefore, we developed an improved protocol for LV delivery to $CD34^+$ cells that simplifies product manipulation, reduces vector consumption and achieves greater vector copy number (VCN) of repopulating HSCs in mouse xenotransplantation assays. Our notable findings include: 1) single-step transduction at higher $CD34^+$ cell density with low MOI conserved LV without compromising transduction efficiency; 2) the VCN of $CD34^+$ cells measured shortly after transduction did not always correlate with the VCN of repopulating HSCs after long-term xenotransplantation; 3) LentiBOOST increased HSC VCN more than prostaglandin E2 (PGE2); 4) while LentiBOOST with PGE2 combination further increased VCN *in vitro*, the VCN observed *in vivo* were similar to use of LentiBOOST alone in our hands; 5) Vector integration site analyses showed a high polyclonal distribution without clonal dominance in transduction enhancer-treated groups. These findings can support to optimize lentiviral gene therapy protocol by increasing the VCN of HSCs for preclinical and clinical study.

985. Epigenetic Downregulation of ApoE4 for Alzheimer's Disease

Natalie Asmus[1], Malik Moncalvo[1], Wendy Dong[1], Ekaterina Ilich[1], Carmel Falek[1], Joseph Rittiner[1], Domenic Tringali[2], Young Yun[2], Jeffrey Gu[2], Angela Wei[2], Anna Yang[3], Misha Angrist[3], Ornit Chiba-Falek[2], Boris Kantor[1]

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ApoE4 has previously been implicated as one of the most significant genetic markers associated with an increased risk of developing late onset Alzheimer's disease (LOAD). In our efforts to develop a novel, targeted gene therapy approach, we have created a series of lentiviral vectors, each carrying the protospacer adjacent motif (PAM) modified dSpCas9 enzyme, VRER-dCas9, fused to a de-novo DNA methyltransferase 3A (DNMT3A) and one of four unique guide RNAs (gRNA). Each gRNA is designed to target a different region of the ApoE promoter, in order to selectively methylate the E4 allele, while also aiming to minimize integration capacity and reduce the risk of potential off-target editing effects (through the use of integrase-deficient lentiviral vectors). The developed system has been validated on human carcinoma kidney (HEPG2) cells, by assessing the relative reduction of RNA and protein levels. We will aim to further validate this approach using neural progenitor cell lines, derived from isogenic iPS cells carrying different ApoE haplotypes.

986. Frequency and Timing of Lentiviral Vector Repeat Dose Delivery Does Not Significantly Alter Long-Term Transgene Expression Levels in Mouse Lungs

Chantelle Carpentieri[1,2,3], Nigel Farrow[1,2,3], Patricia Cmielewski[1,2,3], Juliette Delhove[1,2,3], Nathan Rout-Pitt[1,2,3], David Parsons[1,2,3], Martin Donnelley[1,2,3]

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Background: Lentiviral (LV) gene vectors are a promising option for treating cystic fibrosis lung disease by delivering a functional copy of the *CFTR* gene into airway epithelial cells. However, achieving high levels of sustained gene expression remains challenging. To provide long-lasting therapeutic gene expression it may be beneficial to produce higher levels of initial gene expression through initial repeat dosing, and to be able to effectively repeat-dose if gene expression wanes over time. It is currently unclear how effective repeat LV dosing strategies can be for increasing or maintaining gene expression levels and which timing strategy is best. The aim of this project was to determine the optimal repeat-dose strategy in normal mice for producing robust and lasting gene expression levels. **Methods:** The lungs of C57Bl/6 female mice were conditioned with 10 µl of LPC, followed one hour later by 30 µl of a VSV-G pseudotyped LV gene vector containing the FLuc-F2A-eGFP bicistronic cassette driven by the EF1α promoter. Mice were separated into five re-dosing schedule groups (n = 12/group). The dosing groups

were: 2 x 1 day apart, 3 x 3 days apart, 2 x 1 week apart, 3 x 1 week apart and 5 x 1 month apart. A single-dose group was included as a control (n = 12/group). Bioluminescence imaging (BLI; Xenogen, IVIS) of mice was performed at 1 week, and then 1, 2, 3, 6, 9 and 12 months after the first LV gene vector instillation to assess gene expression over time. Blood samples were collected via the submandibular vein at every imaging time-point to assess immunological responses to the transgene or LV vector system (analysis in progress). **Results:** Lung Luc luminescence was detected in all animals in all groups at the 1 week and 1 month imaging time-points. At 1 week, transgene expression was significantly higher in the 2 x doses delivered 1 day apart group compared to the control group ($p<0.0001$, one-way ANOVA), while at 1 month, transgene expression was significantly higher in the 2 x doses delivered 1 month apart compared to the control group ($p<0.02$, one-way ANOVA). For the remaining imaging time-points, lung luminescence became undetectable in some animals across the re-dosing and control groups. At the 12 month imaging time-point, the re-dosing group that received 5 x doses 1 month apart had only one animal with detectable levels of transgene expression, and the level of transgene expression in that animal was significantly lower than the control group ($p<0.01$, one-way ANOVA). For all other groups at 12 months, and at all earlier time-points, there were no significant differences in transgene expression across the groups. **Conclusions:** The results demonstrated that a VSV-G pseudotyped LV vector can be successfully readministered to the lung using different dose timings. At the 12 month time-point, the 5 x doses delivered 1 month apart group had undetectable levels of transgene expression in all animals except one, suggesting that this dosing schedule is not suited for long-term gene expression. Overall, repeat dosing appeared to confer no advantage over the standard single-dose method. Further studies and immunological analyses will provide insight into the role of immune responses against vector constituents and the transgene following repeat dosing. **Acknowledgements:** Study funded by the Women's and Children's Hospital Foundation, South Australia. C Carpentieri supported by the MS McLeod PhD Scholarship, with a PhD top-up award from Cystic Fibrosis South Australia. N Farrow supported by the MS McLeod Post-doctoral Fellowship.

987. Bacterial Artificial Chromosomes for Rapid Generation of High Titre Stable Suspension Producer Cell Lines for Lentiviral Vector Manufacture

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Supply of lentiviral vector at the large scales required to treat patient populations remains a significant challenge for ensuring patient access to gene therapy medicines. To date most clinical grade lentiviral vector manufacturing has used transient transfection of plasmid DNA into adherent 293T cells grown in cell factories. This manufacturing process entails high costs and long lead times for sourcing clinical grade plasmid DNA and is limited by poor scaleability of adherent cell factory processes beyond low tens of litres. To meet the need for large scale lentiviral vector manufacture GSK has established a method to rapidly generate stable lentiviral vector producer cell lines. This process is based on stable transfection of a single Bacterial Artificial Chromosome (BAC) construct encoding all lentiviral vector components as individual expression cassettes. We have demonstrated that when grown in 50L stirred tank bioreactors these cell lines produce volumetric titres equivalent to current widely-used cell factory processes. Through extended cell culture we have demonstrated that the cell lines are sufficiently stable to be expanded up to manufacturing processes at 2000L bioreactor scale. We will present recent experiments of side by side comparison of stable and transient production including cell full transcriptome RNAseq and functional and physical titration.

988. The Establishment of High Titer Protocol for Baboon Envelope Pseudotyped Lentiviral Vector Focusing on Syncytium Formation Phenomenon

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Introduction: Since Baboon envelope pseudotyped lentivirus (BaEV-LV) receptors, ASCT-1 and -2, highly express on the surface of hematopoietic stem cells (HSCs) without cytokine cocktail pre-stimulation, BaEV-LV has been reported as an alternative transduction tool for HSC gene therapy. The HSCs derived from DNA repair disorders such as Bloom syndrome have a propensity to become apoptotic state during pre-stimulation. Therefore, BaEV-LV could be a candidate for those disorders. However, the difficulty of generating high titer BaEV-LV hampers moving forward to clinical trials. Here, we have established the high titer BaEV-LV production protocol. Methods: Lentiviral vectors encoding green fluorescent protein under the control of spleen focus foamy virus promoter (LV-SFFV-GFP) were pseudotyped with Baboon envelope or VSV-G envelope. Titration was performed on 293T cells by GFP positivity using flow cytometry analysis. The basic protocol was defined as follows. 293T cells were cultured on tissue culture treated plate around 70% confluence. Either Baboon envelope plasmid (BEP) or VSV-G plasmid (0.44 μg per well of 6-well-plate) were co-transfected with LV-SFFV-GFP, REV and RRE plasmid. The viral supernatants were harvested at 48 hours post-transfection. Each factor was optimized as follows. Results: 1. The amount of BEP: Various amount of BEP has been used in previous reports. The virus titer of BaEV-LV and VSV-G-LV with basic protocol were 1.68×10^3 and 2.59×10^6 IU/mL, respectively. Then, we optimized BEP amount (from 0.044 μg to 1.21 μg) and compared the titer. According to the data, 0.528 μg of BEP increased the titer to 12 times higher (2.02×10^4 IU/mL) than basic protocol. 2. Syncytium formation phenomenon: We had observed syncytium formation in 293T cells after co-transfection of LV-SFFV-GFP, REV, RRE and BEP. In detail, time-lapse analysis revealed that syncytium formation and exfoliation began at 8 hours and 48 hours post-transfection, respectively. Interestingly, single BEP transfection induced syncytium formation as well. 3. Harvest timing: Next, we considered that syncytium formation might affect the viral production. Therefore, we optimized the harvest timing from 24 to 48 hours post-transfection. The titration data showed that the best timing was 48 hours post-transfection despite the exfoliation of 293T cells (1.16×10^3 IU/mL (24 h), 5.27×10^3 (48 h)).

4. Culture plate surface: Finally, we considered that the exfoliation of 293T cells might reduce the viral productivity. Therefore, we compared the titer of with or without poly-l-lysine (PLL) coating. The titration data showed that PLL coated plated double the titer of BaEV-LV (1.16×10^4 (with PLL), 5.27×10^3 IU/mL (without PLL)). Conclusions: According to these results, using 0.528 μg of BEP (per well of 6-well-plate), harvesting virus supernatant at 48 hours post-transfection and using PLL coated plate enhanced the titer. The establishment of our high titer protocol could move forward to prevent developing Bloom syndrome associated hematologic malignancies by BaEV-LV gene transfer.

989. The Role of Rev and the RRE on HIV-1 Genome Encapsidation

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HIV-1 genome encapsidation is mediated by interactions between unique structures on the gRNA and the nucleocapsid domain of Gag. The gRNA elements essential for its packaging have been primarily mapped to the highly conserved and structured 5' UTR. However, the presence of elements outside of this region and their role on packaging is unclear. Since packaging elements are dependent on conformation, mutations in the elements or in neighbouring sequences can disrupt packaging, as well as the synthesis of viral proteins. One solution to this problem is to analyse these elements in the setting of a lentiviral vector, while providing the necessary viral proteins in-*trans* from separate constructs. The HIV-1 viral protein Rev is known for its well characterised role in nuclear export of viral RNAs. By binding the Rev responsive element (RRE), Rev permits the nuclear export of unspliced gRNA and singly spliced mRNAs into the cytoplasm. We have observed that the deletion of the RRE from lentiviral vectors, or the absence of Rev, severely reduces lentiviral vector titres as well as genome encapsidation into virus like particles. Interestingly, we have only detected minor effects on cytoplasmic gRNA abundance in the absence of Rev/RRE. Furthermore, replacing the RRE with the constitutive transport element (CTE) completely restores cytoplasmic gRNA but not packaged gRNA levels. Our data suggests Rev/RRE plays an important role in the gRNA encapsidation of HIV-1.

990. Development of Lentiviral Platform for Allele-Specific Targeted Down-Regulation of APOEe4 Expression: Epigenetic Therapy for Precision Medicine in Alzheimer's Disease

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The e4 allele APOE gene is the strongest and most established genetic risk factor for late onset Alzheimer's disease (LOAD). It has been shown that reduction of APOE4 levels decreased LOAD pathology. DNA-methylation regulates APOE expression and differential methylation levels were reported between disease and controls. Here, we developed a novel CRISPR/dCas9-editing tools to enhance targeted methylation to specific downregulate of the ApoE4 expression. The strategy is based on SNP-rs429358 defining E4 that creates protospacer adjacent motif (PAM) recognized by a newly engineered VRER-Cas9 protein. We demonstrated that VRER-dCas9 can specifically and accurately discriminate against the new PAM motif using in vitro system.

991. Generation of Stable Producer Cell Lines (PCL) of Lentiviral Vector (LV) Using PiggyBac Transposase

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Lentiviral vector is one of the most effective and popular delivery vehicles in cell and gene therapy as the therapeutic gene delivered by LV can be maintained for the lifetime of patients. In addition, LV delivers its cargo into both dividing and non-dividing cells, due to which LV has broad applications *in vivo* and *ex vivo*. The demand for lentiviral vectors is higher than ever with the recent success of many CAR-T therapies as well as other clinical trials for genetic diseases. Traditionally, the production of lentiviral vectors in a large scale uses transient transfections where adherent or suspension cells are transfected with multiple plasmids. However, this approach is constrained by its limited scalability and high cost. Furthermore, a large quantity of plasmid DNA is required in the process, adding more challenges in logistics, quality control, safety, and process robustness. To overcome these problems associated with transient transfection, Lonza has generated a stably transfected, inducible producer cell line (PCL) for the large-scale production of lentiviral vectors in suspension culture. For lentiviral vector production, one needs to provide vesicular stomatitis virus G protein (VSV-G), the regulator of virion expression (Rev) of human immunodeficiency virus-1 (HIV-1), and group-specific antigen and polymerase (Gag-Pol) of HIV-1. The sequences of VSV-G and Rev were codon-optimized to allow their higher expression. Each gene above was driven from a tetracycline-inducible, cytomegalovirus (CMV) immediate early promoter. To facilitate efficient, site-specific integration of plasmid DNA into the host cell chromosome, we have adopted PiggyBac transposase technology (exclusively licensed to Lonza) and all of the genes above were cloned into PiggyBac transposon, making a packaging plasmid. Green fluorescent protein (GFP) was chosen for our gene of interest (GOI) and put into a separate PiggyBac transposon, making a transfer vector plasmid. These two plasmids were transfected into 2G7 cell (Lonza's proprietary cell line for LV production) followed by antibiotic selection to obtain the pools of stable producer cell lines. In this process, we tested different ratios of plasmids, type of transfection agents, and concentration of antibiotics to find the optimal condition for PCL generation. Out of these pools generated from different conditions, Pool-2 produced one of the highest LV titers (2~4E6 TU/mL by flow cytometry) upon induction. Single-cell cloning was performed by doing limited dilution using the cells from pool-2. During this process, the growth of cells in each well of the 96-well plate, from a single cell to patches, was monitored and documented by using a CloneSelect Imager. Several dozens of single-cell clones were isolated, amplified, and suspension-adapted before

banking in this process. The productivity of each isolated clone was measured after thawing them at the same time from the frozen vials. Upon induction with doxycycline, some of the PCL clones produced significantly higher titers (~2E7 TU/mL by flow cytometry) than the parental pool-2. The stability of these PCL clones was assessed by maintaining them through multiple passages, and LV titers were measured across the passages after induction. In addition, the copy number of packaging and transfer vector plasmids integrated into the host cell chromosomes was measured by ddPCR for the selected PCL clones. Thus, Lonza has established a procedure to generate a highly productive PCL for a large-scale, suspension production of lentiviral vectors.

992. Continuous Perfusion with a Stable Producer HEK293 Cell Line for Scaling Up Lentiviral Vector Production

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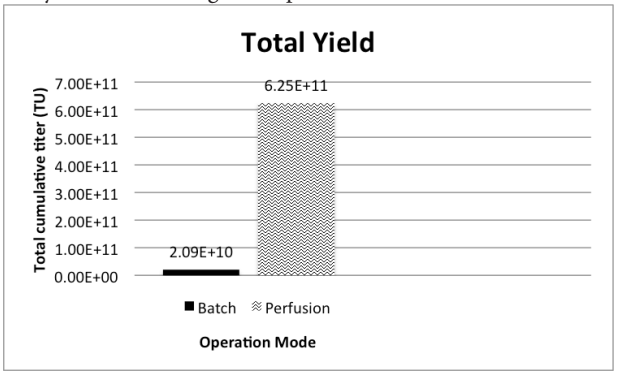
Lentiviral vectors (LVV) represent a key tool for gene and cell therapy applications. The production of these vectors in sufficient quantities for clinical applications remains a barrier, driving the field towards the development of cell suspension processes which are more amenable to large scale production. Stable producer cell lines derived from HEK293 cells grow well in suspension at cell densities of 10 million cells/mL thus offering direct scalability. Furthermore, the production of LVV is induced by the addition of two small molecules (cumate and doxycycline) avoiding the need for plasmids and transfection reagents. Lentiviral vectors can thus be produced in the 10^7 TU/ml in bioreactors operated under batch or perfusion mode. The perfusion bioreactor was run at 40% DO, 37 °C and pH 7.1 for 4 days post induction. In all perfusion runs harvests were collected and the LVV-containing supernatant was kept on ice at -4 degrees centigrade until clarification (once daily). This study demonstrated that LVV production using a novel and scalable perfusion process increased viral titers 30-fold compared to batch processing and reached a cumulative total yield of 6.25 x 10^11 TU in a 3L bioreactor as shown in Figure 1. This increase in yield is the result of increased volumetric output as well as improved specific productivity when operating in perfusion compared to batch mode. The perfusion approach presented here is easily amenable to large scale production of LVV.



993. Development of Droplet Digital Polymerase Chain Reaction (ddPCR) Assay for a Measurement of Infectious Titers of Lentiviral Vectors in a High Throughput Format

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Lentivirus (LV) is one of the most popular delivery vehicles in cell and gene therapies. The accurate measurement of LV infectious titer is an absolute requisite in the process of manufacturing, purification, and application of Lentiviral vectors. Conventional assay methods measuring LV titers, such as flow cytometry or quantitative polymerase chain reaction (qPCR), have some major drawbacks. For these assays, one needs to have a reporter or a specific antibody for the measurement of infectious titers. In addition, it is necessary to optimize the primers, probes, and standards in the qPCR assay before putting them into the assay, which is a very cumbersome process. Droplet digital polymerase chain reaction (ddPCR) has emerged as a reliable, cutting-edge technology to quantify the absolute copy number of any gene of interest without using a standard curve. The RNA genome of LV is first reverse-transcribed to its cDNA before it integrates into the host chromosome. Therefore, infectious titers of LV can be determined using ddPCR by measuring the integration frequency of the transgene into the chromosomes of target cells. However, current methods to determine LV titers by ddPCR are rate-limited due to the tedious process of genomic DNA isolation, which involves extracting chromosomal DNA from a large number of transduced cells. Here, we developed a new, high-throughput method, which enabled us to skip genomic DNA extraction during sample preparations for ddPCR applications. To avoid the tedious DNA extraction process, which commonly involves detergent-mediated cell lysis and column purification of the DNA thereafter, we mechanically disrupted the cells using glass beads. The crude lysates prepared from the cells transduced with LV (encoding green fluorescent protein, GFP) were applied directly to ddPCR assay. To compare and validate our new approach with conventional methods, DNA was also isolated from transduced cells by using a commercially available kit from Qiagen (QIAamp DNA Blood Mini Kit). The primer-probe sets specific to long terminal repeat (LTR) region of LV and beta-actin sequence of the host were used to amplify the target sequences. To calculate the infectious titers, we have compared the following three methods among each other; 1. Sample DNA for ddPCR was isolated using a Qiagen kit. The cell number in the corresponding sample was calculated from the copy number of beta-actin in the same sample, based on which LV titer was calibrated. 2. Sample DNA for ddPCR was isolated using a Qiagen kit. The cell number in the corresponding sample was calculated from the DNA amount in the same sample, based on which LV titer was calibrated. 3. Crude cell lysates were prepared by disrupting the cells using glass beads and directly applied to ddPCR. The cell number in the corresponding sample was directly counted before the disruption of cells by using ViCell, based on which LV titer was calibrated. The infectious LV titers calculated from the above three different methods were comparable to each other for any given sample, indicating that crude cell lysate prepared by bead beating is sufficient for direct ddPCR application. It enabled us to eliminate the time-consuming DNA extraction step during the sample preparation in the ddPCR assay. Through this approach, it was possible to handle

and process large numbers of samples in a relatively short time with minimal hands-on time. Thus, Lonza has developed a high throughput format of ddPCR assay for the measurement of infectious LV titers, which will greatly support the LV manufacturing process in cell and gene therapies.

994. Flow Cytometry-Based Lentiviral Vector Transduction Assay in 96-Well Plates versus 12-Well Plates

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Flow cytometry-based methods are broadly utilized to titer viral vectors that carry transgenes which can be monitored by fluorescence (such as GFP, RFP, etc.) or immunofluorescence (such as membrane proteins/receptors). We have previously established a flow cytometry-based transduction assay by transducing HT1080 cells in 12-well plates (wps) with GFP-containing lentiviral vectors and detaching transduced cells for high-throughput FACS analysis. However, for a relatively large number of samples (> 20) generated in an end-to-end process, 12-wp format is very low efficient since an operator can only process at most 4 wells at one time and needs to handle more than a dozen plates. As for most assays, 96-wp or 384-wp format is preferred in terms of high-throughput processing. Here, we developed and optimized the flow cytometry-based transduction assay in 96-wp format. We have shown that for HT1080 cells and this specific LV vector, smaller virus-containing transduction volume (higher virus concentration), higher cell seeding density and longer transduction time are important for transduction efficiency thus functional titer on both 12-wps and 96-wps. Also, higher cell seeding density are required for 96-wp format to achieve comparable titers observed in 12-wp format. To obtain minimal variations from time to time, it is also critical to use manual pipetting instead of aspirating by a vacuum pump during any supernatant removal on 96-wps as accurate cell counting is essential for titer calculation.

995. Development and Scale-Up of Bioreactor Process for Transient Lentivirus Production Using a Suspension-Adapted HEK293T Clone

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In recent years, the use of lentiviral vectors for gene therapy applications has become increasingly popular. As a result, there has been an increased demand for manufacturing of these viral vectors in large volumes for clinical trials. Current production processes are labor intensive and make use of adherent, flat stock cultures. Bioreactors enable production of large volumes of viral vectors with improved environmental control (pH, dissolved oxygen, mixing and temperature) and reduced production costs. Here, we describe the development and scale up of a suspension-based, transient lentivirus production process. The effects of pH, dissolved oxygen and mixing on growth of the VirusExpress™ 293T Lentiviral Production Cells and viral vector production were tested in 3 L Mobius® Single-use Bioreactor. Optimal process parameters were defined for the cell growth and lentivirus production phases, and the bench-scale bioreactor lentivirus production process was successfully scaled up in 50 L Mobius® Single-use Bioreactor.

996. Optimization of Vector Design and Preparation Conditions to Improve the Lentiviral Vector Productivity

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Lentiviral vector is an important viral vector for delivering target gene into human cells. It provided a stable system for long term gene expression and is widely applied in the Chimeric Antigen Receptor (CAR) T cells preparation. However, the viral vector titer may not be very high when carrying large size of gene of interest. Here, we intended to optimize the design of transfer plasmid and the production condition to increase the viral vector productivity. First, the spatial effect of cPPT/CTS and RRE was examined to evaluate its benefit of viral vector titer. Second, the transfection reagent and plasmid ratio were optimized to improve the virus productivity. Up to now, the titer of viral vector was increased to 8 fold of initial titer. Further optimization would be performed to improve the lentiviral vector productivity, leading to reduce the production scale and required consumables.

997. Scaled-Down, High-Throughput Optimization of Transfection for Lentivirus Production for Use as Gene Therapy Vectors

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Lentiviral vectors (LVVs) are a promising tool for gene and cell therapy because of their ability to deliver genetic payloads to both dividing and non-dividing cells. Hence, they possess significant potential for introducing therapeutic genes to multiple different cells of interest. LVVs are typically produced by transfecting envelope, packaging and transfer plasmids into Human Embryonic Kidney 293 (HEK293) or other suitable cell lines. Nevertheless, several challenges remain in the production process including low titers and a lack of suitable scale-up production. To address these concerns, we developed a high-throughput microwell platform for upstream transfection optimization leading to higher yields of LVVs. Several commercially available cell media were evaluated for their ability to support high cell densities. BalanCD - a commercially available HEK293 suspension cell medium was able to support both high cell densities and generate high transfection efficiencies in 96-deep well plates, and was, therefore, selected for further screens. To optimize transfection, several screens were performed in 96-deep well plates to establish optimal amount of DNA/cell for multi-plasmid delivery, viable cell density at time of

transfection, optimal ratios among packaging, envelope and transfer plasmids, and the ideal transfection reagent to DNA ratio. Comparisons of optimal conditions across scales were then made for both batch and perfusion scale-up modes. The results of the screens were then used to guide lentiviral production to generate higher LVV yields in perfusion bioreactors.

998. Approaches to Innovation in Lentiviral Vector Process Development

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The number of exciting and high profile products based on gene and cell therapy has increased dramatically over the past few years. Consequently, advanced therapeutics now attract significant interest from the wider biotech/Pharma and investment communities. For over 20 years, Oxford Biomedica (OXB) has been a pioneer in the development of products based on lentiviral vectors, with the company being responsible for several firsts in clinical studies based on these vectors. OXB has used this broad CMC, clinical and regulatory experience and know-how to facilitate development of a scalable, serum-free suspension manufacturing process utilising stirred tank bioreactors, both for the company's pipeline products and those of our strategic partners. The maturity of the industry has resulted in a rising demand for vector product and current technologies struggle to keep pace with the expansion of therapies from ultra-rare to larger indications. This has driven the need for further innovation in vector production platforms as the success of the industry hinges, in part, on consistently generating sufficient vector quantities with the desired purity and potency. To meet the forecast on vector demand for gene and cell therapies, OXB has adopted a number of strategies to develop the next generation manufacturing processes yielding higher quantities of vector with suitable product quality attributes and acceptable cost of goods in order to maximise capacity and advance development of a diverse product portfolio in therapeutic areas which currently present significant challenges. These include process intensification through continuous processing, novel methods for vector purification, a transition towards producer cell lines, utilisation of high throughput methodologies including automation and digitalization for data management. These innovative technologies provide opportunities to deliver sufficient vector quantities suitable for commercial manufacture for all indications.

AAV Vectors - Virology and Vectorology

999. Cloud-based Software for NGS Data Management and Analysis for Directed Evolution of Peptide-Based Delivery Vectors

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Adeno-associated viruses (AAVs) are widely used gene delivery vectors due to their ability to transduce dividing and non-dividing cells, their long-term persistence, and low immunogenicity. However, natural AAV serotypes have a limited set of tropisms. Directed evolution has been used to engineer recombinant AAVs to target specific cell types and tissues, leveraging next generation sequencing data. The deluge of data from these deep sequencing experiments has brought about data management and analysis challenges, for which there are no current commercially available solutions. Furthermore, classical approaches to analyzing data from directed evolution heavily involves manual inspection, and often overlooks patterns present in the larger datasets. To address these challenges, we developed robust cloud-based software that provides central management for next generation sequencing data, extracts variants, performs structural modeling, and can be extended to incorporate machine learning models to make predictions for variants with specific properties. The software is composed of a set of interconnected discrete components: a modern web user interface implemented in JavaScript with React, a relational database, a distributed task queue, task workers, and a Django-based API. This architecture allows computationally intensive tasks such as alignments, structural modeling, and machine learning to scale from a single machine to hundreds of machines, with minimal configuration. The software automatically imports and manages sequencing data from several different commercial and in-house sequencing providers. When the data is imported, sequence quality metrics are automatically generated and presented to the user. Variants are extracted by performing pairwise alignments between the natural serotype and the sequencing reads. The variants are further encoded into embeddings, grouped into families, and are analyzed for prevalent sequence motifs. We use the Rosetta software libraries to perform comparative modeling simulations on selected variants. Finally, we are developing and have extension support for Pytorch-based machine learning models to generate novel variants with desirable properties as well as to select candidate variants for additional rounds of optimization and characterization. This software represents a general tool for simple, scalable, and centralized analyses of next generation sequencing data for protein engineering by directed evolution, and could be generalized for all projects with large-scale deep sequencing datasets in the future.

1000. Characterization of GBoV1 Capsid for Gene Therapy Applications

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Human bocavirus 1 (HBoV1) has garnered attention as a gene delivery vector due to its ability to infect polarized human airway epithelia (pHAE) from the apical membrane as well as its large 5.5kb genome packaging capacity. However, this vector is highly seroprevalent, at up to 100% in the human population, posing a problem as the viral vectors will likely be neutralized upon host infection. The first discovered non-human primate bocavirus, Gorilla bocavirus 1 (GBoV1) shares an 86% amino acid sequence identity with HBoV1 and is likely not

a human pathogen. Thus, understanding of the GBoV1 capsid will provide insights into optimizing bocaparvoviruses as potential gene delivery vectors. Here, we report the capsid structure for GBoV1 determined at a resolution of 2.76 Å via cryo electron microscopy and 3D image reconstruction. GBoV1 has a similar surface morphology to that reported for other parvoviruses, with a depression at the 5-fold symmetry axis, protrusions around the 3-fold axis, and depressions at the 2-fold axis. Compared to the HBoV1 capsid structure, differences are localized to protrusions around the 3-fold icosahedral symmetry. We hypothesize that it is these structural differences that are responsible for a lower immunogenic profile of the GBoV1 capsid compared to HBoV1. We tested monoclonal antibodies previously generated against the HBoV1 capsid 5-fold axis (15C6) and protrusions around the 3-fold axis (12C1, 4C2, 9G12). We report that the GBoV1 capsid indeed escapes 4C2 and 9G12 but is cross-reactive to 15C6 and 12C1, for which we determined capsid-antibody structures. Furthermore, we screened human sera for recognition of the HBoV1 and GBoV1 capsids and while cross-reactivity was observed, GBoV1 is less immunogenic. High immunogenic profiles against parvoviral vectors in the human population is a significant barrier to developing an efficient gene delivery system. This study is a step towards optimizing bocaparvoviruses as viral vectors with antibody escape properties.

1001. Novel AAV ITR Sequencing and AAV Plasmid Preparation Protocols to Advance Research and Therapies Using AAV Vectors

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Inverted terminal repeats (ITRs) of Adeno-associated virus (AAV) play a key role in virus replication and encapsidation, and also play a role in viral genome integration and excision from the host genome. Integrity of the ITR regions in AAV transfer plasmid is crucial for rAAV production as truncated ITRs can reduce the yield of full viral capsid production and increase generation of undesirable non-rAAV encapsidated DNA. Unfortunately, due to their high GC content and secondary structures, AAV plasmid ITRs are frequently mutated during plasmid production in *E. coli.,* reducing experiment reproducibility and causing delays if ITR mutations are left undetected. However, traditional methods to assess ITR integrity including commercially available Sanger cycle sequencing kits are typically not effective at reading through ITR regions. In addition, restriction digestion with SmaI cannot detect variants where small deletions occur outside of restriction recognition sites. To overcome this significant challenge, GENEWIZ has developed a new ITR sequencing technology that can sequence through the ITR regions without significant loss of signal, achieving a read length comparable to non-AAV sequencing reactions. Moreover, GENEWIZ has recently developed a proprietary AAV plasmid preparation process, which significantly improves the chance of maintaining ITR integrity, even when other commercially available competent cells growing in low temperature failed. This process can also isolate clones with full length ITR from a sample mixture containing intact and truncated ITRs. Our proprietary AAV ITR sequencing and AAV plasmid preparation services significantly advance the analysis of ITR and improve AAV vector manufacturing for research and gene therapy.

1002. Development of qPCR Analytical Package for AAV Vector Characterisation: Focus on Multiplex qPCR

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Innovation, CobraBiologics, Stoke On Trent, United Kingdom

The manufacture of therapeutic AAV vectors must be coupled with robust analytical methods to ensure quality and efficacy of the product prior to entering clinical development. Unlike conventional biologic therapeutics where a more complete understanding of functionality may be present, viral vectors comprise both protein and nucleic acid components, which further complicate the analytical challenges. Due to the variability in AAV serotypes, changing properties depending on the production scale and the differences in approaches to common methodologies such as qPCR for viral genome quantification, it has become increasingly challenging to develop consistent analytical methodologies for product release and characterisation. A widely accepted method for viral genome titration relies on the use of quantitative PCR for determining the number of AAV genomes present in AAV preparation. This method relies on specific amplification of the inverted terminal repeat (ITR) regions or a transgene specific region to determine the levels of packaged AAV genomes. In addition, the utility of qPCR approaches extends to estimating levels of DNA impurities mis-packaged into the particles during the production and assembly process, residual levels of mis-packaged DNA may lead to unwanted immunological reactions in the patient and must be controlled. Controlling the level of these impurities through the upstream process design has become one of the key focus areas in our research towards developing an AAV manufacture platform process. Our earlier work has determined that a large proportion of the DNA mis-packaged into AAV consists of sequences derived from the plasmids used in the manufacturing process. Here, we describe development of a multiplex qPCR-based AAV genome-containing particle titration assay. Additional multiplex qPCR assays will look at residual levels of DNA deriving from the Helper plasmid; due to potential oncogenicity of DNA sequences from helper genes such as E2a and E4. This would provide a means for high throughput characterisation of the plasmid DNA-derived sequences that can be mis-packaged into AAV particles. The multiplex assay allows for the normalisation of the mis-packaged and correctly packaged amplicons to a virus titre, thereby generating a correlation between the levels of genome to ITR to mis-packaged amplicons within a sample. The use of these assays with in-process samples would provide a means to monitor the packaging of DNA amplicons in the AAV particles throughout the purification process, thereby facilitating focussed AAV purification development.

1003. Novel Machine Learning Algorithms Improve the Design of Synthetic Promoters Resulting in More Selective and Higher Levels of Gene Expression in Muscle and Liver Cells

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Precise, controllable and reliable gene expression is an essential requirement to develop safe and effective gene therapies. AAV vectors have emerged as the vector of choice to deliver therapeutic gene cassettes, but their small size limits the amount of cargo that can be delivered to the target tissue. Moreover, although different AAV capsids can mediate some degree of tissue tropism, more effective cell-type selectivity of therapeutic gene expression is better achieved through transcriptional targeting. Therefore, there is a requirement to develop short, cell-selective gene promoters that can drive expression of therapeutic genes to levels required to correct disease phenotypes. We have focussed on developing computational methods to identify cell-selective and regulatable transcriptional control elements from the human genome that can be used as parts to build synthetic promoters and therapeutic gene expression cassettes. Moreover, we have developed machine learning algorithms that are trained to identify key functional regions within isolated enhancer regions from the genome and which allow us to rationally assemble enhancers and promoters, derived from different gene loci, to create short synthetic promoter assemblies; capable of driving efficient gene expression selectively in CNS, muscle or liver cells. The short synthetic promoters range in size from 200bp to 300bp and are ideal for use in AAV vectors. We will present data showing how we identify key regulatory elements from the genome and the approaches we use to train machine learning models to identify key sequences within those elements. We show that these machine learning tools can improve promoter function by: (a) predicting single base pair changes to known enhancers that will improve activity, (b) detect which sequences within the context of larger enhancers contain short functional elements responsible for controlling transcription, (c) enable the assembly of disparate genetic elements to create rationally-designed synthetic promoters, and (d) identify regions within designed synthetic promoters that can be removed to create shortened gene expression cassettes ideal for AAV vectors. Finally, we will present data showing the in vivo activity profile of CNS-selective, muscle-selective, liver-selective and small molecule regulatable promoters that have been rationally designed using this computational platform, and which demonstrate substantially increased activity in target tissue types compared to the current promoters that are widely used in gene therapy studies. Our work reveals that data from large-scale genomic meta-analyses from different studies can be successfully re-purposed to aid in the design of more effective gene therapy expression cassettes by employing engineering biology principles and using AI tools to learn from these datasets.

1004. Effect of Human Sera on the Transduction Efficiency of AAVs

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Adeno-associated viruses (AAVs) are currently one of the most widely used vehicles for therapeutic gene delivery. Their ability to introduce foreign DNA safely into a cell to express a transgene, without initiating a pathogenic response, has made the AAVs desirable vectors. Currently, three therapeutics that utilize AAV as a vector, have been approved.Despite these successes for AAV in the gene therapy field, preexisting immunity, specifically neutralizing antibodies, pose a threat to full success. For clinically relevant AAV serotypes, such as AAV2 and AAV9, seroprevalence in healthy individuals is estimated to be approximately 70 and 50% respectively. In addition to seroprevalence, a previous published study estimated that approximately 60% of human sera contained neutralizing factors against AAV2 and 35% for AAV9. The current study investigated the effect of various human sera samples on the transduction efficiency of AAV1-13. While some human sera samples enhanced transduction efficiency, we show that 7/20 samples tested were able to neutralize AAV2 (35%), while 5 neutralized AAV9 (25%). Interestingly, those sera neutralizing both AAV2 and AAV9, also neutralized most of the other AAV serotypes. To further analyze this neutralization phenotype, IgGs were purified from the human sera samples by a Protein G column and used in subsequent neutralization assays alongside the flowthrough. Surprisingly, the neutralizing effect of purified IgG were lower compared to whole human sera and flowthrough, suggesting additional neutralizing components in these samples. Native dot immunoblot showed that neutralizing whole human sera and purified human IgGs react with AAV2 and AAV9 capsids. The future goal is to map and eliminate the binding site for these factors on the AAV capsid to help establish a pipeline for structure guided design of patient specific AAV capsid vectors for personalized gene delivery.

1005. Development of a Sensitive and Robust Cell-Based Assay for Measuring Potency of the NAV AAV8 Vector-Derived RGX-314 Gene Therapy Product for the Treatment of Wet Age-Related Macular Degeneration

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Wet age-related macular degeneration (wet AMD) is characterized by excessive blood vessel formation and fluid leakage in the retina leading to vision loss. Treating wet AMD using anti-VEGF therapies requires repetitive and inconvenient intraocular injections to maintain drug levels and thus efficacy. REGENXBIO has developed RGX-314, a novel one-time subretinally-administered NAV AAV8 gene therapy designed to deliver a gene encoding an anti-VEGF monoclonal antibody fragment to treat wet AMD. In accordance with ICH and regulatory requirements, a well-designed, robust potency assay is needed to aid in the determination of the conformity, comparability, and stability of products used during all phases of clinical investigation and following

marketing approval. To represent the complex mechanisms of action, *in vitro* potency assays for AAV gene therapy products should measure the ability to deliver the transgene DNA sequence (infectivity) and the biological effect of the expressed transgene sequence (transduction and biological activity). The development, optimization, and qualification of an *in vitro* relative potency method for RGX-314 is described. The method is specific for RGX-314, precise, accurate, and linear between 25-200% relative potency. The assay also detects changes in potency due to degradation, allowing it to play a key role in advancing drug product formulation development. Various formulations and storage conditions have been tested using this *in vitro* potency method, leading to the rapid screening and selection of potential viable drug product formulations for RGX-314.

1006. Accurately Quantifying Transduction within Barcoded AAV Capsid Libraries via Tracking of Single-Molecule ID Tags

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Multiplexed barcoded capsid libraries have emerged as a powerful tool for high-throughput AAV engineering. Barcoded studies facilitate the accurate measurement of tropism across multiple capsids within a single animal, which is essential to understanding why certain capsids better transduce key delivery targets in vivo. However, many technical and biological artifacts complicate the reliable measurement of critical capsid properties, such as genome packaging and in vivo transduction efficiency. Notable artifacts include cross-packaging, template switching, and errors in DNA synthesis, which substantially reduce the quality of barcode-based library measurements, even when rare. To address these issues, we developed a paired library construction and data normalization strategy that flags potential "decoupling" between barcode identity and variant behavior. Incorporation of random DNA ID tags enables tracking of barcodes with synthesis errors, template switching, or cross-packaging followed by detection of outliers for each barcode-based measurement. Removal of barcode-ID pairs that significantly reduce the coefficient of variation in packaging-efficiency estimates then reliably prevents decoupled barcodes from contaminating subsequent analysis. Correcting barcode counts with this removal procedure, together with normalization according to internal positive and negative controls, improved the accuracy of packaging estimation on a reference set of wild-type and VP3-stop mutants. Furthermore, preventing decoupled barcode-ID pairs from propagating to downstream assays, such as in vitro and in vivo transduction, significantly sharpened these measurements. For example, in an in vitro transduction assay, filtering these decoupled barcodes reduced the false positive transduction rate among VP1 stops and narrowed the set of high-performing variants without impacting wild-type controls. This widely applicable approach significantly improves estimation of packaging and transduction in large, barcoded AAV libraries. Additionally, ID tracking enables estimation of new quantities of interest, such as the numbers of transfected and transduced cells per capsid variant. This improved estimation is highly relevant for the engineering of improved capsids, since reducing false positive rates substantially strengthens the power of models built with this data and increases the efficiency of later-stage capsid validation efforts.

1007. Structural and Biochemical Characterization of Potentially Under-Utilized Gene Therapy Vector AAV7

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The use of adeno-associated viruses (AAV) as gene delivery vectors has vast potential for the treatment of many severe human diseases. The NAV platform of AAV vectors comprises over one hundred naturally existing AAV capsid variants, which were classified into phylogeny clades based on their sequences (Gao *et al*. 2002). Several NAVs, including AAV8, AAV9, and AAVrh.10, and other intensively studied capsids have been propelled into pre-clinical and clinical use, and more recently, marketed products; however, less-studied capsids may also have desirable properties (i.e. potency differences , tissue tropism, reduced immunogenicity, etc.) that have yet to be described. Within this under-studied capsid pool is AAV7, a potentially therapeutic capsid for which tropism studies are limited and direct, head-to-head comparisons with well-described capsids are often not performed. AAV7 appears to transduce the CNS well in a macaque model after direct injection to the brain (Samaranch *et al*. 2013) and transduces human hepatocytes in a mouse xenograft model after IV administration at a similar level to AAV8 (Shao *et al.* 2019). Its capsid protein has an 87.8, 81.6, and 88.4% identity of amino acids compared to AAV8, AAV9, and AAVrh.10, respectively, and is readily produced in HEK293 systems (Lock *et al.* 2010). In this study we sought to more fully characterize this capsid by evaluating its biochemical properties, atomic structure, and biodistribution. As glycan receptor identification for AAV7 failed previously (Mietzsch *et al*. 2014), we assayed 300 biologically relevant glycans in array for capsid binding. We also present the structure of AAV7 capsid by single particle cryo-electron microscopy at 2.7Å. Finally, AAV7 and AAV9 biodistribution were compared directly in a C57BL/6 mouse model via IV administration. The two serotypes shared very similar tissue tropism with only significant differences ($p < 0.05$) found in muscle (forelimb bicep) and lung. Muscle transduction was thirteen times higher in mice dosed with AAV9 compared to AAV7, and lung transduction by AAV7 showed a five-fold increase over AAV9. Interestingly, AAV7 achieved comparable transduction levels to AAV9 in the brain. This data will help build a broader structure-function knowledge base in the field, present capsid engineering opportunities, and enable the use of novel capsids with unique properties.

1008. Elongation of the Rep-Cap Cassette with a Cellular Intron Reduces Reverse-Packaged Rep-Cap *Trans* Plasmid Sequences and Increases Therapeutic Vector Genome Packaging in a HEK293 Triple Transfection rAAV Vector Production System

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Reduction in the levels of co-packaged host cell DNA and reverse-packaged plasmid DNA remains as a priority in manufacturing AAV-based clinical products. Although the mechanisms involved in the co-packaging phenomenon are still under investigation, it is known that impurities are packaged at low levels and that certain impurities have been detected in cells following AAV transduction. Previous attempts to reduce reverse-packaging of Rep-Cap sequences have targeted inherently sensitive regions harboring Rep and Cap RNA splice sites. Here, utilizing multiple naturally occurring alternative consensus splice acceptor and splice donor sites in the Cap coding sequence, we have inserted cellular introns into the VP3 region of both AAV8 and AAV9 Cap genes within the *trans* plasmid utilized in a HEK293 triple transfection system. Importantly, this novel approach would be predicted to have no effect on Rep and Cap RNA splicing nor on generation of Rep and Cap protein isoforms. Production of research grade Factor IX transgene-containing vector genome with the AAV8 Cap version of this novel system demonstrated a 2-8 fold reduction in reverse-packaged levels of both Rep and Cap sequences. Surprisingly, concomitant with the significant reduction of reverse-packaged Rep and Cap sequences, we observed up to 5-fold higher levels of packaged vector genome. Two additional vector genomes containing different regulatory elements and transgene sequences were produced to assess robustness of this modified *trans* plasmid system. Interestingly, similar trends of reduced Rep and Cap co-packaging alongside increased packaging of vector genome were observed with both vector genomes. Application of this technology to an AAV9 Cap *trans* plasmid produced near identical results to the modified AAV8 Cap *trans* plasmid, further demonstrating the strength of this approach.

1009. High-Resolution Profiling of Inverted Terminal Repeat Sequences in Packaged AAV Vectors by Single Molecule, Real-Time Sequencing Reveals Structural Heterogeneity that May Influence Replication

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The use of single molecule, real-time (SMRT) sequencing and adeno-associated virus genome population sequencing (AAV-GPseq) was previously shown to be able to profile the entire recombinant adeno-associated virus (rAAV) vector genome from inverted terminal repeat (ITR) to ITR for self-complementary AAVs (scAAVs). Unlike scAAV genomes, single-strand AAV (ssAAV) vectors are not readily adapterable as solitary genomes on their own. We have since established the ability to adaptor annealed plus- and minus-stranded ssAAV genomes for SMRT sequencing, and are now able to obtain high sequence resolution from ITR to ITR for all current AAV vector platforms. This ability has opened the door to query the composition of a range of clinically relevant AAV vectors on a single-particle scale. Previous studies have shown that foreign DNA elements, truncated genomes, and other anomalous sequences were packaged into vector genome during AAV production. This has raised further concerns for the safety of rAAVs for gene therapy and have prompted higher standards for AAV production, bio-analytics, and quality control. In this study, we demonstrate through the quantification of ITR configuration and heterogeneity, that rAAVs can be packaged with mutated ITRs that are not by design. It is known that the ITR plays central roles in AAV replication, vector genome packaging, formation of episomal species necessary for stable transduction, and drives host cell genome integration. However, it is believed that these functions are just a part of its role during the AAV life cycle. As the last viral elements retained in AAV vectors, full understanding of these structures is intrinsically important. Discoveries by us and others have shown that mutations can occur within ITR regions at the levels of both the plasmid and the vector genome, offering further rationale for investigating these fascinating structures. Since SMRT sequencing relies on an isothermal strand-displacing polymerase for sequencing, it has high sequence resolution over ITR structures, which are by nature, very thermostable. To profile ITR composition, we developed bioinformatics pipelines to examine AAV ITR sequences and structures. Namely, we can accurately quantify ITR heterogeneity in packaged vectors, determine flip and flop configurations, and detect region deletions. Using SMRT sequencing, we have begun to explore the repair of mutated ITRs. It has been shown that if one of the two ITRs in AAV vectors are mutated, that the mutated ITR can be repaired by intramolecular replication using the intact ITR as template. Our study has revealed that, after triple transfection into 293 cells, a vector genome harboring a mutated ITR can be rescued from the cis-vector plasmid and can undergo replication. We also show that more than 98% of the packaged genomes have undergone self-repair and contain wild type ITRs at both ends. These findings demonstrate that ITR repair can be quantified and this technique can provide insight into the structural integrity of ITRs in gene therapy vectors. Our interpretations on ITR diversity observed in packaged virions, and its implications for vector production and potency will be discussed. The ability to profile ITR composition allows for more robust AAV vector designs that can drive the gene therapy field to develop better treatments for patients.

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1010. Characterization of AAV Provector Targeted Delivery through Scar Optimization

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Adeno-Associated Viruses (AAVs) have been a prevalent platform for gene delivery due to their simple structure and genome, lack of pathogenicity, and low immunogenicity. However, like other viral vectors, AAV's transduction is heavily dictated by the capsid's natural tropism. As such, a significant portion of AAV research has been mutating the AAV capsid's tropism to target certain organs more specifically, or inclusion of activatable elements in the AAV capsid to target disease states such as cancer or heart disease. To this end, our lab has developed protease-activatable AAV vectors, known as provectors, to target sites of enzyme overexpression, such as Matrix Metalloproteases (MMPs), Caspases, or Cathepsin B, which occur in various disease states. These vectors work through the insertion of 'peptide locks' composed of cleavable sequences flanking a tetra-aspartic acid inactivating sequence that blocks viral binding to its respective glycan. When the peptide locks are in place, the vectors are in the OFF state, unable to deliver genes. Once the cleavable locks are removed specifically by extracellularly expressed enzymes, the provector's tetra-aspartic acid motif is released from the capsid, and the vector turns ON, able to transduce cells. The 12 amino acids of our activatable insert that cannot be cleaved from the capsid after turning ON, positioned with 6 N-terminal and 6 C-terminal of the cleavage sequence, are referred to as the 'scar.' Previous work from our lab has found that provectors target locations of protease overexpression in models of ovarian cancer (Tong et al., Journal of Controlled Release, 2019) and heart disease (Guenther et al., Molecular Therapy, 2019). However, these activatable inserts produce a vector that has ON transduction efficiency of ~30% of AAV9 capsid's ability to transduce cells. We believe the composition of the scar is crucial in levels of ON and OFF transduction. In order to improve the provector platform, this work explored both inserting and replacing amino acids in the provector scar to improve ON transduction without sacrificing activatable behavior, low OFF transduction, or formation of the capsid. For the insertion analysis, we developed a small library of 40 provector mutants, each with an amino acid inserted on either the N-terminus or the C-terminus of the scar region. For the amino-acid replacement analysis, four amino acids, two on each side of the scar, were replaced with seven possible amino acids of different properties for a sequence space of 2401 mutants. Both libraries were tested to interrogate the effect of the scars on the formation and activatability of the provector. Using a pairwise analysis based on the enrichment and depletion of different amino acid compositions of the scar, we discovered beneficial residues for capsid formation such as arginine and glutamic acid. To test the modularity of the improved scar sequence, we incorporated these changes into a provector with a different proteolytic sequence and characterized the resulting vector's formation, MMP activation, and cellular binding properties. Results from this study will help optimize provectors and further support their use in treating diseases with elevated extracellular proteases.

1011. AAVHSCs Transduction Does Not Significantly Elicit p53-Mediated Apoptosis or Alter Cell Cycle in Human iPSCs and Primary Cells When Compared to Non-Clade F AAV Vectors

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Adeno-associated virus (AAV) has been successfully used in the clinic to deliver functional copies of genes to treat various diseases. There have been efforts to gain further understanding about their tropism, cellular trafficking, and mode of actions to help identify the most safe and efficacious capsids to be used in humans. A new family of AAVs was isolated from human hematopoietic stem cells (HSCs), termed AAVHSCs. These Clade F AAVs have broad tissue tropism across different cell types and species. To further characterize the functional properties of AAVHSCs, commercially available AAVs 1-9 and AAVHSC1/4/6/7/8/9/13/15/16/17 capsids containing a self-complementary CBA-GFP payload were used to transduce four different human iPSC lines. We observed several differences between AAVHSCs and the commercially available non-Clade F AAVs (AAV 1-8). Specifically, AAV12/3/6/8 induced apoptosis, with AAV2/3/6 being the most potent that elicited irreversible cell death in iPSCs when compared to AAVHSCs at multiplicity of infection (MOI) of $1x10^5$. This finding was driven by a p53-dependent pathway as shown by p53 upregulation and its downstream targets including p21, caspase-3, and PARP after transduction. Cell death with the non-Clade F vectors was also observed at low MOI of $2x10^3$, independent of vector genomes, and it was rescued by transient p53 knockdown prior to AAV transduction. By contrast, no or little upregulation of p53 was detected when iPSCs were transduced with AAVHSCs at MOI of $5x10^5$. Upregulation of proteins involved in the DNA damage repair (DDR) pathway, such as the activation of CHK2 at threonine 68 and H2AX at serine 319 following AAV1/2/3/6/8 transduction, was also observed. Knockdown of CHK2 was insufficient to prevent apoptosis in iPSCs upon transduction, while double knockdown of p53 and CHK2 rescued cell death and the cell proliferation was enhanced at 24 hours compared to p53 knockdown alone following the non-Clade F vector transductions. This suggests that CHK2 and p53 are involved in different pathways acting synergistically to regulate cell cycle progression and apoptosis. To determine the effects AAV has on cell proliferation, BrdU was added to culture medium after AAV2 or AAVHSC15 transduction. The cells were subsequently stained for apoptosis and cell cycle markers to visualize the cell cycle distribution in a given cell population by flow cytometry. At MOI of $1x10^5$, AAV2 arrested the transduced iPSCs at G_2/M phase with a concomitant decrease in the proportion of cells in G_0/G_1 and S phase while AAVHSC15 did not impact the distribution of cells within G_0/G_1, S, and G_2/M phases. Similarly, AAV2 also disrupted the normal cell cycle distribution in primary human fibroblasts and skeletal myoblasts/myotubes. Taken together, the results from this study suggest that AAVHSCs have different properties than AAVs from other Clades such as AAV1-8. These differences found in human

iPSCs and primary cells appear to be mediated through the activation of p53 and DDR pathways that are crucial for cell cycle regulation and programmed cell death.

1012. Investigating the Role of DNA Replication in the Establishment and Maintenance of Recombinant Adeno-Associated Viral Vector Genomes

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Over the last 35 years, adeno-associated virus (AAV) has been developed as a recombinant vector for gene transfer. Recombinant AAV (rAAV) vectors consist of a transgene cassette flanked by the viral inverted terminal repeats encapsidated in a non-enveloped icosahedral capsid. rAAV vectors have been used successfully in multiple clinical trials and two commercial products, but there are still aspects of the biology of the vector that remain unknown. Upon infection with rAAV, the vector is trafficked through the cell and delivered to the nucleus, where the vector genome persists. Following a single administration, these vector genomes can persist *in vivo* and maintain stable transgene expression for years in large animal models and in humans. It was previously thought that integration of the vector genome into the host cell chromosome was responsible for this long-term persistence. However, it has been demonstrated that rAAV vector genomes persist in the host cell nucleus as episomal, chromatinized circular monomers and concatemers in skeletal muscle and liver, and these forms are most likely responsible for the observed long-term expression of the transgene. While there is sufficient evidence for the presence of these episomal circular forms, it is unknown if replication is required to establish and maintain them over time. To elucidate this aspect of the vector life cycle, the role of DNA replication in the establishment and maintenance of episomal circular rAAV monomers and concatemers was investigated. Briefly, replicating DNA was labeled with bromodeoxyuridine, isolated by immunoprecipitation, treated with plasmid-safe DNase, and analyzed by qPCR and electron microscopy. In this study, it is shown that, following second-strand synthesis, rAAV vector genomes undergo limited DNA replication in the absence of helper virus and AAV viral genes in both actively dividing and terminally differentiated cells. This phenomenon represents a unique intermediate mode of replication that is neither representative of a latent nor lytic AAV infection. Rather, the data suggest the mechanism observed plays a role in the maintenance and stability of rAAV vector genomes over time. Because rAAV vectors are devoid of viral genes, these replication events should rely on cellular DNA repair factors and host cell replication enzymes. Further studies are necessary to investigate this phenomenon *in vivo*, as well as to determine the specific mechanisms and cellular factors involved.

1013. RNA-Based AAV Capsid Libraries Selection for Improved AAV Bioengineering via Directed Evolution

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The power of AAV Directed Evolution for identifying novel vector variants with improved properties is well established, as evident by numerous publications reporting bioengineered AAV variants over the last decade. However, most capsid variants have been identified using either replication competent selection platforms or PCR based recovery methods. Those strategies can bias the selection towards efficiently replicating viruses or vector variants that are unable to complete the intracellular journey leading to productive transgene expression, respectively. The aim of this study was to develop and validate a novel AAV selection platform, which allows for rapid identification of novel highly functional variants based on transgene expression in target cells. To achieve this goal, we developed and validated two AAV selection platforms, named Functional Transduction platform (FT, in which capsid expression is driven by a SFFV/p40 hybrid promoter) and High Targeted Expression platform (HTE, in which capsid expression is driven by the SFFV promoter), both encoding a reporter transgene in addition to the AAV capsid library. Thus, in contrast to other platforms, our designs allowed for selection of capsid candidates based on transgene expression (function) in the target cells. When combined with cell-type specific antibodies and fluorescence activated cell sorting (FACS), this platform design allows to perform AAV selection in individual cell types in complex tissues. Furthermore, since in contrast to the replication competent (RC) platform, FT and HTE platforms do not require co-infection with wild-type Adenovirus, the selection can be performed in cells/tissues resistant to Adenoviral infection. As part of the validation process, we performed side-by-side comparisons of the new selection platforms with the conventional AAV2-based RC platform. The engineered platforms were all able to package AAVs efficiently and with high capsid-to-genome correlation (low cross-packaging). However, only the FT platform was able to produce vector yields as high as the RC platform, suggesting that in contrast to *cap* gene expressed from the SFFV promoter alone (HTE platform), capsid expressed from the SFFV/p40 hybrid promoter were able to assemble into functional AAV particles more efficiently. To enable stringent comparison between the three library platforms, we performed a surrogate library selection by cloning capsids from AAV2, AAV8 & AAV-DJ into each of the selection platforms and performing direct competition on HuH-7 cells. Based on DNA/RNA NGS data from HuH-7 cells (Westhaus et al., Human Gene Therapy Methods, 2020, in press), AAV2 > AAV-DJ >> AAV8 at cell entry, while AAV-DJ > AAV2 >> AAV8 at transgene expression. Thus, a platform that positively selects for AAV-DJ over AAV2 and AAV8 would therefore be considered the most stringent selection platform. The study showed that FT > RC > HTE at selecting the most functional variant

(AAV-DJ). Based on these results we are currently comparing RC and FT platforms on primary human hepatocytes in a xenograft murine model of the human liver. The results of the comparison between the individual platforms will be presented. The outcomes of this study will influence the future of gene therapy by identifying the most powerful AAV selection platform for bioengineering of novel AAV variants for clinical applications.

1014. Resolving the Adeno-Associated Virus Packaged Genome Population through Long Read Next Generation Sequencing

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Successful clinical trials using Adeno-associated viruses (AAVs) prompted biotechnology and pharmaceutical companies to invest in gene therapy programs. However, commercialization of gene therapy involves large scale production of stable AAV and reliable packaged DNA vectors. Degraded AAV and chimeric DNA vectors negatively impact the efficacy and safety of gene therapy products. For packaged genomes, many analytical tools are being used to assess their integrity. One of the frequently used methods to characterize and profile intact DNA molecules from AAV is by single molecule, real-time (SMRT) Next Generation Sequencing. Here, extracted DNA genomes were not processed by fragmentation, in order to capture truncated and/or chimeric sequences packaged by AAV. This approach can reveal the relative distribution of genome populations in vector production. In addition, it can also uncover large truncation or insertion regions in the packaged transgenes due to replication or packaging errors that may otherwise not be detected using Illumina sequencing. Based on these results, we observed that ~75% of the genome populations are the expected DNA genomes with correct length. In addition, similar to previous studies, truncation hotspots are observed within the inverted terminal repeat (ITR) regions. Chimeric sequences are also present and come from host-cell genomes, plasmid backbone, packaging and helper plasmids. The results also show that DNA sequences with repetitive patterns are prone to skipping errors or deletions during replication. This next generation sequencing approach coupled with a systematic bioinformatics analysis pipeline can reveal undesirable DNA replication and packaging attributes, which may be useful in maximizing vector purity and potency.

1015. Abstract Withdrawn

1016. Abstract Withdrawn

1017. Abstract Withdrawn

1018. Routine Transduction Assays for rAAV Products - Is It Possible?

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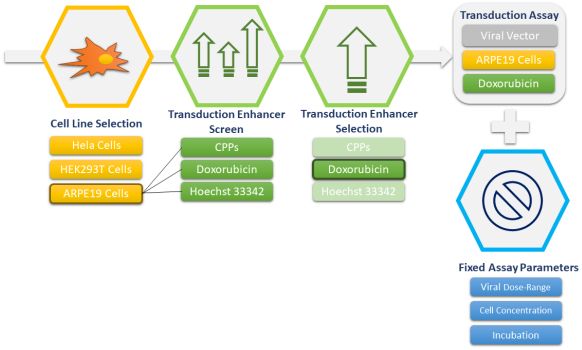
As cell and gene therapy becomes a driving force in the industry's growth, there is a demand for the development of bespoke assays to support novel gene therapies through development. A repertoire of assays can be developed to provide drug safety, quality and efficacy data, an important method being relative potency assays which demonstrate the specific mechanism of action of a chosen biopharmaceutical. Recombinant Adeno-associated viruses (rAAVs) are a popular vector choice for gene therapy as the different serotypes can infect a variety of human cell lines, and are most importantly, non-pathogenic. To assess the relative potency of an rAAV, the protein of interest output must be measured against a reference. In order to achieve this, there are two essential steps; 1. Perform a transduction assay; 2. Perform an endpoint assay. As rAAV transgene inserts are varied, the endpoint assays cannot be defined for all gene therapy products. For example, some rAAVs require ELISA potency assays, and others require enzyme activity assay endpoints. Therefore, we propose the question of whether it is possible to simplify transduction assays by using a novel "Build an Assay" system (BanA) (Figure 1) for efficient rAAV transduction. RAAV cell specificity, transduction efficiency and viral titre can all delay development. AAVs have proven challenging to manufacture at high titres, therefore increasing the transduction efficiency of the vector is vital. An appropriate cell line should be chosen relative to the rAAV serotype and indication, this can involve a significant amount of assay optimisation, thus delaying development timelines. The recent discovery of two receptors involved in AAV cell entry include KIAA0319L, the AAV receptor, and the G protein-coupled receptor 108. These receptors are expressed at sufficient levels in both Hela and HEK293 cell lines, which are routinely used for both AAV transduction and manufacture, demonstrating their suitability for a platform transduction method. Some AAV serotypes and transgenes target retinopathies, therefore other cells lines such as the human retinal pigment epithelial cell line (ARPE19) could be more appropriate. Other factors to consider are the virus addition method and the dose range. Viral titre is a limiting factor for rAAV transduction assays, therefore dose-response analysis models such as log-log and semi-log fit models should be considered. The BanA system is a modular customisation approach that involves selecting components and running screening assays. For example, a cell line would be chosen, followed by a screening selection of a transduction enhancer such as doxorubicin, cell permeable peptides (CPPs) or Hoechst 33342, to determine the optimal transduction conditions from the BanA system. The incubation times post-transduction would be set at 48-72 hours; preferably 72 hours for maximum transgene expression.



Following transduction, the development of an endpoint assay relevant to the rAAV transgene expression is required. The BanA system involves limited optimisation resulting in the majority of development time focusing on the endpoint assay, thus reducing the overall assay development time. The BanA system does not propose a one-size-fits all approach, as cell based relative potency assays are complex in nature, however having a modular approach whereby cell lines and transduction enhancers can be substituted and rearranged provides a flexible but structured method to successfully transduce all rAAV products.

1019. The Use of Low Endotoxin ClearColi K-12 Electrocompetent Cells During rAAV Vectors Production Results in Decreased Immune Responses Both In Vitro and In Vivo

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Background: Recombinant adeno-associated virus (rAAV) gene therapy products require large amount of plasmid DNAs. Those DNAs usually come from gram-negative *Escherichia coli* (*E. coli*) bacteria. The lipopolysaccharide (LPS) in the cell wall of gram-negative bacteria is the major source of endotoxin (Int J Mol Sci. 2017. 18(12): 2737). Residual endotoxin in the rAAV vector may mediate substantial immune response of mammals (Hum Gene Ther. 2015. 26(1): 59-68). ClearColi K-12 is a genetically engineered *E. coli* strain for the purpose of generating low endotoxin plasmid DNAs. These genetic modifications remove six acyl chains of the LPS and disable the endotoxin signal. In this study, to improve the safety of rAAV gene therapy products, we compared the use of ClearColi K-12 and DH5α (a frequently used *E. coli* strain) in rAAV vector production. **Methods:** Both ClearColi K-12 and DH5α cells were prepared as chemically competent and electrocompetent cells, in which rAAV-producing plasmid DNAs were transformed. Using enzymatic digestion to detect the loss of inverted terminal repeat (ITR) sequences from ten individual colonies of each. Then, plasmids isolated from ClearColi K-12 and those from DH5α were transfected into HEK293 cells to produce K-12-rAAV and DH5α-rAAV vectors, respectively. Both plasmids and rAAV vectors were subjected to the Limulus amebocyte lysate (LAL) assay for endotoxin detection and were used to treat mouse microglia BV-2 and human leukemic monocyte THP-1 cell lines to test inflammation-related gene expression. Finally, rAAV vectors were subretinally injected in the eyes of C57BL/6 mice at a dose of 10e9 vg/mouse, followed by investigation of microglia activation and inflammatory responses. **Results:** Transformation of DH5α with plasmid DNAs could be conducted through chemical transformation and electroporation, whilst only electroporation of ClearColi K-12 was successfully performed. The transformation efficiency of ClearColi K-12 was slightly lower than DH5α. Nevertheless, both *E. coli* strains exhibited similar protection of the ITR sequences in the rAAV genome plasmids. It was evident that plasmid DNAs from either ClearColi K-12 or DH5α cells generated similar amounts of rAAV vectors, with equal vector-mediated transduction efficacy in various cultured cell lines determined by flow cytometry assay. LAL assay indicated that both ClearColi K-12-derived plasmids and rAAV vectors had lower endotoxin, compared to their DH5α-derived counterparts. In addition, DH5α-rAAV, but not K-12-rAAV vectors, remarkably augmented expressions of inflammatory factors in cultured cells. Furthermore, K-12-rAAV vectors resulted in less immune responses in mouse eyes *in vivo*. **Conclusions:** Taken together, we demonstrated that using low endotoxin ClearColi K-12 electrocompetent cells during rAAV vectors production induces decreased immune responses both *in vitro* and *in vivo*. We believe that the ClearColi K-12 bacterial strain may have commercial and clinical values for rAAV-related gene therapy products.

1020. Capillary Electrophoresis as a Tool to Assess Multiple Attributes of AAV Based Therapeutics

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Adeno associated virus-based therapeutics have the potential to treat a range of disorders, many of which have unmet needs. Production and purification of these complex structures inherently generate a product profile heterogeneity requiring a matrix of analytical tools. Several instruments and techniques are necessary to monitor and resolve process-associated attributes and impurities at various stages. Of these techniques, Capillary electrophoresis (CE) is a well-established approach having sufficient versatility across many applications in biopharmaceutical development including mAbs, ADCs and fusion proteins. Here, we sought to characterize the structure and occupancy of the AAV capsid and payload using four different CE modalities. A robust CE-SDS platform method was developed and optimized to separate and characterize viral proteins VP1, VP2 and VP3 by ratio and size. Further examination for viral protein heterogeneity was explored using capillary isoelectric focusing (CIEF) toward the measurement of charge state using pI values as a proxy. Further, utilizing both laser-induced fluorescence (LIF) detection and a DNA intercalating dye, the ratio and relative size of the payload DNA conformers could be measured. We have determined that although targeting DNA measurements through a CE approach required manipulation of the gel/buffer sieving matrix and electrophoretic parameters, this method was able to tease apart DNA speciation under forced degradation conditions, highlighting the tunability and sensitivity of this platform. Lastly, the application of mobilizing additives and varying electrophoretic conditions suggests capillary zone electrophoresis (CZE) can be utilized for unorthodox separations, such as quantification and proportional levels of empty and filled AAV capsid ratios.

1021. Considerations in the Use of Analytical Ultracentrifugation for Characterization of AAV Gene Delivery Vectors

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Analytic Ultracentrifugation (AUC) in the biopharmaceutical industry has traditionally been employed in the analysis of aggregation and higher order structure in protein drug products. More recently, gene delivery vectors have opened new avenues for AUC-based characterization and QC lot release methodologies. We discuss here the parameters of an AUC method which conform to the objectives of an ICH/cGMP validation, and suggest gap-bridging strategies to maintain a high-quality AUC platform for use in AAV programs.

1022. Characterization of Immune Response Against AAV in Immune Deficient Mice

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Although adeno-associated virus (AAV) is considered to have low immunogenicity, even a single dose of AAV vectors is sufficient to generate a robust antibody response that can prevent future treatments. The ability to re-administer therapy is important for achieving optimal dosage and for bolstering the effect of previous treatments, particularly in children where therapy must last an entire lifetime. Thus, the formation of anti-AAV antibodies by the host immune system presents a major hurdle in achieving predictable and efficacious results in AAV based gene therapy. Many potential solutions have been proposed to circumvent capsid neutralizing antibodies, such as serotype switching or the creation of novel AAV capsids that are not recognized by previously generated immune response. However, these methods are labor intensive and can be challenging to implement in clinical settings. Immunosuppression can also decrease the extent of anti-AAV immune response, but broad immunosuppression has dangerous side effect. Hence, it is desirable to achieve maximum suppression of anti-AAV immune response with minimal immunosuppression. Our goal is to explore the relative importance of various immune components in generating antibodies against AAV, and to identify key players in antibody generation that, when suppressed, would allow for AAV readministration. To do so, we use a panel of mice with deficiencies in various immune components. While historically there is a greater focus on the adaptive immune response to AAV, there is emerging evidence that the innate immune response also plays an important role in the overall reaction against AAV. Thus, both the innate and adaptive immune systems are considered in our study. So far, our panel includes mice with genetic knockouts in *Rag2/IL2rg* (R2G2), *Ighm* (muMT), *IL15ra* (IL15), *C3*, *MyD88*, and others. Additionally, macrophages/neutrophils are depleted using clodrosome. We injected AAV9 capsid carrying one reporter via tail-vein injection at time 0 weeks. At 3 weeks, we injected AAV9 capsid carrying a different reporter. Each week, we measured the expression of the reporter genes, the neutralizing antibody titer (NAb) against AAV9 capsid using an in vitro cell assay, and the total anti-AAV9 IgG using ELISA. At 6 weeks, mice were taken down and the transcription of cytokines in the liver and spleen were measured using RT-qPCR. Administration of AAV vector induces robust antibody generation in wild-type mice. Antibody generation is undetectable in some immune deficient mice (R2G2, muMT), and detectable but decreased in others (MyD88). Interestingly, NAb seems to be increased in clodrosome treated mice. Antibody generation stabilizes around 2-3 weeks after injection. Upon readministration, expression of the second reporter in wild-type mice is undetectable. Readministration in R2G2 mice, on the other hand, results in transgene expression at levels comparable to that of single injection. Some immune deficient mice, such as MyD88, have undetectable gene expression after readministration despite having decreased antibody formation. Other groups, such as muMT, have some gene expression but at a level significantly lower than that of R2G2. Experiments are being conducted to expand the panel to include other immunodeficient mice. Additionally, we plan to employ our findings to achieve efficacious re-administration in a mouse model of hemophilia A. These results provide valuable information regarding how immune cell populations contribute to the immune response against AAV and identify crucial targets for the development of immune evasive strategies for AAV.

1023. Seeing the Light Again - Next Generation AAVs to Treat Inherited Retinal Dystrophies

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Introduction: Gene therapy is at the forefront of treatments for inherited retinal dystrophies (IRDs) and has already yielded an FDA/EMA approved drug, Luxturna. Adeno-associated virus (AAV) vectors are the preferred vehicles for gene therapy to treat IRDs. Some natural AAV variants have proven useful in preclinical/clinical studies, however they only achieve local effects after an invasive subretinal administration. To expand therapeutic impact, novel AAV variants with improved properties are engineered. Ideally, engineered AAVs should penetrate all retinal layers after a less invasive intravitreal administration and/or spread laterally to the entire retina after subretinal administration. One approach to achieve this is to modify the capsid of AAVs, which is comprised of viral proteins (VP) 1, 2 and 3. The variable region VR-V3 present in all three VPs is susceptible to modifications and allows for changes in the binding capacity of AAVs. **Methods:** By employing a peptide diversification strategy, random 7-mer sequences were inserted at position N587 of AAV2. WT VR-V3 to reprogram the native tropism. Three stringent *in vivo* screening rounds were done in mice, where AAV libraries were injected intravenously and candidates were distilled only from the tissue (retina) and cells of interest (photoreceptors). **Results:** Two novel capsid variants are presented herein, termed AAV2.GL/NN, which are capable of panretinal transduction and lateral spreading after intraocular administration. Characterisation studies show that these novel capsids are superior to the AAV2.WT capsid and the current state of the art AAV2.7m8 following intravitreal

administration in mice. Most importantly, although the screening was performed in mice, AAV2.GL/NN are capable of crossing interspecies barriers. Retinal photoreceptors were successfully infected after a single intravitreal injection in multiple species *in vivo* and human retinal explant cultures *ex vivo*. Preliminary data using mouse models of IRDs, such as *CNGA3*$^{-/-}$ and *CNGB1*$^{-/-}$, show that gene supplementation using AAV2.GL manifests in a fast therapeutic effect as evaluated by electroretinography. AAV2.GL/NN are currently being compared with the AAV variants used in clinical trials for IRDs. These next-generation AAV capsids expand our toolbox for gene therapy applications and offer a solution to cases where the risks of subretinal administration prevent treatment.

1024. Novel, Versatile Viral Vector Cross-Linking Platform for Targeted, Localized and Sustained Gene Delivery

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The success of viral vector gene therapy depends largely on the vector's capability to target specific cells and sustained gene expression afterward. Despite great success, there still exist some unmet needs in achieving spatial and temporal control of Adeno-associated virus (AAV) vector-mediated gene delivery. In this study, a simple, versatile AAV conjugation platform using a cross-linker is proposed. Depending on the concentration of the cross-linking agent used, the cross-linked AAV complex conjugate can either be further linked with specific ligands or relevant biomolecules for targeted gene therapy applications or form an implantable complex for implantable, solid-phase gene delivery applications. At lower concentrations of cross-linkers used, AAV complex was further conjugated with several ligands, one of them being an important ligand in pancreatic cancer cells, as a proof of concept. As a result, gene delivery level was significantly enhanced, up to a 4-fold increase in pancreatic cancer cells. Therefore, we show the feasibility of surface display of pathologically relevant biomolecules to AAVs and subsequent targeted gene delivery through the use of the AAV cross-linking platform. At higher concentrations of cross-linkers used, visible, solid-phase AAV complexes were formed. Using this phenomenon, a simple and easy way to fabricate a novel, implantable AAV complex was developed for localized and sustained gene delivery to the desired site. Structural analysis of AAV complexes reveals that the complex is composed of AAVs in a net-like, porous structure. *In vitro* and *in vivo* functional characterizations reveal that the AAV complex demonstrated sustained, continued gene expression. This system will be able to deliver genes to soft tissues such as the pancreas and brain, as well as organs that require local and sustained gene delivery for a certain period of time.

1025. Abstract Withdrawn

1026. AAV Vector Mobilization is a Concern for AAV Gene Therapy

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Purpose Replication dependent and independent recombinant adeno-associated virus (rAAV) mobilization is thought to cause off-target transgene expression via vector transduction of unintended cells, organs, and individuals; however this theory has never been investigated in a human context. The aim of this study is to quantitatively assess the efficiency of rAAV mobilization *in vitro* and *ex vivo* in a human organ. **Methods** Individual or competitive wild type (WT) AAV and rAAV vector production was examined by alkaline gel electrophoresis and probe-based qPCR to determine the packaged genome integrity and the titer, respectively. Southern blotting of Hirt DNA was performed to compare the WT and rAAV replication efficiency. The infectivity of benzonase resistant rAAV vectors harvested from infected 293 cell or human corneal lysates were examined by infection of new cells (defined non-replication-dependent mobilization). Nascent rAAV particle production post-super-infection or co-infection with WTAAV and Ad5 (defined as replication-dependent mobilization) was also evaluated by detection of benzonase resistant rAAV particles via qPCR and the mobilized rAAV transduction efficiency was examined by luciferase reporter activity. In the *ex vivo* experiments, rAAV mobilization from human corneas after super-infection of WT AAV and WT Ad was assessed by secondary 293 cell transduction. **Results** Alarmingly, rAAV vectors produced following the most commonly used triple transfection protocol all contained WTAAV contamination ranging from 0.8-1.7% of the total rAAV prep depending on the transgenic sequence. rAAV was produced at lower vector yields than WTAAV (3-10 fold), in a transgene dependent manner. However, following co-transfection of plasmids containing the WTAAV genome and rAAV genome in the presence of an Ad helper plasmid, rAAV and WTAAV were produced at similar levels. This result was shown to be independent of the transgenic sequence of rAAV vectors. In a transduction context, about 1% of rAAV particles from the rAAV infected 293 lysate were still benzonase resistant and maintained the infectivity to transduce new cells (non-replicative mobilization). In cells infected with rAAV and then super-infected with Ad, replication-dependent mobilization was observed for rAAV, indicating that WTAAV contamination in the rAAV stocks is sufficient for nascent rAAV production. In rAAV infected cells treated with Ad and WTAAV, rAAV particle production was observed as high as 100-fold compared to the non-replication mobilized vector titer. These replication-dependent mobilized rAAV vectors resulted in 1,000-fold increase of transduction efficiency in subsequent cells. In addition, the ratio of the original input of WTAAV and rAAV played an important role in the competing yields of replication-dependent mobilization of WTAAV and rAAV. Specifically, there is a bias toward the rAAV production if the original input of rAAV is significantly more than WTAAV. The rAAV production is as efficient as WTAAV if the input virus is about the same. **Conclusions** The collective results demonstrate that: 1) at transfection level, there was little to no bias toward WTAAV during "competitive" virus production, 2) non-replicative rAAV mobilization is substantial and therefore raises off-target transduction concerns, 3) in the presence of WTAAV and Ad helper virus, transduced rAAV can replicate to produce infectious

particles in human cells as efficiently as WTAAV depending on the administered dose, 4) WTAAV contamination of rAAV stocks were sufficient to produce AAV vectors in transduced cells super-infected with Ad. The data generated herein highlight the potential of AAV gene therapy treated patients serving as rAAV bioreactors and thereby stress the need for safer, mobilization resistant vectors.

1027. Abstract Withdrawn

1028. Abstract Withdrawn

1029. Unraveling the Uncoating, Capsid Stability, and Aggregation of AAV with High-Throughput Screening on Uncle

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Optimizing the stability of AAV capsids is a critical step in gene therapy development but characterizing uncoating, disassembly, and aggregation behavior is challenging and time-consuming. Uncle is a biologics stability platform able to fluorescently detect AAV uncoating and capsid disassembly as separate events, reflecting the thermal stability of a virus particle. These characterization methods allow for the identification of the most stable vectors and the impact of buffers on stability. In addition, monitoring viral particle size by DLS shows sample quality at room temperature or can be used to track aggregation from heat stress. Uncle measures these parameters for up to 48 samples at a time with 9 µL of volume and AAV titers as low as $5x10^{11}$ vg/mL.

1030. Triple AAV Delivery Modes by Transforming Heparin-Catechol Conjugates

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Adeno-associated viral (AAV) vectors show great potential in gene therapy applications, due to their efficient performances in treating a variety of incurable diseases. To accelerate the successful transition of gene therapy into clinical trials, developing viral gene delivery systems capable of efficiently targeting desired tissues is required. The typical administration of gene vectors by direct injection can cause systemic spread of the vectors, off-target gene expression, and immune responses against the vectors, possibly reducing therapeutic efficacy in the targeted region. Thus, adjuvant systems capable of providing various delivery modes other than systemic administration are necessary for enhancing therapeutic efficacy. The introduction of the capability of responsiveness and flexible adaptability to environmental changes to AAV-mediated gene delivery can be one option. In particular, the catechol groups in mussel adhesive proteins can induce an apparent transition owing to the pH dependence of their properties. In this study, we developed a single system that can diversify delivery modes depending on the catechol-derived transition in given conditions. The mixtures of AAV and catechol conjugated heparin were fabricated into multiple forms, such as liquid formulations, reversible aggregates, and cross-linked film, in order to induce diverse delivery modes dependent on the ratio of mixtures and the external pH. At nano scale, liquid formulations showed ratio-dependent transition of transduction efficiencies in direct injection modes. At micro scale, pH-dependent structural reversibility of heparin catechol efficiently protected AAV from degradation by extreme environments, represented by acidic pH, while maintaining the innate properties of viral vectors. Furthermore, oxidized catechol cross-linked the heparin chain to fabricate the macroscale AAV adsorbed heparin film, making it suitable for application as a localized AAV delivering tool. In conclusion, novel technologies for the fabrication of functionalized scaffolds suitable for particular conditions can modulate in vivo introduction routes and release profiles at target sites to widen the scope of bioengineering targets in AAV-mediated gene delivery.

Gene Targeting and Gene Correction

1031. Transiently Expressed Cell Surface "Tags" for Identification/Selection of Nuclease-Edited Cell Products

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Gene therapy using engineered or guided nucleases for targeted gene editing of cells is a promising strategy for the treatment of inherited and acquired diseases. However, challenges remain for translation into the clinic of efficacious gene-edited cell therapies. Improvements in gene editing efficiency due to advancements in nuclease design and delivery have been reported, but there is need for strategies that allow for the rapid identification and selection of gene-edited cells. Here, we present a clinically-relevant, rapid, transient mechanism to detect and enrich for nuclease edited cells. In this system, a signaling incompetent truncated human epidermal growth factor receptor (tEGFR) protein sequence is fused to the sequence for a CCR5 gene-targeting MegaTAL (MT) by a 2A ribosomal skip site – MT-tEGFR. This allows for concurrent expression of the nucleus homing endonuclease and the cell surface targeted tEGFR. Using an mRNA vector, the tEGFR "tag" is observed on the cell surface at 6 hours after transfection with maximal tEGFR expression at 18 hours. In CCR5-overexpressing CEM cells (CEM.NKR CCR5+; at least 5 copies of the CCR5 gene) transfected with MT-tEGFR, sorted tEGFR+ cells showed consistent increases (77%) in CCR5 indels as measured by Tracking of Indels by DEcomposition (TIDE), in comparison to unsorted cells. Despite the forced expression of CCR5, MT-tEGFR-treated CEM.NKR CCR5+ cells sorted for tEGFR showed marked decrease in the expression of surface CCR5 (36%), which is consistent with enrichment of cells with successful MT activity. We then evaluated the MT-tEGFR system in primary human CD4+ T-cells, to evaluate whether this approach could protect cells from HIV-1 infection. In CD4+ T-cells activated with CD3/CD28 beads, we observed tEGFR expression in 70-85% of cells following that MT-tEGFR treatment. Enrichment of tEGFR+ cells further improved nuclease efficiency as determined by TIDE, as well as decreased CCR5 surface expression by at least 2-fold, in comparison to unsorted MT-tEGFR-treated CD4+cells. Using a weaker T cell activator, treatment with MT-tEGFR resulted in lower levels of

tEGFR expression (24-37%). Importantly, the sorting of tEGFR+ cells consistently improved the efficiency of CCR5 disruption (2 to 7-fold; assessed by TIDE), and was often comparable to the levels observed in sorted tEGFR+ cells from CD4+ T-cells activated with CD3/CD28 beads. These observations suggest that: 1) the detected Tag expression generally reflects the delivery efficiency of the MT construct and, 2) sorting for cells expressing the Tag should provide an enriched gene-edited cell product. Finally, we challenged the MT-tEGFR-treated CD4+ T-cells with R5-tropic HIV-1 virus to assess their sensitivity to infection. Cells treated with MT-tEGFR (unsorted and tEGFR+ sorted) showed increased resistance to HIV infection, whereas their tEGFR- sorted and untreated counterparts were sensitive to infection. Although in this work we have focused on a tagged CCR5 targeted megaTAL, this tagging system can be expanded to other nucleases and has broad applications to other cell types and indications. Furthermore, our studies suggest that our taggable nuclease design has potential for clinical translation, as a strategy for rapid assessment of efficiency of nuclease delivery to target cells (i.e., as a release test), as well as for the enrichment of gene-edited therapeutic cells during the manufacturing process.

1032. A Universal Method to Enrich Therapeutic Relevant Cells with Precise Genome Editing *In Vitro* and *In Vivo*

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In contrast to traditional medicines such as small molecules and antibodies, cell and gene therapy empowers genetically modified somatic cells to fight cancer or restore missing functions. In 2017, the FDA approved the first gene therapy to treat congenital blindness and the first cell therapy to treat B cell malignancies in the United States of America. Since then, increasing numbers of pre-clinical and clinical programs are under development to further expand the applications of these novel therapies to treat other complex diseases, such as solid tumor. This requires more advanced precise genome editing techniques. Nevertheless, the efficiency of precise genome editing or multiple genome editings in somatic cells is generally low and limits their therapeutic potentials. A method to select cells with the desired modifications in this scenario becomes important. In this study, we developed a selection method that can be applied to specifically eliminate non-edited cells and protect edited cells. Unlike existing selection methods, our method requires no external selection marker and introduces no random insertions or deletions within the endogenous genes, which is highly desired in therapeutic applications. We customized this method to enrich precise genome editing events, including base editing and homology-dependant repair (HDR) events, and observed a dramatic increase in editing efficiency in selected cells, up to 60-fold. For the generation of knock-in cells, we compared our method with traditional methods based on antibiotic selection, and found that a homogenous bi-allelic population of precisely knocked-in cells can be generated with our method while not with the standard traditional method. Finally, we demonstrated that the application of our method is not limited to cancer cell lines, it can be applied successfully *ex vivo* in therapeutic relevant cells such as primary T cells and inducible pluripotent cells (iPSCs) as well as *in vivo* in humanized mice models.

1033. PEGylated KL4 Peptides as Promising Vectors for mRNA Delivery

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Introduction The clinical use of messenger RNA (mRNA) for the prevention and treatment of various diseases such as cancers and infections was extensively investigated. However, safe and effective mRNA delivery vectors are still under development. KL4 is a synthetic cationic peptide that was investigated for RNA delivery. It could form complexes with the negatively charged RNA by electrostatic interactions. The high leucine content of KL4 peptide renders it poorly soluble. To improve its solubility, PEGylation strategy, in which the hydrophilic polyethylene glycol (PEG) is covalently attached to the KL4 peptide, was adopted. In this study, three PEGylated KL4 peptides with different molecular weights (MW) of PEG namely PEG_LKL4 (low MW), PEG_MKL4 (medium MW) and PEG_HKL4 (high MW) were investigated and compared. **Methods** mRNA transfection - Peptide/mRNA complexes were prepared at ratios from 5:1 to 30:1 (w/w) in OptiMEM I reduced serum medium containing 1 μg mRNA per well. The complexes were added to A549 cells (human alveolar epithelial adenocarcinoma) after 30 min of incubation. At 24 h post-transfection, the luciferase expression was detected with luminometer (SpectraMax L Microplate Reader, Molecular devices, CA, USA). The results were expressed as relative light unit (RLU) per mg of total protein. Flow cytometry - A549 cells were transfected with naked mRNA and peptide/mRNA complexes at 10:1 ratio (w/w) in OptiMEM I reduced serum medium containing 1 μg of cyanine-5 labelled EGFP mRNA per well. After 4 h of incubation, the fluorescence intensity of the cells was analyzed by flow cytometry (BD FACSCantoII Analyzer, BD Biosciences, CA, USA). Transmission Electron Microscopy (TEM) - The peptide/mRNA complexes were prepared at 10:1 ratio (w/w) and stained with 4% (w/v) uranyl acetate. The morphology of the complexes was visualized by TEM (FEI Tecnai G2 20 S-TWIN, FEI company, Hillsboro, Oregon, USA) at a voltage of 100 kV. **Results and Discussion** After PEGylation, PEG_MKL4 and PEG_HKL4 peptides were soluble in water. For the mRNA transfection study, the transfection efficiency of all three PEGylated peptide/mRNA complexes increased significantly compared with KL4 peptide (Fig. A). Improvement of transfection could be observed when the ratio increased from 5:1 to 10:1 (w/w) and there was no significant increase of luciferase expression at higher ratios. Therefore, ratio 10:1 was chosen for subsequent studies. Flow cytometry study showed that cellular uptake increased significantly when the PEG MW increased, which was consistent with the transfection study (Fig. B). The highest uptake could be observed in cells transfected with PEG_HKL4 peptide, with more than 60% of cell uptake. It could be seen from the TEM images that the KL4/mRNA complexes appeared as large aggregates (Fig. A) with free KL4 peptides observed (as filiform structure). The

complexes formed by all PEGylated KL4 peptides appeared to be smaller in size and more compact. The increase of cellular uptake of peptide with higher PEG MW could be due to the smaller particle size. **Conclusions** Overall, all these PEGylated KL4 peptides are promising mRNA vectors with improved cellular uptake and in vitro transfection compared with the non-PEGylated KL4 peptide.

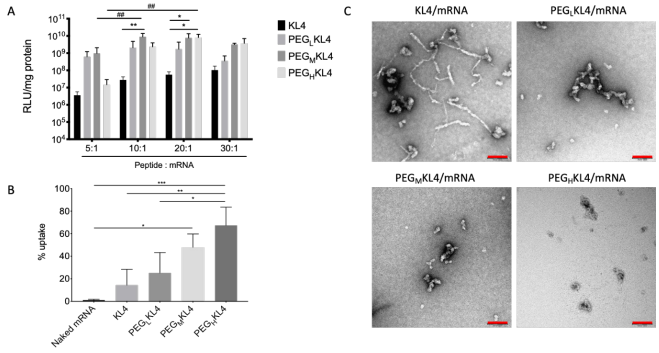

Figure (A) Luciferase mRNA transfection on A549 cells with peptide/mRNA complexes prepared at different weight ratios. The data were analyzed by one-way ANOVA followed by Tukey's post hoc test. *p<0.05, **p<0.01 when compared among peptides at the same ratio; ##p<0.01 when compared the same peptide at different ratios. (B) Cellular uptake study of cyanine-5 labeled mRNA using flow cytometry on A549 cells. The data was analyzed by one-way ANOVA followed by Tukey's post-hoc test. *p<0.05, **p < 0.01, ***p < 0.001 (n = 3). (C) Transmission electron microscopy (TEM) images peptide/mRNA complexes prepared at 10:1 ratio (w/w). Scale bar = 100 nm.

1034. Nuclease-Free Engineering of B-Cells Against HIV

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Life-long treatment with antiretroviral drugs (ART) is the standard of care for HIV infections as it allows control over the viremia. However, the requirement for daily dosing and the associated side effects may lead to reduced compliance and to a consequent development of resistant strains. Combination therapies of broadly neutralizing antibodies (bNAbs) can suppress viremia and provide an alternative to ART, but bNAbs too may have to be chronically administered, and at a higher cost than ART. Constitutive expression of bNAb genes from non-lymphoid tissues has been demonstrated, but such antibodies do not undergo class switch recombination (CSR) nor somatic hypermutation (SHM) and subsequent affinity maturation. bNAbs expressed from non-lymphoid tissues may further be prone to anti-drug antibodies (ADA), due in part to improper glycosylation. To overcome these limitations, expression of bNAbs from the natural Ig loci of primary human B-cells was achieved using CRISPR/Cas9-mediated integration. In addition, in immunocompetent mice, adoptive transfer of B cells engineered to express HIV-bNAbs facilitated the production of HIV-neutralizing antibody titers. Expression of single-chain anti-RSV antibodies from the immunoglobulin heavy chain (IgH) loci of murine B-cells provided protection from infection. More recently, we have shown that CRISPR-engineered murine B-cells were able to undergo antigen-induced activation *in-vivo*, leading to immunological memory, CSR and SHM. B-cell engineering via the CRISPR/Cas9 technology is thus a promising avenue in the fight against the rapidly evolving HIV. However, engineering using CRISPR/Cas9 may be associated with genotoxicity. In particular, CRISPR-induced DSBs may lead to mutations and InDels. In addition, CRISPR breaks may lead to oncogenic translocations with CSR-induced and CSR-independent breaks in activated B cells. Here, we developed site-specific B cell engineering without nucleases, based only on the natural CSR-induced breaks. In particular, we use recombinant adeno-associated viral vectors (rAAVs) to introduce genes coding for the anti-HIV bNAb 3BNC117 and GFP into CSR induced breaks at the IgH locus of B cells (Fig. 1). In lipopolysaccharide-activated primary murine B-cells, a 3BNC117 cassette that lacks the heavy chain constant exons allowed us to detect accurate splice junctions between the 3BNC117 variable part of the heavy chain and either the endogenous IgM or IgG1 exons. The introduced cassette was encoded under a derivative of a murine IgH promoter, which is active only upon on-target integration in proximity to the endogenous enhancers. Next, we used both GFP and 3BNC117, encoded under the modified IgH promoter, to test for expression of 3BNC117 as a membranal B-cell receptor (BCR) on edited cells. Our cassette contained a GFP open reading frame, followed by a 2A peptide and a single-chain version of the 3BNC117 bNAb, with only the variable part of the heavy chain provided. A full, membranal BCR presentation would therefore be possible only upon successful integration into the IgH locus. Engineered splenic B cells were GFP-sorted and propagated on feeder cells expressing CD40L and BAFF, for activation and proliferation. One-week post-sorting, 30% of the cells expressed GFP, out of which 17% bound the HIV antigen gp120, uniquely demonstrating efficient nuclease-free B cell engineering. Our method may provide a safe alternative to the rising field of CRISPR-based B-cell editing against HIV.

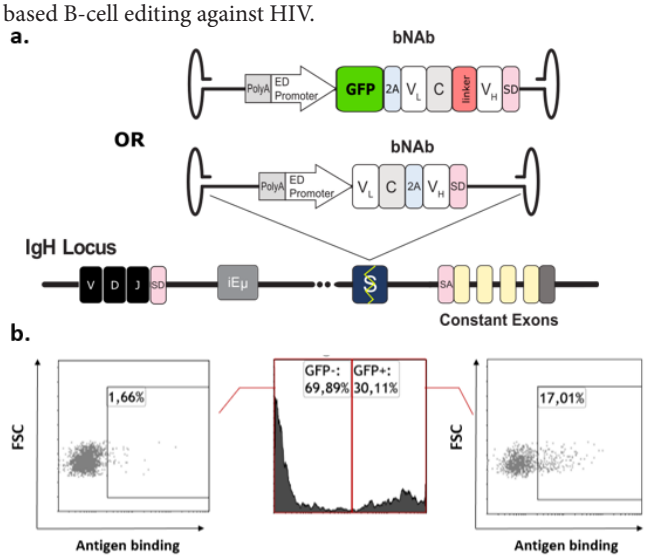

Fig. 1. Nuclease-independent targeting of anti-HIV bNab cassettes into CSR-induced breaks. (a) The targeting Scheme. (b) Flow cytometry analysis of engineered primary murine B-cells, assessing binding to the HIV antigen gp120 of GFP+ versus GFP- cells.

1035. Simultaneous In Vivo High-Throughput Evaluation of Cas9 Toxicity, Mutagenesis and Off- Targeting in Zebrafish Larvae

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The recent findings in CRISPR-Cas9 highlighted the need of safer and more efficient gene and cell therapies approaches. Thus, it became imperative the development of standardized methods capable to provide quantitative readouts describing Cas9 endonuclease efficacy, potential cellular/in vivo toxicity and off-target activity. Several distinct advantages positioned Zebrafish among the ideal animal models to study the molecular and cellular biology of the CRISPR/Cas9 technology. First, the cellular machinery involved in DNA repair is fully conserved among species with strong homology to the mammalian one. Second, protocols to assess pharmacological safety in zebrafish have been already established and multiple assays allow the evaluation of acute and chronic aa well as general or organ-specific toxicity. Third, the zebrafish genome is fully sequenced, allowing to precisely quantify the mutagenesis efficacy of the Cas9 enzymes on both on-target and off-target sites. Finally, the possibility to generate robust and high-throughput data from multiple conditions with a better time/cost profile then higher vertebrates like rodents. These procedures allow to estimate the toxicity of CRISPR and therefore, to use the gathered information towards the prediction of potential adverse events in humans.

1036. Large Insertions Mediated by CRISPR/Cas Editing via Improved HDR Templates, and Comprehensive Characterization of Editing Events with Long Read Sequencing

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Specific genome editing outcomes can be achieved via homology-directed repair (HDR). This repair mechanism is exploited when a repair template, homologous to the genomic sequence adjacent to the double-stranded break (DSB), is delivered at the same time that the DSB is generated. HDR repair outcomes with CRISPR/Cas systems are most efficiently driven by single-stranded DNA (ssDNA) templates when small insertions (up to ~120 bp), deletions, or SNP changes are desired edits. Larger insertions can be incorporated via HDR using enzymatically-generated ssDNA or double-stranded DNA (dsDNA) donor templates. Here, we present work demonstrating that improved efficiency in repair by large insertions is obtained when dsDNA donor templates include novel end-modifications. These modifications improve the frequency of HDR and abrogate blunt insertion repair outcomes. Using Alt-R modified dsDNA templates, we observe more than a 4-fold increase in the ratio of HDR:blunt repair outcomes as compared to an unmodified dsDNA template. In addition, off-target editing analysis shows that the repair has improved specificity with these modifications. Characterizing the integrity of these insertions while maintaining phasing of the DNA sequence can be achieved with long-read sequencing. Long-read sequencing technology allows for a more comprehensive analysis of the outcome of large insertions or deletions created by CRISPR/Cas9 genome editing. Here, we describe a target enrichment approach to selectively sequence a region of interest (ROI) around the CRISPR edited site to measure the rates of precise insertion by HDR.

1037. High-Throughput ELISA Screening for Mechanisms of Non-Enzymatic PNA-Mediated Gene Editing

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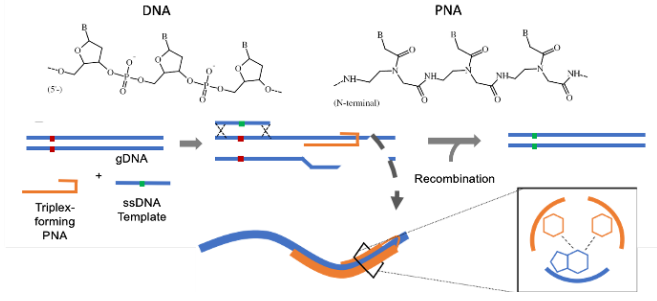
Peptide nucleic acid (PNA)-mediated editing technologies are a mechanistically distinct, non-enzymatic approach to gene editing. Instead of relying on enzymatic activity on DNA substrates, PNAs bind directly to a single strand of genomic DNA in a sequence-specific fashion to form recombinogenic triplex structures. When encapsulated into polymeric, biodegradable nanoparticles, PNA reagents and ssDNA donors have been used to correct disease-implicated mutations in multiple tissues in mice with therapeutically consequential effects - *ex vivo*, *in vivo*, and *in utero*. Here we explore the mechanisms by which PNA:DNA heterotriplex structures achieve site-specific editing without the introduction of an exogenous enzyme. To investigate this phenomenon, we created a high-throughput ELISA-based platform to interrogate factor binding to PNA triplex structures anchored to the bottom of 384-well plates. Wells within triplex-coated plates were exposed to 293T cell lysates expressing FLAG-tagged factors and interrogated for differential binding as compared to duplex dsDNA conditions. A screening effort encompassing 497 known DNA-binding factors, of which ~80% were DNA repair-related proteins, revealed 47 factors that differentially bound PNA triplex vs. duplex structures (z-score cutoff > 5). Screen hits featured multiple interesting factors sharing common pathways. Factors implicated in nucleotide excision repair (NER), structure specific recombination machinery, and single strand annealing (SSA) repair pathways were identified. The functional consequence of select hits on PNA editing are being validated by siRNA factor knockdown and quantitative editing readouts. Notably, many these processes are not known to be shared by any other current approaches to gene editing and suggest a mechanistically novel means of achieving therapeutically relevant site-specific recombination.



1038. The Role of ssODN and rAAV6 Donor Template Delivery in Altering the Long-Term Engraftment and Fate of *Ex Vivo* Edited-HSC and Its Role in Correcting Sickle Cell Disease

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Sickle cell disease (SCD) is caused by a single nucleotide transversion in exon 1 of the *HBB* gene. This genetic change increases the hydrophobicity of adult globin (β^A), leading to substantial morbidity and a reduced life-span in affected subjects. Gene editing utilizing co-delivery of a designer nuclease in the presence of a DNA donor template provides the potential capacity for precise homology-directed repair (HDR). *Ex vivo* gene editing and subsequent engraftment of gene corrected HSC could provide a therapeutic alternative to HLA-matched bone marrow transplantation in SCD. In previous work we have demonstrated that single stranded oligo-nucleotide (ssODN) outperforms recombinant adeno-associated 6 (rAAV6) donor template delivery *in vivo* when introducing a sickle mutation in healthy human mobilized peripheral blood stem cells (mPBSCs). In the current study, we extensively explored the role of donor template delivery in altering the *in vivo* fate and long-term engraftment of edited human mPBSCs and their erythroid progeny. We compared the use of ssODN vs. rAAV6 delivery to introduce a codon-optimized change following Crispr/Cas9-mediated cleavage of *HBB* in healthy human mPBSCs. Using optimized media, low-density culture conditions and RNP-donor co-delivery using Lonza nucleofection system, we achieved high levels of HDR *in vitro* in input cells (RNP + ssODN HDR: 34.6 ± 14.4%, RNP + AAV HDR: 24.3 ± 9.4 %, n = 3 transplant studies using 2 independent donors). We similarly observed high levels of human $CD45^+$ engraftment within the bone marrow (BM) of NBSGW recipient animals at 16 weeks post-transplant for both template delivery methods ($hCD45^+$: RNP + ssODN = 55.5 ± 30.2 %, n = 15 animals); RNP + AAV = 55.1 ± 14.7 %, n = 16 animals). Notably, recipients of ssODN-modified HSC exhibited a significantly higher proportion of HDR-modified cells within the bone marrow. This increase was consistent across total human cells (18.7 ± 11.7) as well as within the $CD34^+$ (23.4 ± 9.7 %) and $CD235^+$ (19.8 ± 12.9 %) compartments compared to rAAV6-modified animals (Total human cells: 11.6 ± 6.1 %, $CD34^+$ = 11.8 ± 4.2 %, $CD235^+$ = 9.5 ± 2.1 %). In parallel, we assessed additional key functional outcomes including RNA transcripts analysis using digital droplet PCR (ddPCR) and globin sub-type expression by high-performance liquid chromatography (HPLC). Our combined findings demonstrate the capacity to achieve clinically relevant HDR editing rates *in vitro* and *in vivo* using either donor template delivery method. Use of ssODN donor template-delivery, however, is consistently associated with higher levels of gene correction *in vivo* as demonstrated by sustained engraftment of HDR-modified HSC and erythroid progeny in the BM at 16 weeks.

1039. Nanoparticles Containing Oxidized Cholesterol Improve mRNA Delivery *In Vivo*

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Introduction: There have been several advances in local and systemic mRNA delivery, especially to hepatocytes. However, dysfunctional liver endothelial cells, Kupffer cells, and other cells within the liver microenvironment likely cause as much disease as hepatocytes. Nanoparticles that target the liver microenvironment are difficult to design, in large part because there is no high throughput method to study nanoparticle mRNA delivery *in vivo*. This universal problem in nanomedicine slows the development of all RNA therapies, since scientists are forced to perform high throughput nanoparticle assays *in vitro*, even though cell culture can be a poor predictor of delivery within a complex *in vivo* microenvironment. Thus, we reasoned that an ideal nanoparticle experiment would study lipid nanoparticles (LNPs) directly *in vivo*. We used FIND, a recently developed functional DNA-barcoding screening platform to quantify how 75 LNPs functionally delivered mRNA (i.e., mRNA translated into protein) to the cytoplasm of 28 different cell types, all *in vivo,* in one group of mice. We then utilized a custom DNA barcode-based bioinformatic pipeline to relate LNP structure to *in vivo* delivery, and found cholesterol structure influenced LNP activity. **Materials and Methods:** Nanoparticles were formulated in a microfluidic device by mixing Cre mRNA, DNA, and LNP components. The nucleic acid was diluted in citrate buffer (Teknova). Nanoparticle materials were diluted in ethanol. The phases were mixed together via microfluidics. Each LNP was formulated to carry a distinct barcode; LNP1 carried Cre mRNA and DNA barcode 1, whereas LNP2 carried Cre mRNA and DNA barcode 2. LNP hydrodynamic diameter was measured using dynamic light scattering. Mice were aged 5-8 weeks, and N = 3-4 mice per group were injected intravenously. Nanoparticle DNA barcode sequencing was performed on MiniSeq using Illumina protocols. **Results and Discussion:** After performing a high-throughput *in vivo* delivery experiment to test our hypothesis, we found that LNPs formulated with cholesterols chemically modified with hydroxyl groups near the D sterol ring - but not LNPs with hydroxyl groups on the B sterol ring - can potently deliver mRNA to cells in the liver microenvironment *in vivo.* Moreover, the 75 LNPs that we analyzed delivered gene editing Cre mRNA much more efficiently to liver endothelial cells, Kupffer cells, and immune cells than to hepatocytes. To validate these screening results, we tested two lead LNPs. Both LNPs delivered mRNA to hepatic endothelial cells and Kupffer cells 5-fold more potently than hepatocytes *in vivo*, as predicted. These LNPs, formulated with modified cholesterol, potently delivered mRNA at doses as low as 0.05 mg / kg. **Conclusions:** LNPs containing oxidized cholesterol can deliver mRNA to cells in the liver microenvironment more potently than to hepatocytes. Given the importance of liver endothelial cells and Kupffer cells in disease, these data suggest that additional advances in delivery could eventually result in protein replacement therapies within the liver microenvironment. In the context of these experiments, cholesterol structure impacts functional delivery. We find it feasible that different protein coronas may adsorb onto LNPs based on cholesterol structure, thereby altering targeting.

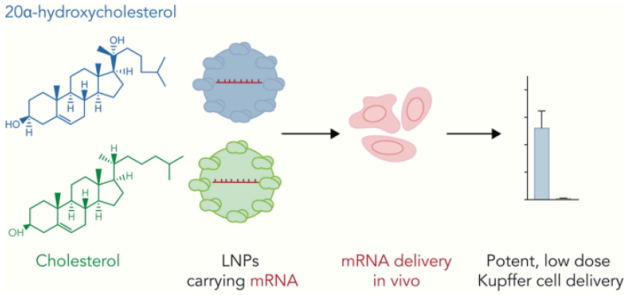

Figure 1. An LNP containing oxidized cholesterol can lead to potent, non-hepatocyte mRNA delivery at clinically relevant doses when compared to the same LNP formulated to carry regular, unmodified cholesterol.

1040. Lentiviral Capsid as a Safe and Efficient Delivery Vehicle for Genome Editing

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Delivery of the CRISPR/Cas9 machinery is still a challenge. Viral vectors have high delivery efficiency but fall short in safety. Delivering Ribonucleoproteins (RNP) is safer but the efficiency needs to be improved. We have developed novel bionanoparticles, hybrid delivery vehicles between viral vectors and mRNA or RNP nanoparticles, for efficient and safe delivery. These bionanoparticles consist lentiviral capsids and Cas9 mRNA or RNPs. The lentiviral capsids mediate efficient cell entry and delivery of the Cas9 mRNA or RNP cargos for efficient genome editing. Due to the lack of DNA, viral genome or packaging cis elements, possible integration is eliminated. The RNA and protein cargos delivered are only transiently expressed. The packaging of cargos in lentiviral capsids is mediated by the specific interactions between aptamers and aptamer-binding proteins (ABP). Aptamers are added in the 3' untranslated region of Cas9 mRNA for mRNA packaging, and in various locations of single guide RNA for RNP packaging; whereas ABPs are added in the nucleocapsid protein of the lentiviral capsid. The system has been successfully used for efficient delivery of SaCas9 mRNA, SaCas9 and SpCas9 RNPs, adenine base editor RNPs and CRISPRi RNPs. Over 50% INDEL rates can be obtained from un-concentrated particle-containing culture supernatants. Multiplex gene editing by the same or different Cas9 variant is possible in a single preparation of multiple-RNP-co-packaged bionanoparticles. Large-scale genome engineering (~18 Mb) is possible by targeting multiple loci on the same chromosome. In summary, our lentiviral capsid based delivery systems offer efficient, safe and flexible delivery of various genome editing machineries.

1041. Aldevron's CRISPR-Associated Nucleases for Gene Editing; Tools to Support Discovery and Therapeutic Programs

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Gene editing using CRISPR-associated nucleases, including SpCas9, offers a novel approach to treat many diseases. SpCas9 now is being evaluated in multiple preclinical and clinical programs. While many vendors offer research-grade SpCas9, these nucleases are not applicable to clinical trials due to construct design and quality grade. Aldevron offers SpCas9 and AsCpf1 nucleases optimized for development and clinical applications. In addition to wild-type SpCas9, Aldevron offers *Spy*Fi™ Cas9 which supports gene editing with significantly reduced off-target effects. Aldevron's gene editing proteins are supported by a robust, scalable manufacturing protocol and an extensive quality package. These proteins are available in a range of quality grades from research grade to GMP, allowing for a seamless transition from research applications to the clinic.

1042. Optimization of CRISPR/Cas9 Mediated Editing in Murine Lineage Negative Cells for SCD

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Previous studies have demonstrated that *in vitro* correction of the sickle mutation in hematopoietic stem/progenitor cells (HSPCs) from sickle cell disease (SCD) patients is feasible using CRISPR/Cas9 ribonucleoprotein (RNP) and a corrective DNA single-stranded oligodeoxynucleotide (ssODN) donor to initiate homology directed repair (HDR). While editing the β-globin gene (*HBB*) resulted in correction in up to 25% of the total alleles, undesired on-target insertions and deletions (indels) due to non-homologous end joining (NHEJ) were also induced in up to 60% of the total alleles. The clinical outcomes of moderate levels of correction accompanied with high levels of undesired indels remains unclear, thus leading to the creation of this project: to study the physiological consequences resulting from different rates of on-target indels induced by the CRISPR/Cas9 system at the *HBB* locus for SCD. To investigate, electroporation protocols will be optimized using murine lineage (lin) negative cells from the Townes SCD transgenic mouse model targeting the *HBB* locus. Edited cells will then be pooled into various HDR to NHEJ ratios and transplanted back into mice to enable observation of differences in phenotype and disease outcome. Preliminary data have shown that the production of NHEJ and HDR alleles at *HBB* in lin negative cells is possible using the Neon Transfection System. Toxicity one day post-electroporation proved to be drastic (viable cell recovery =16 ± 8%), with nearly a 4-fold decrease in percentage of viable cells compared to mock-treated samples (62

± 2%). Despite high toxicity, High Throughput Sequencing (HTS) analysis revealed 79 ± 7% indel formation at the *HBB* locus. To then test for gene correction potential, a ssODN donor template containing the corrective *HBB* gene was co-delivered with the RNP reagents, resulting in only 3.7 ± 0.6% of HDR alleles. Further experiments utilized AAV6 as a DNA donor template to initiate HDR at the *HBB* locus using various multiplicities of infection (MOIs). The average viability twenty-four hours later was 31.2 ± 0.09%, demonstrating that toxicity was not affected by MOI. HTS data proved AAV6 to be a more effective DNA corrective template to induce HDR than ssODN *in vitro*, with a five-fold increase in HDR alleles (61.7 ±13.1 %). Other transfection systems were tested for gene correction potential at *HBB*. Experiments were conducted in the Maxcyte ATX Transfection system using electroporation protocols with varying voltages. HTS revealed that variations in voltage did not affect indel formation (93.7 % ± 0.75). However, increasing voltage led to a 1.5-fold increase in the creation of HDR alleles using the ssODN donor from 2.31 ± 0.08% at the lowest setting to 3.75 ± 0.03% at the highest setting. These data suggest that future experiments utilizing even higher voltages should elevate the frequencies of HDR at *HBB*. Further experiments will be performed using AAV6 as a DNA donor template in the Maxcyte ATX Transfection system in attempts to replicate the high levels of HDR achieved using AAV6 in the Neon System. Following electroporation optimization experiments, mice will be transplanted with varying ratios of HDR and NHEJ edited cells and observed for differences in SCD disease and other hematological outcomes. This study will enable insight about the role of on-target indels on clinical outcomes for the treatment of SCD using gene editing techniques.

1043. Validation of CRISPR Gene Knockouts in U2OS Cell Lines with Global Proteomic Analysis Using Data-Independent Acquisition Mass Spectrometry

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Background CRISPR-Cas9 is an essential tool for gene modification in cellular systems, with applications in functional screening, target validation, and generation of disease models. The ease of using CRISPR-Cas9 for genome editing has made it possible to assay the functions of multiple genes in one experiment. Here we use an optimized RNP delivery protocol to engineer gene knockouts (KOs) which are functional at the pool level. In order to confirm protein depletion, the results of a CRISPR gene modification are commonly validated using readily available off-the-shelf antibodies which may not always be available, or custom designed assays that are time-consuming and expensive. Here, we investigate the utility of quantitative global proteomic characterization using data-independent acquisition mass spectrometry (DIA-MS) to validate knockout of protein expression levels in U2OS cells. The global proteomic data set not only enables rapid validation of target knockouts but also enables the characterization of secondary consequences of gene modification. **Methods** To create KO cell pools using CRISPR, a wild type U2OS cell line was nucleofected using pre-complexed ribonucleoproteins (RNPs) of Cas9 and custom designed synthetic guide RNAs targeting: COA3, ENSA, GRB2, MAPRE1, PPP1CC, RAP2A, TOMM5, TPM1, TPM2 and UQCRQ. U2OS cells were dissociated and 100,000 cells were added for RNP assembly reaction. Nucleofections were performed on a Lonza 4D Nucleofector with the CM-104 program. Three days after nucleofection cells were lysed for genotyping. PCR amplicons between 400 - 800bp with both primers at least 100bp from any of the guides were generated and analyzed by Sanger sequencing (Genewiz). Sanger data and guide sequences were analyzed to determine editing and to quantify generated indels (Hsiau). On day 15 after nucleofection, ~1e6 cells were collected, washed with PBS, pelleted, and stored at -80°C for proteomics analysis. Cell pellets were prepared for mass spectrometry using standard procedures. All samples were analyzed using 2-hour gradients on a C18 column coupled to a Thermo Scientific Q Exactive HF mass spectrometer in data-independent acquisition (DIA-MS) mode. Quantitative protein data was extracted in Spectronaut X (Biognosys) using a hybrid sample-specific sample and directDIA™ peptide assay library. **Results** A peptide spectral library was generated from a pool of all samples covering 9'720 proteins with 182'605 peptides. Following data extraction, 8,002 proteins were quantified across all samples (peptide and protein FDR < 0.01). Among the genes tested, 8/10 (all but ENSA and RAP2A) were verified to have clear depletion of target protein concentrations by between 5 and 85-fold (median = 13-fold). In addition to gene specific knockouts, between 28 and 847 proteins were differentially expressed with respect to a non-targeted control condition (log2 fold change > 0.58, q-value < 0.05). In the knockout of cytochrome c oxidase assembly factor 3 homolog (COA3), which is an upstream translational regulator for cytochrome c oxidase (complex IV), we observed co-depletion of multiple proteins from the mitochondrial respiratory chain complex IV including: MT-CO2, NDUFA4, COX5B, COX6A1, COX6B1, COX6C, COX7A2, and COX7C. These data highlight the importance of fully characterizing the global proteomic changes due to CRISPR based gene modification. **Conclusions** DIA-MS enables deep characterization of global cellular proteomes and is ideal for rapid validation of gene knockouts, owing to the lack of need for protein/gene specific assay development. Furthermore, capturing global proteomic changes enables characterization of downstream protein network regulation.

1044. Abstract Withdrawn

1045. CRISPR/Cas9-Disruption of Non-Pathogenic SNPs in Human Rhodopsin In Vitro as Part of an Allele-Specific Strategy to Treat Autosomal Dominant Retinitis Pigmentosa

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Dominant mutations in rhodopsin (RHO), a light-sensitive protein found in rod photoreceptors in the retina, cause retinitis pigmentosa. This disease affects roughly 1 in 16 000 individuals and causes progressive loss of photoreceptors over several decades, resulting in blindness. Successful gene therapy treatment of this condition will require allele-specific knock down of the mutant rhodopsin strand, which can be achieved using the CRISPR/Cas9 technique. This

technique uses a DNA-cleaving enzyme Cas9 which is directed to regions that share sequence homology with a guide RNA (gRNA). As there are over 150 identified RHO mutations, the ideal treatment strategy should be mutation-independent. Common, non-pathogenic single nucleotide polymorphisms (SNPs) present on the mutant RHO strand can be targeted with CRISPR/Cas9. The absence of this SNP on the wild-type strand will create a 1 bp mismatch between the gRNA and DNA which may be sufficient to prevent Cas9-driven gene disruption. The human RHO gene was screened for common SNPs, which occurred either in the gRNA binding region, or generated or removed a PAM site (a PAM site is a DNA sequence necessary for Cas9 binding). Seven SNPs and one 10 bp deletion were identified that enabled the design of 10 gRNAs (RHO_1-10). Encouragingly, the SNPs occurred in the gRNA "seed region", the 8-10 bp at the 3' end of the sequence where gRNA:DNA mismatches are least tolerated, in all gRNAs except RHO_7. The 10 bp deletion spanned the gRNA binding region of gRNA RHO_3 and removed the PAM site from gRNA RHO_4, making these the most promising candidates for allele-specific disruption of RHO. HEK293-EGFP cells were genotyped and found to be homozygous for all SNPs. gRNAs targeting the SNPs present in HEK293-EGFP cells were cloned into an all-in-one plasmid expressing both SaCas9 and gRNA. 48 hours post transfection the cells were harvested. Tracking of Indels by DEcomposition (TIDE) analysis was conducted on 7 of the 10 gRNAs, which had rhodopsin disruption levels of 16.4 % with gRNA RHO_2 to 44.2 % with gRNA RHO_7. The remaining 3 gRNAs could not be assessed by TIDE analysis as highly repetitive regions adjacent to the cut site prevented successful Sanger sequencing (a requirement of TIDE analysis). Instead these were subcloned into the pGEM-T Easy vector and sequenced. This method detected higher editing efficiencies of 66.6 % to 73.3 %. Selected gRNAs were subsequently tested for allele-specific knockdown using a dual luciferase assay. All gRNAs were able to disrupt rhodopsin in HEK293-EGFP cells in vitro with varying efficiencies from 16.4 % to 73.3 %. The subcloning technique appears to detect a higher rate of CRISPR-mediated gene disruption than TIDE analysis. Assessments of allele-specific knockdown revealed varying degrees of specificity depending on the gRNA/target.

1046. Dual Guide CRISPR/Cas9 Editing of the CCR5 Gene Provides Complete Protection Against HIV in Humanized Mouse Models

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Hematopoietic stem cell transplant (HSCT) with *CCR5*$^{d32/d32}$ defective stem cells has resulted in long-term remission of HIV infection in three patients ("Berlin", "Dusseldorf", and "Oxford") that received allogeneic HSCT for co-occurring malignancies. However, the scarcity of HLA-matched, *CCR5*^{d32} homozygous stem cell donors represents a significant hurdle to more widespread adoption of HSCT for treatment of HIV infection. The ability to effectively edit the *CCR5* gene in autologous, mobilized, CD34$^+$ hematopoietic stem progenitor cells (mobHSPCs) would overcome this hurdle and provide a path toward a cure for HIV infection. Guides were screened for editing efficiency by CRISPR/Cas9 ribonucleoprotein (RNP) nucleofection of primary human CD4$^+$ T cells. Edited CD4$^+$ T cells were then stimulated and challenged with R5-tropic HIV. A dual guide approach resulted in the highest level of *CCR5* gene editing and complete protection from high titer HIV challenge *in vitro* and was selected for HSPC editing and transplant. Dual guides were then tested for editing efficiency and HIV protection *in vivo*. Our dual guide approach resulted in 81-92% *CCR5* gene editing frequency in mobHSPCs from an anonymous HIV-negative donor (guide 1: 70%; guide 2: 58%; deletion: 36%; total: 81-92%). Transplantation of *CCR5*^{edit} HSPCs into NSG mice resulted in normal hematopoiesis with efficient human immune cell reconstitution. The populations of human monocytes, B cells, and T cells were comparable to the control sham (GFP guide) edited mice. High frequency *CCR5* gene editing was detected in descendant monocytes, B cells, and T cells (median 89%), and the frequency of circulating T cells expressing CCR5 on the cell surface was <0.25% compared to 57% in the sham edited controls. Importantly, *CCR5*^{edit} mice were completely resistant to an infection challenge with an ID_{100} of a CCR5-tropic HIV (0/5 *CCR5*^{edit} mice infected), which was able to infect all 8/8 control mice. Furthermore, *CCR5*^{edit} mice were also able to resist a challenge dose of 50x ID_{100}. In contrast, subsequent intraperitoneal challenge of a *CCR5*^{edit} mouse with a CXCR4-tropic HIV strain resulted in robust infection and plasma viremia confirming CCR5-specific protection. In conclusion, this work demonstrates that high frequency CRISPR/Cas9-mediated editing of the *CCR5* gene in human HSPCs is achievable and is sufficient to prevent infection during multiple, high dose exposures to a highly pathogenic strain of HIV. These results suggest that an autologous transplant could provide a functional cure for patients with chronic HIV infections.

1047. Biomedical Reporter Pigs for Testing Somatic Cell Gene Editing Tools

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Translating gene editing tools like CRISPR and base editor from basic research tools to therapeutics will require extensive testing to ensure their safety and efficacy. Large animal models are uniquely applicable to preclinical studies due to their similar anatomy, physiology, and genetics of human patients. Further, the short gestation period (<4 months), large litter size (>6), and ability to easily create transgenic and gene-edited offspring gives them a significant advantage over nonhuman primates. We are developing a suite of biomedical research pig lines, with reporters at safe-harbor loci, that will detect gene editing activity as part of the NIH Somatic Cell Genome Editing consortium. Various lines are being developed that can detect double-strand break activity, differentiate between non-homologous end joining and homology directed repair, or detect base editor activity. The desired editing outcome will activate a fluorescent marker, so analysis can be done on tissues using IHC or in single cells using FACS or sequencing. We are also developing technology that will allow reporter activation to be detected non-invasively. These biomedical reporter pigs will allow for the analysis of optimal routes of administration, delivery vehicles, and/ or gene editors at the tissue-, cell-, and genome-levels for novel gene editing therapeutics.

1048. An Expanded Repertoire of ZFN Architectures for High-Precision Therapeutic Genome Engineering

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We have previously shown that diversification of zinc finger nuclease (ZFN) architectures enables high density targeting of therapeutic loci in the human genome at high efficiency in clinically relevant cell types[1]. Here we have further extended this work, via two distinct approaches. First, in the context of the canonical ZFN dimer we identified ZFP-FokI linkers that enable efficient cleavage for a wider range of gap spacings between ZFN subsites. In a typical canonical dimer the ZFN binding sites are separated by a five or six base pair gap and each ZFN contains an identical ZFP-FokI linker to span this gap. However, as the gap spacing widens, the entropic penalty incurred by use of progressively longer linkers on each ZFN leads to a decrease in ZFN editing efficiency. To overcome this limitation, we modified the linker configuration such that one ZFN in the dimer contained a standard, short ZFP-FokI linker, while the second ZFN contained an extended linker capable of spanning the larger gap sizes. Identification of such linkers was accomplished via directed evolution using a cleavage-based bacterial selection system. This selection identified linkers capable of spanning seven or eight base pair gaps yielding ZFN dimers that cleave with high efficiency. Second, using phage display we were able to select linkers between otherwise adjacent zinc fingers that enable skipping of two base pairs of DNA. Combined with our previously developed single base pair skipping linkers, these linkers allow the use of a much more diverse set of zinc fingers within the zinc finger array while maintaining cleavage at the same location. Relative to previous architectural improvements[1], we estimate that our new options increase targeting capabilities a further 6-fold, enabling design densities that are >400-fold higher than would be otherwise achievable using the archetypal ZFN structure[2]. These improvements substantially enhance our ability to identify ZFN dimers with the desired combination of efficiency and specificity for any given clinical application. 1. Paschon, D.E. et al. Diversifying the structure of zinc finger nucleases for high-precision genome editing. *Nature Commun*. **10**, 1133 (2019).2. Urnov, F.D. et al. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* **435**, pages 646-651 (2005).

1049. GeneRide-Encoding hUGT1A1 Rescues Phenotypes of a Mouse Model of Crigler-Najjar Syndrome

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Crigler-Najjar (CN) Syndrome is an ultra-rare, neonatal onset monogenic disease caused by the loss of function of a liver-specific enzyme, UDP-glucuronosyltransferase family 1 member A1 (UGT1A1). The complete loss of UGT1A1 function in CN type I patients leads to hyperbilirubinemia that causes irreversible neurological damage and even death. The standard of care for CN type I patients consists of daily cycles of >12 h blue light phototherapy, which is associated with poor compliance and loss of efficacy as the patients grow. Recent progress in AAV gene therapy has shown great promise in numerous monogenic diseases. However, the durability of treatment remains a concern of this approach for pediatric patients as liver growth leads to episomal dilution/degradation that results in loss of efficacy. GeneRideTM is a novel AAV-based, promoterless, nuclease-free, genome editing technology that leverages the natural process of homologous recombination to insert, site-specifically, a copy of therapeutic transgene into the genome (Barzel A, et al. 2015 Nature). For liver targeting indications, the therapeutic transgene is inserted into the Albumin locus, the highest expressed gene in hepatocytes. In this study, we evaluated the therapeutic efficacy of GeneRide vector carrying human UGT1A1 transgene (GR-hUGT1A1) in a mouse model of CN syndrome (B6.129-Ugt1tm1Rhtu/J). The homozygous knockout (KO) mice show jaundice at 24 hr post birth and usually die within 7 days if not treated (Nguyen N, et al. 2008 JBC). By employing 12 hr/day blue light therapy, a fraction of KO mice can be rescued (~17% survival by 1 month of age). Interestingly, surviving KO mice rarely die despite of hyperbilirubinemia (total bilirubin > 10 mg/dL) and discontinuation of 21-day phototherapy. We further treated the KO mice with GR-hUGT1A1 intravenously and observed rescue of neonatal lethality as well as significant reduction of bilirubin. The administration of GR-hUGT1A1 also induced dose-dependent increase of circulating biomarker (Alb-2A) as well as human UGT1A1 as detected by western blot. In conclusion, we have demonstrated that GeneRide is capable of rescuing the lethality and improving hyperbilirubinemia in treated CN mice. Such results support the development of GeneRide-mediated expression of UGT1A1 as a therapeutic strategy for this devastating disease.

1050. Allele-Specific CRISPR-Cas9 via Dual AAV-Mediated Gene Editing Rescue Retinal Degeneration in a Humanized RHO-P23H Mouse Model

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The c.C69A p.Pro23His (P23H) mutation in the Rhodopsin gene is the most common cause of autosomal dominant RP (adRP) in USA. This *P23H* mutant allele produces a misfolded rhodopsin protein that plays a dominant negative or gain-of-function role in photoreceptors, resulting in gradual rod degeneration followed by progressive visual loss. Our previous studies on a *Rho-P23H* knock-in mouse model had showed that allele specific gene editing specifically ablates the P23H mutant allele, leading to a significant decrease of the expression of *Rho-P23H* transcript, as well as reduction of photoreceptors death. In this study, we further evaluated the CRISPR/Cas9-based allele-specific approach to edit the P23H mutation of human *RHO* gene in a

humanized mouse line, in which the endogenous mouse *Rho* gene was replaced with a human *RHO* gene with P23H mutation and a fusion RFP at the C-terminal. The humanized mice that carries a wild type human *RHO* gene and a GFP fusion was used as controls for specificity testing. Both mouse lines were gifted by Dr. Theodore Wensel from Baylor College of Medicine. We tested a series of SaCas9 sgRNA in cells and packed the most efficient SaCas9-sgRNA component into a dual-AAV vectors: AAV2/5-pRK-SaCas9 and AAV2/5-pU6-sgRNA-pCMV-BFP. We delivered the AAV-CRISPR components into the retinas of *mRho/hRHO*-P23H-RFP heterozygous mice at P14-P18 by subretinal injection and evaluated the editing efficiency, specificity and efficacy at 4 months of age. We observed effective targeting of the human P23H allele with cleavage frequencies up to 22% in BFP+ transduced retinal cells. Less than 10% of cleavage was detected targeting wild type *RHO* allele in the *mRho/hRHO*-GFP heterozygous mice. Immunohistochemical analysis in treated regions of retinas revealed significant reduction of the *RHO-P23H-RFP* signal in the inner segment and outer nuclear layers, compared to the adjacent untreated areas. Measurements of the retinal function with electroretinography (ERG) showed that the amplitude of a-wave of scotopic ERG in treated eyes is significantly greater than that of untreated eyes at 16 weeks post injection. The thickness of outer segment, the cell numbers and rows of outer nuclear layers are significant preserved in the treated region of retinas. Collectively, our results demonstrate that AAV-mediated allele-specific CRISPR/Cas9 gene editing approach could effectively rescue photoreceptor cell death by selectively ablating the expression of human RHO-P23H allele. This provides strong evidence that allele-specific editing approach can be an effective therapy for *RHO-P23H* associated adRP.

1051. Targeting Various Genomic Loci using AAV-GeneRide Results in Similar Genome Editing Efficiencies but May Affect Translation of the Chimeric mRNA Transcripts

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Gene editing can provide sustained therapeutic gene expression and can directly or indirectly complement disease-causing mutations. As such, these strategies can be used as a single therapeutic treatment for patients with genetic disorders of all ages. In this regard, gene editing via adeno-associated virus (AAV) targeted integration into the host genome via homologous recombination, also referred to as AAV-HR, is a promising strategy for *in vivo* therapy. A specific form of AAV-HR called GeneRide was developed by our lab, in which a therapeutic coding sequence preceded by the P2A ribosomal skipping site is flanked by host genomic sequences. By targeting integration just upstream of the stop codon of an endogenous gene, a chimeric mRNA encoding both the endogenous and therapeutic gene is produced and translated into two distinct protein products. This technology does not rely on designer nucleases nor does it include exogenous promoters, representing a paradigm shift in safety. However, little is known about the characteristics and mechanisms of the newly developed GeneRide technology. We evaluated three molecular parameters associated with GeneRide based genome editing. First, we examined endogenous target site gene expression patterns and locales of integrated cells *in vivo* to determine if target site expression is stochastic (as therapeutic gene expression is controlled by the target site's endogenous promoter in GeneRide strategies). Secondly, we sought to examine the feasibility and characteristics of targeting alternative loci than our original integration target site of *Albumin*- the most abundantly expressed gene in the liver. Lastly, we sought to determine if the chimeric P2A-transgene mRNA possesses similar stability and translatability compared to the endogenous untargeted protein. Using RNAScope *in-situ* hybridization, we show that the production of chimeric mRNA, resulting after successful gene targeting, is not geographically restricted to a single region of hepatocytes in the liver, but does show differences in zonal patterns of expression. Next, we determined that targeting integration to 4 distinct genomic loci (*Albumin*, *Gapdh*, *ApoE*, and *ApoA2*) resulted in sustained human factor IX (hFIX) RNA and protein production *in vivo.* In general our results to date show that the number of targeted hepatocytes determined by RNA detection was similar regardless of which locus was targeted. Moreover, the expression of plasma factor IX roughly paralleled the relative expression from the endogenous targeted gene. We also investigated if the chimeric mRNA produced by GeneRide technology had an altered half-life compared to endogenous untargeted mRNA. To do so we transfected plasmids expressing the native *mAlb* mRNA or chimeric *mAlb-P2A-hFIX* mRNA, under the control of the constitutive CAG promoter into 293T cells. We found similar amounts of steady-state wildtype and chimeric mRNAs were produced suggesting a comparable mRNA half-life. This was further supported when we inhibited transcription with actinomycin D and found an equivalent falloff in chimeric and wildtype mRNA levels. Unexpectedly, up to 10-fold lower mAlb protein accumulated from the chimeric mRNA compared to wildtype mRNA following transfection. Together our data suggest, the chimeric mRNAs may be less well translated. Ribosomal profiling studies are underway to confirm this hypothesis. Uncovering the mechanism for this limitation in translation (or other factors) may allow us to increase transgene expression from AAV- GeneRide vectors making it a more versatile platform for genome editing.

1052. Comparison of Two *Campylobacter jejuni* CRISPR Cas9 Orthologues in Active and Deactive Forms

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CRISPR-Cas9 offers genome editing possibilities and therefore potential as a future gene therapy. However, with current gene therapies using adeno-associated viral (AAV) vectors, transgene delivery is limited by the ~5kb packaging size of a single AAV capsid. Here, we investigated the use of *Campylobacter jejuni* Cas9 orthologues, due to their relatively small gene size of ~2.95kb and unique PAM site. Two different versions, px404 Campylobacter jejuni (px404) and pRGEN-CMV-CjCas9 (pRGEN), of the *C. jejuni* Cas9 (CjCas) were compared, pRGEN being codon optimized for human cells. The CjCas9 genes have 80% sequence alignment, but 100% amino acid alignment. However, 11% of the difference is attributed to a greater -GC content in the pRGEN variant, possibly enabling greater stability. Given the differences between the two orthologues, we sought to compare them

to determine which may have more potential in future in vivo testing. We targeted 8 sites in GFP and one validated site targeting AAVS1 in HEK293-EGFP cells. The editing rates of both CjCas9 variants were equivalent, with no significant differences in the editing rates identified. Given the irreversibility of Cas9 cleavage, we deactivated both *C. jejuni* Cas9 variants (dCjCas) to enable inhibition of transcription without inducing DNA changes. Both orthologues were catalytically inactivated at previously investigated sites: D8A and H559A. Cleavage activity was confirmed to be abolished with both deactivated CjCas9 variants using 3 selected GFP targets and the AAVS1 target. The ability of the dCJCas9 variants to inhibit gene expression was then assessed using a luciferase assay. The AAVS1 target was inserted between the SV40 promoter and the *Renilla* luciferase coding sequence in the commercially available Psi-CHECK2 plasmid. It was observed that both dCjCas orthologues enabled significant blocking of *Renilla* expression, with px404 and pRGEN achieving 70.1% and 75.8% knockdown, respectively. Both CjCas9 orthologues achieved approximately equivalent knockdown, so no significant difference in the extent of knockdown was observed between orthologues. These results encourage further research of both active and inactive *C. jejuni* Cas9 orthologues for possible eventual therapeutic applications.

1053. Epigenetic Regulation of Gene Structure and Function with Cell-Permeable Cas9 Nuclease (CP-Cas9) for Gene Editing

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The clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 system is highly specific RNA-guided genome modification composed of Cas9 nuclease and single stranded guide (sg) RNA. Delivery of these two components has been achieved with electroporation or microinjection. However, these methods are limited in their application due to safety and efficiency including cell damage and uncontrolled integration of external genome, and also unable to apply in animal. To address these issues, cell-permeable (CP) Cas9 recombinant protein has been developed by fusing sequence-optimized hydrophobic cell-penetrating peptide (CPP) namely advanced macromolecule transduction domain (aMTD) to deliver Cas9 directly into cells and tissues. In T7 endonuclease assay, cells treated with spontaneously induced CP-Cas9-sgRNA complex and cells pre-treated with CP-Cas9 followed by adding lipofectamine-coated sgRNA did not exhibit any activity; however, cells pre-treated with lipofectamine-coated sgRNA followed by CP-Cas9 demonstrated cleavage of target DNA. Furthermore, cells pre-treated with lipofectamine-coated sgRNA followed by various concentration of CP-Cas9 (0.025-0.1 μM) had significant activity but treating spontaneously induced CP-Cas9-sgRNA complex did not have any activity even at much higher concentration tested (0.5 μM). These results directly indicate that sgRNA must be delivered into nucleus first in order for CP-Cas9 to carry out its activity. Moreover, it is essential that not only Cas9 but sgRNA and/or dsDNA (desired nucleic acid to be integrated) to be conjugated to aMTD peptide, an efficient CPP, to utilize Cas9 system for gene editing. This method will enable Cas9 system to work in animals and ultimately in clinical setting to cure genetic diseases. Taken together, this study provides a new approach of utilizing aMTD-fused Cas9 and aMTD-conjugated sgRNA and/or dsDNA as a therapeutic gene editing module that is practical to target human genetic diseases via genome modification.

1054. Activation of Homology Directed DNA Repair Plays Key Role in CRISPR Mediated Genome Correction

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Approaches to gene therapy for the cure of inborn errors of metabolism (IEM) to complex liver disease such as hepatocellular carcinoma have produced encouraging results, however high rates of off-target effects and overall inefficacy of fully differentiated adult hepatocytes for genome manipulation pose significant challenges from applicability standpoint. In this study, we provide evidence that in utero genome editing in a mouse model of hereditary tyrosinemia type-1 (HT1) can repopulate the liver with corrected hepatocytes and provide stable cure of the disease phenotype. An extensive gene expression analysis revealed the inherent characteristics of fetal liver versus adult liver that allows for such genomic correction. Fetal liver is comprised of proliferative hepatocytes with abundant expression of components that are involved in homology directed repair (HDR) of DNA double-strand breaks, which also serves a key role during genome correction by CRISPR editing. Homology directed genome correction requires the replicative phase of an actively proliferating cell that is abundant in the fetal liver. Injury to adult liver via controlled partial hepatectomy can trigger similar cell proliferative activity and expression of HDR components, thereby allowing successful HDR mediated genome correction following CRISPR editing in adult hepatocytes. This study signifies that triggering cell proliferation and HDR activity through partial hepatectomy may serve as a tool to improve CRISPR-mediated genome correction and provide the potential for curative therapy of complex liver disease in adults.

1055. In-Silico and Experimental Detection of Personalized CRISPR Off-Targets

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The specificity and efficiency of CRISPR-Cas9 systems are highly dependent on the guide RNA (gRNA) designs. Current gRNA design tools are based on the assembled reference genomes, which could be different from the haplotypes of individual persons. The impact of this difference on CRISPR-Cas9 off-target profiles can be significant. Hence, we developed a bioinformatics and web-based tool that is capable of designing gRNAs with personalized haplotypes, aiming to achieve a more accurate prediction of the potential off-target sites. The differences in off-target profiles using reference and personalized genomes were analyzed using both *in-silico* and experimental approaches. Our results demonstrated the importance of using personalized haplotypes in gRNA design for therapeutic genome editing applications.

Both the personal genome sequence and sample cell lines were obtained from the 1000 Genomes Project. We first performed Principle Component Analysis (PCA) on 2504 individual VCFs (Chromosome 22), in which the clusters correlated well with super population labels (Fig.1A). Individual genomes and cell lines used in the downstream analysis were therefore randomly selected from different super populations for higher diversity (Fig. 1B). Sequences of 13,807 gRNAs mapping to 3452 genes were collected from Addgene pooled CRISPR gRNA library. The gRNAs were screened in 18 individuals' haplotypes and hg19 reference genome. About 28% of gRNA gained or lost perfect match sites when comparing their target site profiles between personal haplotypes and reference.

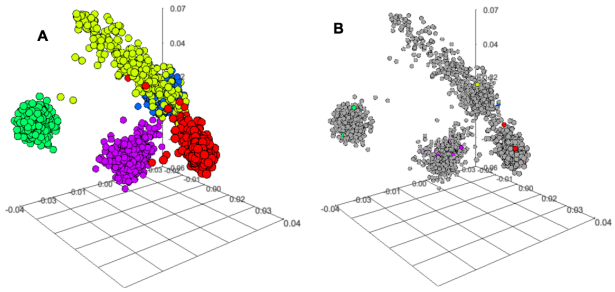

Figure 1: Principle Component Analysis (PCA) on 2504 individual VCFs (Chromosome 22) collected from the 1000 Genome Project. **A:** PCA with super-population color label. **B:** Individuals used for *in-silico* gRNA screening. Individuals from different super-populations were selected for Circle-seq analysis of CRISPR/Cas9 target site screening.

We further validated that personal genome variants could change the target site profile of gRNAs using Circle-seq, an unbiased genome-wide nuclease activity detection assay. Two gRNAs targeting HBB and CFTR, respectively, were selected for experimental whole-genome off-target screening on different individual sample cell lines. As shown in the Venn Diagram (Fig.2), while individuals shared over one hundred off-target sites, each cell line had its unique off-target profile. These unique sites appeared due to Single Nucleotide Polymorphisms (SNPs) that only existed in a subset of the populations.

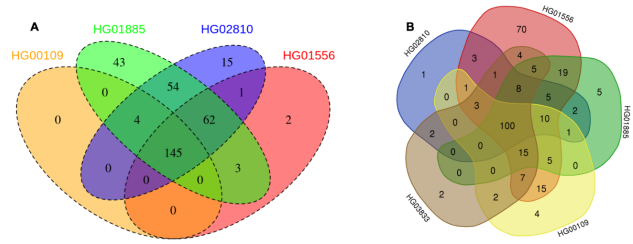

Figure 2: Venn Diagram of individuals' target site profiles with gRNA targeting HBB (**A**) and CFTR (**B**) using Circle-seq. Circle-seq was performed on Lymphoblastoid Cell Lines from the 1000 Genome Project, and only mapped sites with over 50 reads number were considered.

It is necessary to use the genetic variance of the individuals as the reference for gRNA design in personalized therapeutic genome editing. Here we introduce DePICT: Detection of Personalized in-silico CRISPR Off-targets, which performs variants-aware off-target screening on individual genomes. It could take user uploaded VCF files and process them into indexed haplotype references. After screening, off-target sites could be scored by a sequence homology dependent algorithm and ranked by risk assessments. This tool could be used to better predict off-target profiles for optimizing gRNA designs, thus facilitate personalized therapeutic genome editing.

1056. Design Considerations Toward the Unbiased Screening and Identification of Genomic Safe-Harbor Sites

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Safe and stable transgene expression is one of the primary goals for gene therapy. To this end, numerous approaches have found success through the use of viral vectors. For example, vector genomes based on AAV possess the ability to maintain high levels of expression through the formation of episomes. Unfortunately, episomes are lost over time in mitotic cells. Other vectors, such as lentivirus, achieve stability through integration within the genome, often in wildly unpredictable fashion, and sometimes disrupting essential gene functionality. Despite AAV vectors' effectiveness in initiating therapeutic gene transfer through infection of target tissues, efficient and targeted genomic integration systems are actively being explored for their potential adaptation or replacement of traditional viral vector delivery platforms. It is widely recognized that precise and targeted editing is key to safety in such systems, but the genomic context in which gene transfer takes place has not been rigorously evaluated. While several criteria have been proposed for the evaluation of genomic loci as candidate safe harbor sites (SHS), no such site has been identified. Traditionally, sites commonly employed for targeted transfer have been identified by integration of genetic elements, often viruses, within or near functional genes. These include *AAVS1*, *Rosa26*, and numerous others that are actively being explored in animal models. The explicit linkage of sites with naturally integrating elements implies an inherent degree of genomic instability, which may compromise safety if applied to the general patient population. More recent approaches have identified sites that are able to be edited efficiently, but fail to meet accepted SHS criteria, threatening their widespread application. As strategies for whole-genome screenings continue to improve, engineered nucleases such as Cas9 offer unique opportunities for SHS identification, vastly increasing access to the genome. In this study, the proposed criteria for SHS identification are evaluated in an unbiased view of the human and mouse genomes, through bioinformatic tools flexibly integrated for CRISPR/Cas9 guide-RNA library construction design. Regions of the genome are first systematically masked, based on established criteria, to avoid targeting of known genes and transcripts, functional regulatory elements, and structurally unfavorable properties such as repeat- or GC-rich DNA. Buffer sequence can be individually added, or applied collectively to masked regions, in order to balance potential impact on neighboring genomic loci with the maintenance of an effective library scale, under the inherent constraints of screening. Through location binning and conservative manipulation of masking parameters, initial designs reveal thousands of regions across all chromosomes of the human and mouse genomes that match all theoretical criteria established for genomic SHS integration. In consideration of the putative gRNA library sizes and technical limitations of screening large pools of cells, further safety restrictions were applied and sites were limited to known DNase hyper-sensitivity clusters. By utilizing targeted

gene transfer techniques that are unencumbered by homology-directed repair, our proof-of-concept library screenings first aim to establish stable transgene expression, followed by evaluation of off-target and regional effects for top hits, then re-evaluation of design parameters. With safety considerations made at each step, effective gene transfer can now be iteratively and empirically determined across multiple cell and animal models, in a manner that minimizes risks associated with the creation of new animal models and clinical gene therapy.

1057. A Gene Editing Approach to Eliminate Hepatitis B Virus Using ARCUS Meganucleases

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Hepatitis B virus (HBV) is a major, worldwide health concern with more than 250 million people chronically infected. Although treatments are available to control HBV infection, there is no cure for chronic Hepatitis B. As a curative therapeutic approach, we have developed HBV genome-specific ARCUS meganucleases to target the viral polymerase gene (ARCUS-POL). Through subsequent rounds of nuclease engineering, we have generated a highly specific and active ARCUS-POL nuclease. After mRNA transfection of cell lines carrying integrated HBV sequence, ARCUS-POL nucleases show robust cutting efficiency. In HBV-infected primary human hepatocytes transiently exposed to HBV-POL nucleases, we achieve significant reduction in covalently closed circular DNA (cccDNA) and secreted viral surface antigen (HBsAg). Furthermore, we demonstrate high level editing *in vivo* using an AAV episomal target sequence and LNP/mRNA-delivery of an ARCUS-POL nuclease. These data support an ARCUS gene editing approach for elimination of cccDNA and an HBV cure.

1058. Transient Global Inhibition of p53 in Edited Hematopoietic Stem Cells Improves Gene Correction

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Gene editing using the Cas9/sgRNA system has emerged as a promising strategy to treat monogenic disorders like sickle cell disease through site-specific gene correction of autologous hematopoietic stem cells. It is possible to obtain upwards of 50% corrected alleles in vitro using AAV6 DNA donor template delivery. However, AAV6 induces a strong p53-mediated response in CD34+ cells, leading to cell cycle arrest and apoptosis *in vitro*, and reduced engraftment in NSG mice, coinciding with a decrease in corrected alleles *in vivo*. Moreover, p53 has been implicated in regulating the DNA repair process by inhibiting HDR directly, thereby promoting NHEJ, an unwanted editing outcome. In this work, we examine the ability of the "p53 genetic suppressor element 56 (GSE56)" to temporarily inhibit p53-dependent transcription of DNA damage response elements (DDR) to prevent AAV-mediated cytotoxicity. Novel Cas9 fusion proteins containing the GSE56 element were designed and tested for their ability to increase gene correction by HDR. Preliminary work suggests that global expression of GSE56 inhibits p53 activity assessed by p21 induction, and improves the HDR/NHEJ ratio of edited PBSCs, but that localization of GSE56 to a Cas9-induced DSB has no effect on HDR.

1059. Leveraging Weak Promoters to Increase *In Vivo* Genome Editing Nuclease Specificity

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Our research group previously tested the efficacy of an engineered ARCUS nuclease for editing the genome of non-human primates (NHP). We did so by intravenously delivering adeno-associated viral (AAV) vectors that encoded a second-generation I-CreI meganuclease that was specific for a 22bp DNA sequence in the coding region of the PCSK9 gene. Vector delivery significantly reduced PCSK9 levels in rhesus macaques. While the on-target editing showed several insertions and deletions (indel%) in the PCSK9 gene, the indel% in some of the identified off-targets was significantly higher than in untreated cells. This indicates that the nuclease specificity can be improved. We aimed to reduce the off-target activity of the nuclease without compromising its strong on-target activity. We hypothesized that 1) high expression of the nuclease in transduced cells is unnecessary for achieving editing of the target DNA sequence; and 2) the off-target activity results from an accumulation of the nuclease in the cell. To reduce nuclease expression, we replaced the highly active liver-specific promoter of the thyroid hormone-binding globulin (TBG) in the AAV expressing the nuclease (AAV.TBG.nuclease) with liver-specific promoters that had lower transcriptional activity. We searched the Human Protein Atlas Database for genes that are liver-specific but transcribed at lower levels than TBG. We selected three genes based on these parameters: CCL16, CYP26A1, and SLC22A9. We produced AAV vectors expressing the ARCUS nuclease under the promoter for those genes (named AAV.CCL16, AAV. CYP26A1, and AAV.SLC22A9, respectively). As an alternative and in parallel, we aimed to develop weaker versions of the TBG promoter by keeping only 64, 113, or 140bp of the 3' end of the annotated TBG promoter (AAV.F64, AAV. F113 and AAV.F140, respectively). We administered an AAV vector expressing human PSCK9 to immunodeficient RAG KO mice. Two weeks later, mice received six AAV weak-promoter constructs. We harvested livers at two or seven weeks after administering the second vector and analyzed the DNA. Only mice treated with AAV.F113 and AAV.F140 presented a reduction in the levels of PCSK9 in serum as well as indels in the target region in the AAV.hPCSK9 transgene. The number of off-targets decreased approximately seven times in mice treated with those two constructs compared to the AAV.TBG.nuclease control group. After analyzing some of the top-ranked off-targets, we found

that in mice treated with AAV.F113 and AAV.F140, the reduction of the indel% in the off-targets was 15-40 times lower than the indel% observed in mice treated with AAV.TBG.nuclease vector. Encouraged by this reduction in the nuclease off-target activity, we assessed whether a similar reduction could be observed in NHPs. Rhesus macaques were intravenously administered with a single dose of AAV.F113. We obtained liver biopsies at d18 and d128. Next, we measured indel% in on- and off-targets, levels of rhesus PCSK9, and liver transaminases after vector administration; we will discuss the results of this NHP study. In summary, we have developed a strategy to reduce the ARCUS nuclease off-target activity in liver while retaining its on-target efficacy. Utilizing weak promoters to reduce the expression of the nuclease and, consequently, increase nuclease specificity is a novel strategy that can be applied to other nucleases as well as other genome-editing nucleases.

1060. Proteins Complex of the Fanconi Anemia Pathway as Determinant of AAV-Mediated Genomic Targeted Integration

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Recombinant AAV (rAAV) vectors are able to perform genomic targeted integration (TI) without DNA double-strand break (DSB), therefore they have been employed for genome editing in absence of nucleases. Our lab has optimized a promoterless nuclease-free approach (AAV-HR) for TI by harnessing the homologous recombination machinery upon AAV transduction (Barzel et al., Nature, 2015). In particular, the coding sequence of human factor IX (hFIX) was flanked by murine *albumin* homology regions in order to integrate, in a non-disruptive manner, the hFIX into the *albumin* locus. Thus, the chimeric mRNA produced by the host transcriptional machinery will generate the albumin protein and the human FIX. This technology, named GeneRide™, has been successfully assessed by various laboratories in various animal models for the treatment of hemophilia B and liver metabolic diseases. In order to better understand the possible mechanism by which rAAV mediates TI and possibly identify determinants that might enhance this process, we performed an unbiased genetic screen in human cells using a AAV-HR vector containing two homology regions to drive genomic targeted integration. Thus, we determined that the inhibition of the Fanconi anemia complementation group M (FANCM) protein enhanced nuclease-free TI in different cultured human cells by ~5-fold. Moreover, the combined inhibition of FANCM and other two proteins from the BLM-TOP3A-RMI (BTR) dissolvase complex, led to the enhancement of nuclease-free TI up to ~17 times. Nuclease (CRISPR/Cas9)-mediated TI was also increased by ~1.5- and ~2-fold in *FANCM* and *RMI1* knockout cells, respectively. Furthermore, knockdown of FANCM in human CD34$^+$ hematopoietic stem and progenitor cells (CD34$^+$ HSPC) increased nuclease-free TI by ~3.5-fold. FANCM and the BTR dissolvase complex have been associated with different aspects of genome maintenance and this study might expand our knowledges concerning the mechanism that governs the AAV-mediated TI while opening new perspectives toward future improvements of AAV-mediated nuclease free genome editing.

1061. Optimization of NLS Architecture to Increase Editing Activity of CRISPR-Cas12a

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Type V CRISPR-Cas12a systems provide an attractive alternative nuclease platform for specific genome editing applications. The most commonly employed Cas12a nucleases from *Acidaminococcus sp* (Asp) and *Lachnospiraceae bacterium* (Lba) recognize TTTV (V = A/G/C) protospacer adjacent motif (PAM) sequences and utilize a single ~40 nucleotide crRNA. Additionally, Cas12a cuts distally from the PAM sequences, generating 4-5 nucleotide 5'-overhangs. These properties, along with the favorable precision of the Cas12a platform, provides potential advantages of Cas12a over Cas9-based systems for therapeutic applications. With *Streptococcus pyogenes* Cas9 (SpyCas9) we found that improved nuclear localization signal (NLS) sequence composition and number can increase their activity when delivered to quiescent primary cells. We sought to translate the same improvements to Cas12a systems. We previously reported that two NLS sequences on the C-terminus of Cas12a (2xNLS-NLP-SV40) result in increased nuclear import and editing efficiency in mammalian cells and zebrafish. Here we describe further improvements to the NLS architecture of the Cas12a system that result in highly-efficient targeted mutagenesis in mammalian and primary cells. We hypothesized that substitution of the less efficient SV40 T antigen NLS with a more efficient sequence, such as the c-Myc NLS, and an increase in the number of NLS sequences from two to three would facilitate more effective nuclear entry and result in a more robust genome editing platform (Figure 1a). After construction and purification of several AspCas12a and LbaCas12a NLS variants, we characterized the activities of these proteins when delivered into human cells as ribonucleoprotein (RNP) complexes by electroporation. We found that the three C-terminal NLS sequences produced a 1.25-to-3 fold increase in editing efficiency of Cas12a in HEK293T, Jurkat, and K562 cells at subsaturating concentrations (Figure 1b). We are currently evaluating the activity and precision of these systems in primary human hematopoietic stem and progenitor cells. These new Cas12a architectures should provide improved platforms for therapeutic genome editing.

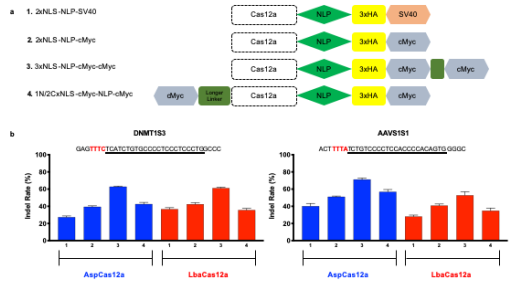

Figure 1. Optimized NLS architecture of Cas12a proteins. a) Original design of 2xNLS Cas12a in addition to new AspCas12a and LbaCas12a NLS variants. SV40 = SV40 Large T-antigen NLS, NLP = Nucleoplasmin NLS, cMyc = cMyc NLS, 3xHA = 3 x Hemagglutinin Tag. b) Cas12a RNP targeting DNMT1S3 and AAVS1S1 in HEK293T cells.

Oligonucleotide Therapeutics

1062. Conditional Deoxyribozyme-Nanoparticle Conjugates for miRNA-Triggered Gene Regulation

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Oligonucleotide-nanoparticle conjugates (ON-NPs) can knockdown gene expression transiently and effectively, making them desirable for gene regulation therapeutics. ON-NPs are constitutively active and are rapidly taken up by most cell types, and thus result in limited control over tissue- or cell type- specificity. A common targeting mechanism involves conjugating antibodies or ligands to the ON or the NP, which leads to enhanced uptake of ON-NPs in cells expressing specific surface markers. However, this strategy fails when the target cells lacks surface displayed biomarkers. Herein, we provide a novel strategy to address the problem by leveraging the natural programmability of nucleic acids to develop "pro-drug" ON-NPs that can only be activated by specific transcripts in the cytoplasm. We incorporate toehold exchange, a versatile molecular programming modality, to switch deoxyribozyme molecules attached on NPs from an inactive state to an active state by binding to a specific RNA input. As a proof-of-concept, we designed conditional deoxyribozyme nanoparticle conjugates (conditional DzNPs) that knockdown tumor necrosis factor-α (TNFα) mRNA when triggered by miR-33, as a potential therapeutic for decelerating progression of atherosclerosis. We demonstrate inhibited activity of the conditional deoxyribozyme in the absence of miR-33, and restoration of deoxyribozyme activity in the presence of the miR-33 trigger. We then optimized the preparation, configuration and toehold length of conditional DzNPs. The results demonstrate that conditional DzNPs specifically and robustly turn on in response to the miR-33 trigger in buffer. Furthermore, we demonstrate substantial uptake of conditional DzNPs and endogenous miR-33 triggered knockdown of TNFα mRNA in mouse macrophages, showing the potential of conditional gene regulation using DzNPs. We envision that conditional ON-NPs have substantial applications in programmable gene regulation by bridging native specific transcripts and emerging oligonucleotide therapeutics.

1063. ENaC Targeted Therapy for Cystic Fibrosis

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Cystic fibrosis (CF) is a rare genetic disorder caused by mutations in the cystic fibrosis transepithelial conductance regulator (CFTR) gene that leads to imbalanced Cl^- ion transport in airway epithelia. The disease condition is accompanied by increased activity of the Na^+ channel ENaC, which results in increased Na^+ absorption followed by water retention in airway epithelia that leads to thickened mucus and decreased airway surface liquid (ASL). This leads to decreased mucociliary clearance on the surface of the epithelium hence increased entrapment of infectious agents along with severe breathing difficulties. Previous trials to treat CF have aimed at increasing CFTR function where CFTR is produced but has reduced function. Potentiator molecules have been shown to improve lung function that work to improve non-functional CFTR but they do not address cases where there is no CFTR made. Such cases would require correction at the genetic level. Reducing ENaC activity presents itself as an alternative approach to treat a wider CF conditions as it could be applicable to patients with all types of CFTR mutations. Use of chemicals to block ENaC activity has been fruitful in terms of decreasing Na^+ intake and increasing airway surface hydration but their effects are very transient. We have previously shown that the use of siRNAs targeting ENaC can lead to decreased ENaC production and reduced activity for up to 8 days post transfection. In this study, we aimed to achieve higher ENaC downregulation by targeting ENaCα subunit and to investigate how it affects subcellular localisation of ENaC to improve new ways to control ENaC activity. We utilised human airway epithelial cells derived from CF patients with Δ508 mutations. Lentiviral transduction of the airway basal epithelial cells with *BMI-1* gene led to a stable *in vitro* CF model that can be maintained in culture for long term. After differentiating these cells in airway liquid interphase cultures (ALI) we have achieved fully ciliated, mucus producing airway cells with decreased CFTR activity compared to wild type cells. Transfection of these fully differentiated cells with siRNAs targeting ENaCα with nanoparticles, we have achieved 80 % downregulation of ENaCα at the transcriptional level that was accompanied by decreased ENaC activity detected by decreased change of epithelial conductance in response to ENaC blockers. Immunofluorescent staining of ENaCα has revealed increased ciliary localisation of ENaC in airway epithelial cells of CF patients compared to that of wild type cells and this ciliary localisation of ENaC has been significantly reduced in siRNA treated cells that showed decreased ENaC activity. This study shows for the first time that ENaC trans localisation into cilia is highly increased in CF and that downregulation of ENaC decreases ciliary ENaC with decreased activity. These findings could lead to the development of alternative approaches to treat CF by decreasing ENaC activity through regulation of its entry into the cilia.

1064. Precise, Non-Viral, CAR-T Engineering with CRISPR sgRNA and ssDNA

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CAR-T cell therapy utilizes engineered T cells expressing tumor antigen-specific CARs for treating cancer. T cell engineering has traditionally been done with viral vectors, but this approach has limitations including knock-in (KI) size restrictions and random insertion. Direct delivery of CRISPR Cas9 protein, synthetic gRNA, and donor DNA has the benefit of both precise CAR KI and simultaneous knockout (KO), and without the need for a viral vector. However, synthesizing sgRNA and long DNA donor template for CRISPR based HDR gene KI has been challenging. GenScript has develop several tools to overcome these challenges. We will show data on how GenScript can efficiently manufacture sgRNA and long single-stranded DNA (ssDNA or ssODN) for precise CAR KI in T cells. With this upgraded capability and recent advances in non-viral delivery of CRISPR components, it will become increasingly possible to efficiently and precisely engineer T cells with minimal off-target impact.

1065. Extracellular Vesicles for Monitoring Therapeutic Antisense Oligonucleotide Drug Activity in Duchenne Muscular Dystrophy

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Introduction: Duchenne muscular dystrophy (DMD) results from frame-shifting mutations in the dystrophin gene that lead to absence of dystrophin protein expression in skeletal muscle. One therapeutic strategy for treatment of DMD involves the use of antisense oligonucleotides (ASOs) to induce removal, or "skipping" of certain exons to restore the reading frame and produce a partially functional dystrophin protein. In clinical trials of ASOs in DMD, mRNA splicing outcomes in serial muscle tissue biopsies are used to monitor therapeutic exon skipping drug activity. However, tissue biopsies are invasive, impractical for long term monitoring of therapeutic response, and require general anesthesia in children. Non-invasive monitoring of ASO drug activity is needed. Eteplirsen (ExonDys 51) is an FDA-approved therapeutic ASO that is designed to induce the skipping of dystrophin exon 51. Extracellular vesicles (EVs) in urine contain mRNA splice products that can serve as powerful biomarkers of disease status in patients with myotonic dystrophy type 1 (Antoury, et al., 2018). Urine also contains dystrophin mRNAs that can reveal cryptic splice sites (Antoury, et al., 2019) or mutation-specific deletions, and can confirm exon-skipping activity of the ASO drug eteplirsen (Antoury, et al., 2018). The analytic and clinical validity of EV splice events for monitoring ASO drug activity is unknown. **Objective**: To develop EVs for monitoring therapeutic ASO drug activity in DMD. **Methods**: We collected urine samples from four DMD patients on commercial eteplirsen treatment, measured EV diameter and concentration using nanoparticle tracking analysis (NanoSight), isolated mRNA from EVs and urine cells, examined RNA size distribution using capillary gel electrophoresis, and quantified dystrophin exon 51 inclusion using droplet digital PCR (ddPCR). Two of the DMD patients have a deletion of exons 45 - 50 and two have a deletion of exon 52. Urine samples from individuals with DM1 and individuals without known muscular dystrophy served as controls. To examine the downstream effects of therapeutic exon skipping on the EV transcriptome, we also developed ddPCR assays for monitoring therapeutic exon 23 skipping in urine and tissues of the mdx mouse model of DMD and in EVs released from cultured myotubes into serum-free tissue culture medium. **Results**: EV diameter and RNA size distribution appeared similar in DMD and non-DMD controls. Exon 51 inclusion ranged from 10% to 69% of dystrophin transcripts (31% to 90% skipped) in EVs and 2% to 52% of dystrophin transcripts (48% to 98% skipped) in urine cells. In one individual, exon 51 skipping in urine was stable at about 90% in EVs and in urine cells over the course of six months. Using ddPCR, we quantified exon 23 inclusion in urine and tissues of mdx mice and in EVs collected from myotube culture medium. **Conclusions**: Urine provides a renewable source of mRNA splice products with the potential to monitor therapeutic response in DMD. Further examination of EVs as non-invasive measurements of exon skipping may facilitate the development of newer and better exon-skipping drugs for DMD, reduce the need for muscle tissue biopsies, and enable longitudinal monitoring during the course of treatment. **Support**: United States Department of Defense; Muscular Dystrophy Association.

1066. Solving Challenges in Manufacturing of mRNA Drug Products: Rapid Development and Scale-Up of a Model mRNA Therapeutic Encoding Erythropoietin

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Messenger RNA (mRNA) is a platform technology for enabling transformative medicines, and it is poised to become a major class of drugs in the next era of biopharmaceuticals. Lipid nanoparticle (LNP) technology has emerged as the preferred approach for encapsulation and in vivo delivery of mRNA. The therapeutic benefit of mRNA LNPs is under evaluation for immunotherapy applications, rapid response vaccines (e.g. influenza pandemic response) and protein replacement therapies, among others. Formulation development and scalable manufacturing of these mRNA drug products are current challenges in the field that need to be overcome to realize the full potential of mRNA. In this work, we showcase the rapid development and scale-up a model mRNA drug encoding for the therapeutic protein erythropoietin (EPO). The LNP formulations selected were the MC3 clinical benchmark, D-Lin-MC3-DMA/DSPC/Cholesterol/DMG-PEG (50:10:38.5:1.5 mol%) and GenVoy-ILM ionizable lipid mix. LNPs encapsulating human recombinant EPO-encoded mRNA were consistently manufactured using a novel microfluidics platform (NxGen) at various lipid to mRNA (N/P) ratios, batch sizes (mL-L) and throughputs (up to 12 L/h). The LNPs were downstream processed using tangential flow filtration (TFF) and terminal sterile filtration, resulting in the final drug product. The LNPs were then i.v. administered to C57BL/6 mice

at 1 mg/kg mRNA equivalent dose and evaluated for EPO production (ELISA) and therapeutic efficacy (hematocrit). Therapeutic levels of EPO were produced following delivery of both GenVoy-ILM and the MC3 benchmark LNPs. Evaluation of the hematocrit conducted 7 days following administration of the LNPs showed similar RBC production between GenVoy-ILM and the MC3 benchmark. Further, cytokine levels 6 and 24 h post administration the LNPs was compared to assess the safety profile of GenVoy-ILM and the MC3 benchmark. We have demonstrated the generation of therapeutic levels of human recombinant EPO in mice using EPO-encoded mRNA LNPs. These LNPs were rapidly developed and scaled-up by leveraging modular technologies for lipid formulations, microfluidic manufacturing and downstream processing. GenVoy-ILM provides comparable performance to the MC3 benchmark, can be used as reference formulation for mRNA drug development, and is accessible for users without prior formulation expertise. NxGen is a disruptive technology that provides reproducible, high-quality production of LNPs. NxGen can scale from mL/min to L/h production rates, allowing for mRNA LNP drug candidates to developed cost-effectively at small-scale, and then rapidly scaled-up for GMP manufacturing to meet clinical requirements. These technologies can help to alleviate key challenges in the field of nucleic acid therapeutics

1067. Myostatin - A Circulating Biomarker Correlating with Disease in Myotubular Myopathy Mice and Patients

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Myotubular myopathy, also called X-linked centronuclear myopathy (XL-CNM), is a severe congenital disease targeted for therapeutic trials. To date, biomarkers to monitor disease progression and therapy efficacy are lacking. The *Mtm1*$^{-/y}$ mouse is a faithful model for XL-CNM, due to myotubularin 1 (*MTM1*) loss-of-function mutations. Using both an unbiased approach (RNAseq) and a directed approach (RT-qPCR and protein level), we identify *Mstn* levels are decreased in *Mtm1*$^{-/y}$ muscle, leading to low levels of myostatin in both plasma and skeletal muscles. Myostatin (*Mstn*,*Gdf8*) is a protein released by myocytes and inhibiting muscle growth and differentiation. Decreasing *Dnm2* by genetic cross with *Dnm2*$^{+/-}$ mice or by antisense oligonucleotides blocked or postponed disease progression and resulted in an increase in circulating myostatin. In addition plasma myostatin levels responded rapidly to treatment, and inversely correlated with disease severity and *Dnm2* mRNA levels in muscles. Altered *Mstn* levels were associated with a generalized disruption of the myostatin pathway in mice. Importantly, in two different forms of CNM we identified reduced circulating myostatin levels in plasma from patients. This provides evidence of a blood-based biomarker that may be used to monitor disease state in XL-CNM mice and patients, and supports monitoring circulating myostatin during clinical trials for myotubular myopathy.

1068. Novel Strategies to Validate Oligonucleotide Pharmacokinetic Methods in Compliance with Updated Regulatory Guidelines

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The research and development of oligonucleotide analytes as pharmaceutical compounds for the treatment of a wide range of disease has been ongoing in academia and the pharmaceutical industry for well over thirty years. Due to the recent approvals of numerous oligonucleotide-based modalities, there has been increased interest in the development of oligonucleotides to treat a wide array of diseases such as cancer, viral infections, and rare or genetic diseases. With the wide and ever-increasing array of oligonucleotide modalities and the advances made in chemical modifications and drug delivery, the appropriate assay platform should be selected for pharmacokinetic (PK) profiling, drug metabolism, and assessment of anatomical compound distribution. Among the favored platforms for PK analysis are hybridization enzyme-linked immunosorbent assays (hELISA) and hybridization electrochemiluminescent (hECL) formats. Given the unique nature of oligonucleotides, as well as the recent updated FDA regulatory guidance that altered the experimental design of core accuracy and precision (A&P), novel strategies that update previously used methods must be put into place to ensure that PK methods are validated to accurately and precisely measure oligonucleotide concentrations. Among the many updates that have been addressed are appropriate strategies to assess fresh and frozen calibrators and quality controls (QC) for core A&P, specificity and selectivity for the parent compound, assessment of metabolite quantitation and interference, and drug verification (i.e., quantitation of total full-length ASO) when the oligonucleotide is conjugated to a specific delivery moiety. In collaboration with Ionis Pharmaceuticals, PPD has implemented a strategy to assess fresh and frozen calibrators and QCs during method development. In validation, we have adopted the use of fresh calibrators and QCs in at least one core A&P run with fresh calibrators and frozen QCs utilized for the remainder of core A&P. One of the drawbacks of using these assay formats is that they may not discriminate between quantitation of full-length oligonucleotides and longer metabolites (e.g., N-1). Due to this, it is imperative to determine during assay development and validation if the hELISA or hECL method is selective for the parent compound or if metabolites potentially quantitate or interfere with ability of the parent compound to quantitate appropriately when present. Further, we have implemented drug verification strategies to assess the ability of the method to appropriately quantitate both the unconjugated and fully conjugated (such as N-Acetylgalactosamine) oligonucleotides indiscriminately. Finally, we have updated our oligonucleotide PK

method validation strategy to assess all experiments set forth by the recently released FDA guidance aimed at addressing assay specificity or selectivity for the full-length parent compound at the lower limit of quantitation rather than the low QC level, including hemolysis, lipemia, metabolite cross-reactivity and drug verification. With the above validation strategies implemented, we have successfully validated multiple hELISA and hECL oligonucleotide PK methods that fulfill the validation requirements and are suitable to measure full-length oligonucleotide compounds in a wide array of matrices.

1069. AAV.U7 Induced Exon Skipping for a Mutational Hotspot (~6%) of the *DMD* Gene Results in Efficient Exon Skipping, Protein Restoration and Force Improvement

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Mutations in the DMD gene cause two disorders, the severe Duchenne muscular dystrophy (DMD), associated with no dystrophin expression and the milder Becker muscular dystrophy (BMD) associated with the production of an internally truncated but semi-functional protein. Genotype-phenotype correlation of DMD and BMD prompted a promising therapeutic strategy named exon skipping that intends to convert a DMD to a BMD phenotype. This strategy makes use of antisense oligonucleotides (AONs) to hide exon definition. This should transform an out-of-frame *DMD* reading frame into an in-frame one, thus leading into expression of an internally truncated but functional protein. AONs are promising but have two major drawbacks 1. Stability and required repeated injections; 2. limited penetration in heart upon systemic administration, one of the main causes of premature death in DMD patients. Alternatively, an antisense sequence can be carried by a small nuclear RNA (in this study, U7snRNA) and delivered using a viral vector such as adeno-associated virus (AAV). This approach (AAV.U7) has been demonstrated by several groups including ours, to overcome AON's drawbacks. In this study, we focused on skipping of exon 44, a mutational hotspot of the *DMD* gene as its skipping could be beneficial to ~6% of patients. Our objective was to evaluate the efficiency of an AAV.U7 exon skipping strategy in this hotspot mutation both *in vitro* and *in vivo*. For *in vitro* studies, we used skin punches from 3 DMD patients and transdifferentiated them into myotubes. Following transduction of 6 different AAV.U7 constructs, we evaluated exon skipping at the mRNA level using RT-PCR and western blot to evaluate protein restoration. For *in vivo* studies, we used a new humanized DMD mouse model that is missing human exon 45. Its absence disrupts the reading frame of hDMD. This new model (referred to as hDMD/mdx del45 mice) does not express neither mouse nor human dystrophins and presents a dystrophic muscle pathology in multiple muscles. We evaluated exon skipping at the mRNA level using RT-PCR and protein restoration using western blot and immunostaining. Restoration of dystrophin associated proteins by immunostaining was also looked at. Several tests which look at muscle function were assessed in this mouse model. Following transduction of 3 DMD transdifferentiated patient cells, we showed that our constructs efficiently mediated exon 44 skipping (at least ≥50%) and induced expression of a slightly truncated dystrophin. *In vivo,* some of these vectors, 1- and 3-months post intramuscular injection, reveal efficient exon 44 skipping (at least ≥50%), expression of a truncated dystrophin (at least ≥50%) and associated proteins. Muscle strength was tested by measuring tibialis anterior specific force and eccentric contraction. Both test demonstrated muscle improvement (by ~50% compared to untreated mice). Based on our previous success with a different AAV.U7 program for DMD, we are currently conducting minimal efficacious dose (MED) studies to determine which amount of vector is required for systemic injections. Altogether, we identified one lead candidate that can induce efficient *DMD* exon 44 skipping, resulting into dystrophin production and muscle strength improvement. This AAV.U7-exon44skipping vector could help ~6% of DMD patients.

1070. Transient CD40L Immune Blockade Prevents Development of Anti-Drug Antibodies Following *In Vivo* Delivery of Xenogenic Human IgG Plasmid DNA-Encoded Antibodies (DMAbs) in Mice

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Synthetic non-viral nucleic acids (plasmid DNA and mRNA) and viral adeno-associated virus vectors (AAV) are rapidly advancing platforms for gene-encoded *in vivo* delivery of monoclonal antibody (mAb) biologics. Like recombinant biologics, the development of anti-drug antibodies (ADA) against mAbs can dramatically impact biologic efficacy, resulting in lower *in vivo* potency and duration, as well as impaired re-administration. Utilizing a synthetic DNA-encoded mAb (DMAb) platform we showed that CD4+ and CD8+ T cell depletion prevents the development of ADA. T cell depletion positively enhances long-term expression of xenogenic human IgG DMAbs in a mouse host, resulting in expression lasting >365 days. However, T cell depletion is not ideal for clinical treatment regimens and alternative solutions to overcome ADA are important for the translation of gene-encoded mAb platforms. To address this, we investigated transient blockade of early innate immune signals in parallel with DMAb delivery including strategies that target early co-stimulation and downstream intracellular signaling pathways. Mice (n=5 mice/group) were administered DMAb in combination with rapamycin, CTLA4-Ig, or anti-CD40L (anti-CD154). Daily administration of rapamycin and CTLA4-Ig both delayed development of ADA, however mouse anti-human DMAb antibodies developed when treatment was stopped. Interestingly, transient blockade mediated by anti-CD40L resulted in suppression of ADA. A single administration provided enhanced human IgG1 DMAb expression with extended duration in circulation of >365 days, similar

to T cell depletion. These results were consistent for DMAbs targeting *Pseudomonas aeruginosa*, influenza virus, and HIV. Taken together, our results demonstrate that CD40L blockade is a simple approach with potential applications for clinical translation. This is an important step to mask gene-encoded antibodies from immune surveillance, with potential application for other gene therapy products.

1071. Exploring the Effects of Intrastriatal AAV5-miHTT Lowering Therapy on Transcriptional Dysregulation, MRS Signal, and Mutant Huntingtin Levels in the Q175FDN Mouse Model of HD

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Introduction: Huntingtin (HTT)-lowering therapies hold great promise to slow-down or halt neurodegeneration in Huntington Disease (HD). We have developed an engineered microRNA targeting human HTT, delivered via adeno-associated viral vector serotype 5 (AAV5-miHTT), leading to efficient HTT-lowering *in vitro* and *in vivo* in rodent models. Here, we explored the use of striatal RNA sequencing (RNAseq) and non-invasive magnetic resonance spectroscopy (MRS) as translational efficacy measures for the assessment of HTT-lowering therapies in HD. **Methods:** Three-month-old homozygous Q175FDN HD mice were injected bilaterally into the striatum with formulation buffer (sham), low (5.2×10^9 gc/mouse) or high (1.3×10^{11} gc/mouse) doses of AAV5-miHTT. Wild-type (WT) mice injected with formulation buffer served as controls. T1-weighted structural MR imaging (MRI) and striatal MRS were performed 3 months after injection, and shortly afterwards the animals were sacrificed to collect brain tissue for protein and RNA. **Results:** Decreased total N-acetylaspartate (tNAA, neuronal integrity marker) and increased myo-inositol (mI, gliosis marker) levels were found in Q175FDN sham-treated mice with respect to WT controls, similarly to previous observations in the putamen of HD patients. These findings were reversed in the Q175FDN high-dose AAV5-miHTT treated mice with higher levels of tNAA and reduced levels of mI compared to sham-treated Q175FDN mice. Dose-dependent changes in AAV5 vector DNA level, miHTT expression and HTT protein were observed in striatum and cortex. Correlations were shown between tNAA MRS levels and HTT protein levels in striatum and cortex, suggesting a direct relationship between HTT lowering and the striatal MRS signal. Striatal transcriptional analysis using RNA seq revealed mHTT induced changes that were partially reversed by HTT lowering. **Conclusions:** Striatal MRS analysis suggests a restoration of neuronal function and partial reversal of gliosis and RNAseq analysis shows a reversal of transcriptional dysregulation following AAV5-miHTT treatment. The results of this study support the use of MRS in HTT-lowering clinical trials and strengthen the therapeutic potential of AAV5-miHTT in reversing striatal dysfunction in HD.

1072. Extracellular Vesicles - A Trojan Horse for Therapeutic Agent Delivery

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Introduction- Extracellular vesicles (EVs) may prove to be one of the optimal payload carriers for therapeutic agents. While they travel through the extracellular space, the EV's lipid membrane layer shields their luminal cargo from deleterious external factors. When autologous EVs are used to protect this therapeutic cargo, little immunogenic effects are expected compared to viral vectors and artificial structures, such as liposomes. Their usage is potentially manifold, and they are ubiquitously present in all body tissues and fluids. The key is to develop a manageable EV loading agent for adoptive transfer therapies. Methods - To exploit the unique properties of EVs, highly positively charged proteins were used to load them with multiple biomolecules, such as a Cas9 protein or Dicer substrate dsRNA as a functional payload and to improve their apparently inadequate natural ability to deposit cargo into the cytoplasm of recipient cells. Results - Highly positively charged proteins can associate with and/or diffuse through a phospholipid bilayer (Thompson et al. 2012). When these kinds of charged proteins are mixed with isolated EVs in vitro, they are loaded into the EVs. The positive charge of the protein has the advantage that it can associate with negatively charged agents, such as RNA species, and aids the associated molecule to also incorporate into the EV. Moreover, the positive charge of the protein helps with cargo delivery, and thus overcoming the bottleneck of the EV's cargo to escape the endosome post-uptake in a recipient cell. Self-quenching fluorescent lipid dyes demonstrated that discharge of the highly positive EV cargo into the cytoplasm is concomitant with lipid mixing between the membrane of EVs and the membrane of the recipient cell. When eGFP-expressing microglia were exposed to EVs loaded with a Dicer substrate dsRNA able to silence eGFP via the positively charged protein, the uptake of Dicer substrate dsRNA was concomitant with a decrease in eGFP expression in the microglia. A similar result was achieved when EVs were loaded with Cas9 protein conjugated to the highly positively charged protein. Post-uptake of these Cas9-loaded EVs, microglia expressing anti-eGFP sgRNA (single guide RNA) lead to decreased eGFP expression.Conclusion - Our EV delivery technology has the capability of delivering multiple biomolecules, such as protein and RNA cargo and demonstrates post-uptake of the EV functionality of the EV delivered cargo in the recipient cell.

1073. How ERbeta Play Vital Role in the Process of Bone Metabolism

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It is well known that estrogen secretion deficient is the main reason causes postmenopausal osteoporosis. Research indicated that estrogen receptor alpha (ERα) and ER beta (ERβ) antagonize each other's action in many tissues and cell lines, but little knowing in bone tissue and bone-related cell line. In this study, we constructed an ERβ knockdown bone mesenchymal stem cell (BMSC) model, evaluating the alternation of osteogenic differentiation of BMSC, as well as using RNA-Seq to detect the different expression miRNA in this process. The results

showed that the osteogenic differentiation ability is weakened in ERβ knockdown BMSC, together with a series of miRNA expression alternation, including 27 up-regulated, and 69 down-regulated. Then we combined the results that we previously reported about the differently expressed miRNA in ERα knockdown BMSC, there are 11 miRNA different expressed in both ERα and ERβ knockdown group, including 7 miRNAs (miR-351-5p, miR-503-3p, miR-293-5p, miR-193-5p, miR-292-5p, miR-331-5p, miR-210-3p) both down-regulated, and 4 miRNAs (miR-6325, miR-10a-5p, miR-99a-5p, miR-139-5p) behaved the opposite trend, we analyzed the GO annotation of those four miRNA and involved signaling, it revealed that miR-99a-5p involved ER signaling transduction; furthermore, the existing research indicated it closely related breast carcinoma. Therefore, we hypothesis that miR-99a-3p is the key regulator of the antagonistic action between ERα and ERβ in BMSC. This research explains the mechanism relationship about ERα and ERβ synergistic and synergistic effects in miRNA level, and provides a new vision about the therapeutic and prevents estrogen receptor-related bone disease.

1074. Targeted Delivery of Oligonucleotide Therapeutics to Muscle Demonstrates Potential to Treat Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disease caused by mutations in the dystrophin gene that leads to disruption in the mRNA reading frame, resulting in lack of dystrophin protein production. It is the most common pediatric onset form of muscular dystrophy and is characterized by progressive muscle degeneration and weakness leading to early mortality as a result of respiratory and cardiac failure. Antisense oligonucleotides (ASOs) are promising therapeutics that can alter the mRNA reading frame through exon skipping to restore a more complete, functional dystrophin protein. However, the efficacy of current exon skipping ASO therapies is limited by poor muscle tissue uptake and biodistribution. Targeted delivery of ASOs to muscle tissue could improve biodistribution and ultimately benefit patients. The FORCE platform, a receptor-mediated targeting technology developed by Dyne Therapeutics, improves the delivery of therapeutic molecules to skeletal, cardiac and smooth muscles. Dyne Therapeutics utilized the *mdx* mouse, a well-established animal model which mimics key aspects of DMD pathophysiology in humans, to evaluate the therapeutic potential of a proprietary antibody-ASO therapeutic. A single dose of the FORCE DMD therapeutic in the *mdx* model significantly increased exon skipping and dystrophin protein production in muscle. More importantly, these changes improved muscle function as measured by multiple functional assessments. These data support the therapeutic potential of muscle-targeted oligonucleotides for the treatment of DMD.

1075. Mild Innate Immune Activation Overrides Efficient Nanoparticle-Mediated RNA Delivery

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Targeted systemic mRNA delivery remains challenging, in large part because the physiologicalfactors that influence delivery in vivo remain underexplored. For example, mRNA delivered bylipid nanoparticles (LNPs) is being considered to treat inflammation, but whether inflammationitself can change LNP delivery remains unclear. Relationships between immunity, endocytosis,and mRNA translation led us to hypothesize that TLR4 activation reduced LNP-mediated mRNAdelivery. After using low dose lipopolysaccharide (LPS) to activate TLR4, we observed a robustreduction in LNP-mediated mRNA delivery, both in mice and cells. By quantifying LNP uptake,endosomal escape, and mRNA translation with and without TLR4 activation, we found that TLR4activation blocked mRNA translation in all tested cell types, without reducing LNP uptake. Notably,we found that inhibiting TLR4 or its downstream effector PKR improved mRNA delivery. Thediscrepant effects of TLR4 on (i) LNP uptake and (ii) translation suggests TLR4 activation can'override' LNP targeting, even after mRNA is delivered into target cells. Given near-future clinicaltrials using mRNA to modulate inflammation, this highlights the need to understand inflammatorysignaling in on- and off-target cells. More generally, this suggests an LNP which delivers mRNAto one inflammatory disease may not deliver mRNA to another.

1076. Development of Novel ASO Therapies to Treat Neurodevelopmental Disorders

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Neuro-developmental disorders (NDD), such as STXBP1-associated disorders, are often caused by genetic mutations that lead to haploinsufficiency, or a loss-of-gene function. Mutations that cause haploinsufficiency of neurodevelopmental genes affect over 200,000 births each year, and commonly result in epilepsy and intellectual disability. While symptomatic treatments exist, for most conditions, there are no treatments that directly correct the reduced levels of the haploinsufficient gene. Recent research by our group and others has revealed that many NDD-causing genes like STXBP1 are normally under active repression by microRNAs. This raises the possibility of removing this microRNA "brake" to increase gene expression and help restore normal function in NDD haploinsufficiency. We hypothesize that the expression of downregulated genes can be restored through steric-blocking antisense oligonucleotides (ASO) that specifically disrupt gene repression by microRNAs. Our bioinformatic analyses and transcriptome-wide profiling of human brain tissue predicted that common NDD-causing genes including STXBP1 are actively repressed by miRs, such as miR-218, miR-424 and miR-338 in the brain. We tested the hypothesis that ASOs could be used for NDDs by first assessing if STXBP1 was under miR regulation using lentiviral vectors

to deliver miR inhibitors to SH-SY5Y cells. Immunoblotting showed that anti-miR-218 triggered an ~2 fold upregulation of STXBP1. MiR regulation of STXBP1 was confirmed by CRISPR/Cas9 deletion of miR-218 in SH-SY5Y cells, with gene deletion again resulting in STXBP1 upregulation. Based on the above results, we designed 3 blocking ASOs to walk each of our identified target sites in STXBP1 and tested their function following transfection into SH-SY5Y cells. Two blocking ASOs upregulated STXBP1 protein levels by more than 50%, with miR-218 more robust followed by miR-424; ASOs designed against the miR-338 site were ineffective. We next developed a high-throughput, light-based STXBP1 3' UTR- reporter assay for future ASO screening. The validity of the reporter was assessed using the lenti-expressed antagomiRs targeting miR-218 or miR-424, with each mediating reporter repression.These studies provide strong support for the use of ASOs as a non-viral therapeutic approach toward correcting haploinsufficiency in leading genetic etiologies of neurodevelopmental disorders.

1077. Delivery of siRNA Targeting Epidermal Growth Factor Receptor (EGFR) as a Therapeutic Strategy Against Non-Small-Cell Lung Cancer (NSCLC)

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Background: Non-small-cell lung cancer (NSCLC) is one of the prominent causes of mortality worldwide. The expression of epidermal growth factor receptor (EGFR) was overwhelming in NSCLC patients. However, conventional treatments such as chemotherapy and radiotherapy lack specificity and provoke side effects. EGFR - tyrosine kinase inhibitor (TKI) is the first-line therapy for advanced NSCLC. Nevertheless, some patients develop resistance and become irresponsive to TKI. Recently, small interfering RNA (siRNA) has been recognized as a new class of therapeutics for a wide range of diseases including lung cancer. siRNA could manipulate specific gene expression via RNA interference (RNAi) by cleaving target mRNA. Here, we aimed to use siRNA to knockdown EGFR expression in NSCLC cells and to compare the transfection efficiency between Lipofectamine 2000 (a commercial transfection agent) and Peptide A (a synthetic peptide developed in our research group). Moreover, we also aimed to evaluate whether the use of siRNA approach, together with first generation TKI, erlotinib could potentiate their therapeutic effects. **Methods:** Three human NSCLC cell lines harbouring different EGFR status were employed, namely H292 (wild-type EGFR), HCC827 (EGFR with exon 19 del mutation) and H1975 (EGFR with L858R/T790M double mutation). The cells were transfected with lipoplexes (Lipofectamine 2000/siRNA complexes) or polyplexes (Peptide A/siRNA complexes) containing 50, 75 or 100 nM pooled EGFR siRNA or scramble siRNA for 48 h. Western blot was performed to evaluate the protein expressions of total EGFR. For combination treatment of EGFR siRNA and erlotinib, the cells were transfected with 100 nM pooled EGFR siRNA using Peptide A. After 24 h, the cells were treated with a range of erlotinib concentration, from 10^{-10} to 10^{-5} M, for additional 24 h. Cell viability was subsequently assessed by MTT assay. **Results:** Western blot analysis showed that the level of EGFR was significantly inhibited by siRNA in all three cell lines investigated. Regarding the transfection efficiency, Peptide A was more effective than Lipofectamine 2000 in H1975, followed by H292 and HCC827 cells, with EGFR knockdown of 90%, 70% and 60% achieved respectively when 100 nM siRNA was used. In addition, cells transfected with EGFR siRNA followed by erlotinib treatment exhibited a lower cell viability than cells treated with erlotinib alone, suggesting a potential potentiating effect of EGFR siRNA on erlotinib. EGFR siRNA potentiated erlotinib only at a low concentration of erlotinib (10^{-10} M) in HCC827 cells. On the other hand, the effect sustained until a higher concentration of erlotinib (10^{-6} M) in H1975 cells. **Conclusion:** Our data reveal that EGFR siRNA could significantly knockdown the expression of EGFR in NSCLC cells and Peptide A was more effective than Lipofectamine 2000. Besides, EGFR siRNA could restore erlotinib sensitivity and potentiate its cytotoxicity effects. Further studies on the effects of EGFR siRNA with second or third generation of TKI as an alternative treatment strategy against NSCLC are warranted.

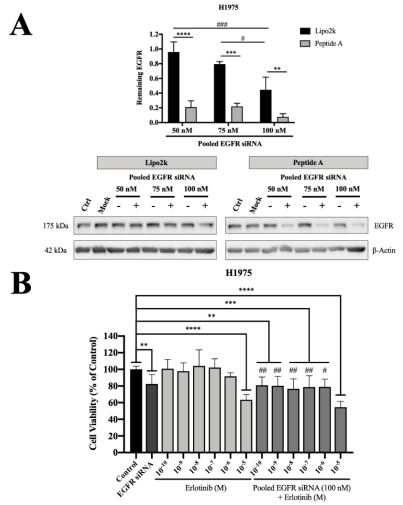

Fig. A. Pooled EGFR siRNA transfection efficiency using Lipofectamine 2000 and Peptide A in H1975 cells. The data were analysed by two-way ANOVA followed by Turkey post-hoc test. #p < 0.05, ####p < 0.001 for comparisons of transfection efficiency using the same transfection agent. **p < 0.01, ***p < 0.001, ****p < 0.0001 for comparisons of transfection efficiency among the two transfection agents under the same concentration (n=3).
Fig. B. Cell viability of H1975 cells at Erlotinib concentration ranging from 10^{-10} M to 10^{-5}M with 24-h pre-treatment of 100 nM pooled EGFR siRNA delivered by Peptide A, or without EGFR siRNA transfection. The data were analysed by one-way ANOVA followed by Dunnett's multiple comparison test when comparing cell viability at various Erlotinib concentrations with control within the same type of treatment, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; and one-way ANOVA followed by Tukey's multiple comparison test when comparing cell viability between treatments at the same Erlotinib concentration, #p< 0.05, ##p< 0.01 (n=3).

1078. Selection of Aptamers Against Chemokine Receptors and characterization of Their Potential Functions in Signaling

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There are over 20 Chemokine receptors, which are cell surface-expressed receptors that respond to chemokines. While normally expressed on healthy tissues, these receptors are also aberrantly expressed on cancer cells. CXCR4 (C-X-C chemokine Receptor type 4) is a Chemokine receptor that is expressed in various cancers, among them are breast and prostate cancers. High expression level of CXCR4 in breast cancer is an indicator for metastatic potential and poor prognosis. Another chemokine receptor, CXCR7, is found in prostate cancers as well. Furthermore, it has been found that CXCR4 and CXCR7 can form heterodimers that can enhance cell migration *in vitro*. Aptamers are short oligonucleotides with high affinity for their targets; they also

possess the ability to transport therapeutic RNA into cells - these dual functions make them ideal candidates for developing targeted therapy for chemokine receptor-overexpressing cancers. Currently, we are selecting RNA aptamers against CXCR4 and CXCR7 through SELEX (Systemic Evolution of Ligands by Exponential enrichment). In particular, we are selecting for aptamers that will bind with high specificity to: (1) CXCR4, (2) CXCR7 and (3) the CXCR4/CXCR7 heterodimer - these candidates will be characterized for functions using assays for Chemokine receptor signaling and the wound healing assay. We performed five rounds of selection to enrich for aptamers that are specific to CXCR4, CXCR7 or the CXCR4/CXCR7 heterodimers. Afterwards, we proceed to two separate selections to further enrich aptamers towards CXCR4 alone and CXCR7 alone. Methods to further enrich for aptamers to the CXCR4/CXCR7 heterodimer will be carried out concurrently. Throughout the selection process, we performed high throughput sequencing and employed bioinformatics approaches to identify aptamer candidates for affinity testing. Apart from selecting aptamers with high binding affinity, we are also exploring the functional properties of our aptamers. To the best of our knowledge, there is little study about their potential roles in receptor signaling, so we are testing our aptamer candidates with a particular focus on this aspect of their function. We are using two platforms for this purpose: (1) the Presto-Tango assay, for measuring β-arrestin recruitment and (2) the Ca^{2+} Influx assay. We are also using the wound healing and cell migration assays to assess the ability of our aptamers to reduce the invasiveness of cancer cells. With the functional characterizations mentioned above, we hope to deepen the therapeutic potentials of aptamers.

1079. Antitumor Immunotherapy of Synthetic Stem-Loop RNA (sl-RNA) Derived from Sendai Virus Genome

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We previously reported that inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) particles called HVJ-envelope (HVJ-E) have multiple-anti-tumor activities. The activities are activation of anti-tumor immunity through NK cell activation, generation of CTL against cancers and inactivation of regulatory T cells. Additionally, cancer selective apoptosis by induction of proapoptotic genes such as TRAIL and Noxa in various human cancer cells but not in normal cells. Most of these anti-tumor activities are conducted by RNA genome fragments of HVJ-E via RIG-I/MAVS (retinoic-acid inducible gene-I, mitochondrial antiviral signaling protein) signal pathway. Among various Sendai virus strains, Sendai virus Cantell strain showed the highest production of IFN-β in dendritic cells and the strongest DC maturation and we found that DI (Defective Interfering) particles of Cantell strain resulted in those immunostimulatory activities. We investigated RNA molecules in Cantell HVJ DI particles, which have no replication activities. The Cantell stain HVJ DI RNA has complementary termini (approximately 100nt) and exhibits the highest binding affinity to RIG-I. DI RNA genome (544 base) was isolated and transferred to human prostate cancer cell PC3. The DI RNA induced higher expression level of those apoptosis-related proteins such as Noxa and TRAIL and more cancer cell death than whole-genome RNA (approximately 15 kb) of complete Cantell strain without DI particles. However, 544 base RNA is impossible to be chemically synthesized. Then, we developed much smaller RNA fragment using the DI particle RNA as a template, and named stem-loop RNA (sl-RNA). sl-RNA has 25-base double strand stem part and 25-base single strand loop part in the secondary RNA fragment structure, which can be synthesized. Moreover, to deliver the RNA to tumor tissue efficiently, we developed pyro-drive jet injector (PJI). The PJI is a novel injector system capable of injection depth adjustment. The sl-RNA was injected to B16F10 (mouse melanoma) tumor by PJI three times. The tumor growth was strongly suppressed in sl-RNA injected group. Chemokine and cytokine array revealed that MCP-2, IP-10, RANTES and MIP-2 secretions were increased in the B16F10 tumor tissues after sl-RNA injections. Additionally, macrophage infiltrations and macrophage polarization to M1 (anti-tumorigenic) were observed in the sl-RNA injected B16F10 tumor sections by F4/80 and NOS-2 immunostaining. These findings provide a novel nucleic acid medicine for the cancer treatment.

1080. Exosome-Mediated Delivery of Antisense Oligonucleotides Reprograms Tumor-Associated Macrophages and Induces Anti-Tumor Responses

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Tumor-associated macrophages (TAMs) are key tumor-promoting mediators of the tumor microenvironment, and thus an attractive target for immunotherapy efforts. To date, macrophage targeting experimental therapies targeting the differentiation, recruitment or survival of TAMs have shown marginal anti-tumor activity. An alternative approach is the induction of macrophage reprogramming from a pro-tumoral, immunosuppressive ('M2') phenotype to an anti-tumoral, immunostimulatory ('M1') phenotype. This can be achieved by inhibiting transcription factors that define the M2 phenotype, such as C/EBPβ, which are undruggable with current technologies. We have designed and developed a new class of therapeutic, exoASO, consisting of proprietary engineered exosomes loaded with antisense oligonucleotides (ASOs) directed specifically against the transcription factors of interest. *In vitro* assays using primary human M2 macrophages demonstrated dose-dependent target gene knockdown (KD) with higher potency (IC_{50}) of exoASO-C/EBPβ vs. free ASO. exoASO-mediated KD resulted in profound changes in gene expression and cytokine secretion profile consistent with reprogramming to a proinflammatory (M1) phenotype. Biodistribution studies in tumor-bearing mice showed that exoASO associates preferentially with macrophages and myeloid derived suppressor cells in the tumor. Local administration of exoASO-C/EBPβ in CT26 tumors resulted in a significantly higher KD of the target gene in tumor-associated CD11b+ cells, compared with an equivalent dose of free ASO (50% vs 20%, respectively). Changes in iNos, CD206 and Csf1r mRNA expression in these cells demonstrated *in vivo* M2 to M1 conversion. Efficacy studies in the CT26 tumor model showed significant anti-tumor activity of intratumorally dosed

exoASO-C/EBPβ as single agent (80% tumor growth inhibition, 60% complete responses), while equivalent doses of free ASOs or standard anti-CSF1R monotherapy did not affect tumor growth significantly. In an orthotopic hepatocellular carcinoma (HCC) model, intravenous treatment with exoASO-C/EBPβ attenuated HCC-mediated increase in liver to body weight ratios (≤ 10%) and showed little to no observable tumor lesions in 50% of treated mice. In contrast, all mice (100%) treated with either free C/EBPβ ASO or anti-CSF1R mAb showed increased liver to body weight ratios (≥ 10%) and observable tumor lesions. Thus, both local and systemic administration of exoASO-C/EBPβ result in potent anti-tumor efficacy as monotherapy. In summary, exoASO is a novel therapeutic that allows selective targeting and specific inhibition of transcription factors in TAMs, promoting M2 to M1 reprogramming, which effectively translates into potent antitumor activity.

1081. Investigation of the Natural miRNA *miR-675* as a Prospective RNAi-Based Gene Therapy Product for Facioscapulohumeral Muscular Dystrophy (FSHD)

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Background: FSHD is an autosomal dominant muscle disease associated with progressive skeletal muscle weakness. FSHD is caused by the aberrant expression of *DUX4* in skeletal muscles in which it is toxic. FSHD severity is often variable, but little is known about the mechanisms responsible for this variability. We hypothesized that microRNAs expressed in skeletal muscles could target *DUX4* mRNA, and therefore, function as modifiers of FSHD severity. Using a candidate approach, we previously identified *miR-675* as a direct inhibitor of *DUX4* expression and DUX4-induced pathogenicity (manuscript submitted). *miR-675* is a muscle specific miRNA that is derived from the long non-coding RNA *H19*. **Objectives:** We aim to test the role of *miR-675* as a modifier of DUX4-induced muscle damage, in a loss-of-function *in vivo* study, using a tamoxifen inducible Cre-DUX4 mouse model (TIC-DUX4), and to use *miR-675* as an RNAi-based gene therapy product for FSHD. **Methods:** To validate *DUX4* as a target for *miR-675*, we used the dual-luciferase assay, western blot, viability assays, qPCR, ddPCR, molecular beacon binding assay and mutational analysis. To optimize *miR-675* inhibition efficiency, we used the dual-luciferase assay and western blots. To validate the role of *miR-675 in vivo*, we crossed a *miR-675* knockout mouse with the TIC-DUX4 mouse model. We administered tamoxifen using oral gavage, and measured behavioral activity (e.g. open field and Rota-Rod), muscle absolute and specific force, and looked for worsening of muscle histopathology. At the molecular level, we looked for an inverse correlation between *miR-675*, *DUX4* and *DUX4*-responsive biomarker. **Results:** *In vitro*, we confirmed the specific targeting of *DUX4* by *miR-675*, and optimized its targeting efficiency by changing sequences within the miRNA stem loop structure. *In vivo*, the induced TIC-DUX4 mice lacking *miR-675* showed earlier deficits in their behavioral activity and reduced specific force of their gastrocnemius muscle compared to the TIC-DUX4 mice induced for 8 weeks using a low dose of tamoxifen. Muscle histopathology was also more severe on the *miR-675* null background, as indicated by increased numbers of centrally nucleated myofibers (regeneration). At the molecular level, *DUX4* expression in the TIC-DUX4 mice was associated with an increase in *miR-675* expression. One explanation is that *DUX4* expression induces a muscle injury response in which *miR-675* expression increases to promote myogenesis, which is a known function of *miR675/H19*. Interestingly, in our study, *miR-675* knockout led to increased *DUX4* expression, which, in turn was associated with the increased myopathic phenotypes. **Conclusions:** We showed that *miR-675* specifically targets *DUX4 in vitro* and in the TIC-DUX4 mouse model, and that its absence can exacerbate *DUX4*-induced skeletal muscle damage. Accordingly, our results point towards a tempering effect of *miR-675* against DUX4-induced muscle weakness, and give evidence for the possible use of *miR-675* as an RNAi-based gene therapy product for FSHD. In the future, we aim to test AAV.*miR-675 in vivo* using the TIC-DUX4 mouse model, and to validate *miR-675* as a gene modifier of FSHD severity using human subject samples. This could have translational implications, as it could help better define and predict disease progression, which could lead to better stratification of patients for clinical trials.

1082. Delivering Epigenetic Silencing Sirna to the Nucleus of Hiv-1 Infected Primary Activated Cd4+ T-Cells via Nanoparticle System

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RNAi-based gene therapy approaches have shown great potential in recent years. However current delivery of siRNA via viral vectors causes safety concerns and issues that need to be avoided for widespread implementation. Concurrently, the use of RNA-directed epigenetic silencing to inhibit HIV-1 virus transcription is investigated as a potential targeted functional cure. We therefore hypothesize the use of novel nanoparticle technology as an efficient method. Delivery of siRNA-based gene therapeutics into primary human cells continues to be a serious challenge, and nanoparticles could be a safe and suitable alternative to overcome the barriers. These findings could be extended beyond the HIV-1 model as a general gene therapeutic delivery method. For these studies we used the epigenetic silencing siRNA siPromA, an HIV-1 5'LTR targeting siRNA, and separately, the siScrambled control to load onto a Layer-by-Layer nanostructured film and treated HIV-1 infected primary activated CD4+ T-cells. To visualize the localization of siRNA inside infected cells, we used fluorescent virus and fluorescent-labelled siRNA, imaged using the fluorescent microscope DeltaVision Elite, and identified delivery into cells and localization of siRNA in the nucleus by Arbitrary line intensity profile and 3D Volume Viewer. To analyse functional delivery, we cultured infected cells over a 16-day time course and performed Reverse Transcriptase (RT) assays and

RT-qPCRs. Entry, detachment and successful delivery into the nucleus of activated CD4+ T-cells was confirmed 48h after Nanoparticles-siRNA were added to the cultures. RT-Assays showed significant reduction of active virus in supernatant of siPromA treated cultures compared to Nanoparticle controls [Buffer ($p<0.0063$), Nanoparticle-alone ($p<0.0122$) and Mock ($p<0.0013$)] on day 16 post NP addition. These results were confirmed via RT-qPCRs, showing significantly lower levels of viral mRNA for siPromA treated cultures compared to the Nanoparticle controls: Buffer ($p<0.0001$), Nanoparticle-alone ($p<0.0263$) and Mock ($p<0.0027$). Here we have shown successful nanoparticle delivery and release of epigenetic silencing siRNA in primary human cells. This study shows a pathway for RNAi gene therapeutic delivery into primary human cells. Successful non-viral delivery via the use of a nanoparticle system provide an important step for the future use of a wide range of gene-therapy agents.

Synthetic/Molecular Conjugates and Physical Methods for Delivery

1083. A Cationic Lipid/Polymer Hybrid Nanoparticle Platform for mRNA Delivery

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Introduction: In recent years, messenger RNA (mRNA) has become a promising therapeutic modality for multiple diseases through its ability to mediate production of biologics *in vivo* for protein replacement therapies and vaccines. mRNA is particularly suited for this role due to its capability to be translated rapidly into protein without the need for transcription, as well as the minimal risk of insertional mutagenesis of the host's genome. Despite the promise of mRNA, a critical challenge to clinical translation is the efficient, robust, and safe intracellular delivery of genetic material to drive the expression of the protein. In this study, we have developed a lipid polymer hybrid (LPH) system to enable *in vivo* mRNA delivery for a wide variety of biomedical applications. **Materials and Methods**: LPH particles were synthesized through microfluidic nanoprecipitation of poly (lactic-co-glycolic acid) (PLGA), helper lipid (DSPC), pegylated lipid (DMG-PEG2000), and cationic lipids (DOTAP and MC3). mRNA was incorporated through encapsulation during the mixing formulation or adsorption to the particle surface post production. Particles with different ratios of cationic lipids were evaluated for *in vitro* transfection through formulation with eGFP mRNA and subsequent incubation with HepG2 cells. *In vivo* biodistribution and mRNA expression dynamics were evaluated with Cy5 labeled and unlabeled fLUC mRNA and subsequent luminescent and fluorescent imaging. Systemic administration was evaluated through intravenous administration and local administration was evaluated via intramuscular administration in a mouse model. **Results and Discussion**: *In vitro* transfection revealed that the DOTAP and MC3 lipids synergize to promote mRNA delivery and adsorbed formulations outperformed encapsulated formulations at higher particle doses. Evaluation of systemic administration in mice revealed that for all formulations tested the particles accumulated preferentially in the liver, but the majority of mRNA expression occurred in the lung and spleen (**Fig 1a-c**). Adsorbed particles led to higher protein expression than the encapsulated in systemic administration. Conversely, local administration in the muscle revealed that mRNA expression was higher for encapsulated mRNA versus adsorbed with detection out to 9-12 days following single injection suggesting a depot effect. (**Fig 1d-e**).

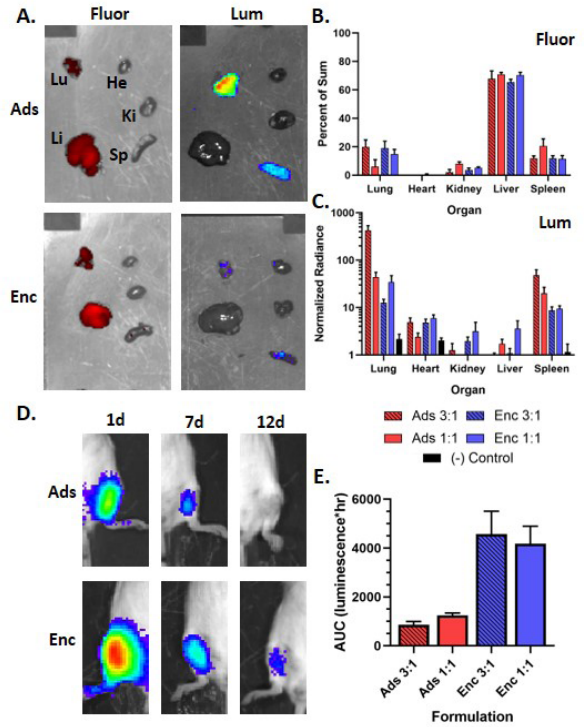

Figure 1. (A) Images and (B) quantification of fluorescence (Fluor) and luminescence (Lum) of major organs (Lu = lung, He = heart, Ki = kidney, Sp = spleen, Li = liver) following systemic administration of adsorbed (Ads) and encapsulated (Enc) particles shows particle accumulation in liver but mRNA expression in lung and spleen. (D) Images and (E) AUC of mRNA expression following local muscle administration of Ads and Enc particles demonstrates higher in Enc groups.

Conclusions: In this study, an LPH system for delivery of mRNA was developed and successfully demonstrated translation *in vitro* and *in vivo*. This platform has revealed a unique synergy between different classes of cationic lipids to enable versatile LPH facilitated mRNA expression via different routes of administration for varied purposes. Continued development of this platform for mRNA delivery may enable the widespread potential of mRNA as a therapeutic for multiple disease applications.

1084. Utilization of High-Throughputin Vivoscreening Technologies for the Development of Lipid Nanoparticles That Can Deliver mRNA in NHPs

Cory Sago
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Lipid Nanoparticles (LNPs) are a class of delivery vehicles that enable repeat administration for a variety of genetic medicines, including siRNA and mRNA, for clinical use. The discovery and validation process of LNPs have traditionally been low throughput with relatively few potential LNPs tested *in vivo*due to technical and practical difficulties of screening large numbers of LNPs *in vivo*. We have pioneered a novel technology allowing for the testing of thousands of LNPs directly *in vivo*. Using an increasingly advanced DNA-barcoded LNP system, we can measure the *in vivo*biodistribution of hundreds of LNPs in a single experimentwith greater sensitivity than fluorescence. Additionally, we can quantify functional delivery of the LNPs genetic payload to dozens of cell types in a single mouse. We tested over 1,000 chemically distinct LNPs with an mRNA payload to mouse hepatocytes *in vivo* over several iterative experiments, and we were able to improve delivery by several orders of magnitude as compared to our initial starting LNP. We subsequently tested three novel LNPs and MC3-LNP for delivery of human EPO (hEPO) RNA at 0.3 mg/kg (IV) both in rats and NHP. We determined that the novel LNPs have a similar or better efficacy (as measured by Cmax) of hEPO as compared to MC3-LNP. Furthermore, we tested for tolerability of these novel LNPs by measuring ALT/AST, cytokines and chemokines including IP-10, MCP-1 and TNFαand determined that the novel lipid LNPs had similar or decreased measurable responses compared to MC3-LNP.In conclusion, we demonstrated that Guide Therapeutics' high-throughput *in vivo*screening method has utility in identifyingefficacious and well-tolerated LNPs for the delivery of mRNA in NHPs.

1085. Liver-Directed Gene Therapy in Pigs via Retrobiliary Hydrodynamic Injection of Plasmid DNA During an Endoscopic Procedure

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Introduction: Monogenic liver diseases encompass a wide spectrum of disorders ranging from metabolic to hematologic disturbances. Gene therapy has been sought as a cure for these diseases, with adeno-associated virus (AAV) being the most popular vector in clinical trials. However, AAV has a number of relative limitations, including limited packaging size for transgene cassettes, susceptibility to immune-mediated clearance of transduced cells, difficulty for repeat administration due to the development of neutralizing antibodies, and relatively high cost of manufacturing. A non-viral strategy of gene delivery for monogenic liver disorders would circumvent these concerns. Hydrodynamic injection through the tail vein is a cheap and efficient way of transfecting DNA into the liver in rodents, but the technique is deemed too dangerous in larger animals due to vascular stress and tissue injury. Attempts to model hydrodynamic injection in local organs in large animals have had some success, but require complex sealing of blood vessels in order to achieve local pressure increase. Moreover, pressure and transfection efficiency has only been modest from this strategy. **Methods:** Herein, we developed an alternative approach to perform hydrodynamic injection into the liver via the biliary tract. Advantages of a retrobiliary approach include the ability through one access point to reach the entire vast network of hepatocytes, while also being less invasive than vascular approaches requiring no incision. We utilized human factor IX (hFIX) as a model of gene therapy. An endoscopic procedure was performed with clinical equipment used in human patients, consisting of ERCP with the catheter being advanced into the common hepatic duct. Sealing a balloon at the hepatic hilum and entrance into liver parenchyma to provide a closed system, 30 mL of saline solution containing 3-5.5 mg DNA was rapidly injected at 2mL/sec with maximal intrabiliary pressure increase of 85 mmHg achieved. **Results:** The hydrodynamic injection procedure was well tolerated in all four female pigs (~40kg) tested, and subsequent labs showed mild-to-no elevation of liver enzymes during the three weeks post-injection. No inflammation was induced by the procedure as determined by a normal and unchanged white blood cell count. Pigs displayed normal behavior and growth pattern, and post-necropsy organs and histology showed no abnormalities. hFIX DNA was integrated into the hepatocyte genome with co-injected piggyBac transposase plasmid and was found in all liver lobes 1 and 3 weeks post-injection, indicating global liver delivery. No human factor IX could be detected in the pig serum, however, suggesting that the plasmid dose was not sufficient to yield therapeutic levels. **Conclusion:** We demonstrate the safety of retrobiliary hydrodynamic injection through endoscopy as a gene delivery vehicle into the liver, which can be performed using FDA-approved tools and techniques that clinicians already use in endoscopic retrograde cholangiopancreatography (ERCP) procedures. We suggest that non-viral endoscopic gene delivery could be an alternative to AAV-based viral vectors utilizing cheaper material in plasmid DNA, and that this strategy merits further investigation. Future studies will continue to optimize transfection efficiency through DNA dose and mechanical injection parameters.

1086. Toxicity Evaluation of Novel Liver-Targeted mRNA Lipid Nanoparticles in Rat and NHP

Milloni Chhabra, Cory Sago
Guide Therapeutics, Tucker, GA

The utility of RNA-based therapeutics was first described nearly 30 years ago and has great potential for treatment of genetic diseases. Delivery of these medicines still remains an unmet need due to limitations of using naked RNA molecules; encapsulation of the RNA is required to avoid rapid degradation. Use of lipid nanoparticles (LNPs) as a non-viral vector for RNA-drugs solves several challenges of delivering mRNA to the intended tissue and specific cell type. Guide Therapeutics' high-throughput *in vivo* screening method is able to simultaneously generate and test hundreds of LNPs directly *in vivo* for delivery to specific cell types using a proprietary DNA-barcoding technology. Using our platform, we have an iterative approach to identify and optimize several hepatocyte targeting LNPs and test

them in rats and NHP for efficacy and tolerability. We tested three novel liver-targeting LNPs and MC3-LNP for delivery of human EPO (hEPO) RNA at 0.3 mg/kg (IV) both in rats and NHP. We determined that the novel LNPs have a similar or better efficacy (as measured by Cmax) of hEPO as compared to MC3-LNP. Furthermore, we tested for tolerability of these novel lipids by measuring ALT/AST, cytokines and chemokines including IP-10, MCP-1 and TNFα and determined that the novel lipid LNPs had similar or decreased measurable responses compared to MC3-LNP. In conclusion, we have determined that we can use the Guide Therapeutics' high-throughput *in vivo* screening method to identify novel lipids for use in generating safe and efficacious LNPs for liver-targeted delivery of mRNA in NHPs.

1087. Targeted Non-Viral Delivery of Mini-Intronic Plasmids for Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is an autoimmune disorder of the joint characterised by inflammation, synovial hyperplasia and increased vascularisation. Synovial fibroblasts are aggressive cells in RA pathogenesis, contributing to inflammation and cartilage degradation. Current treatment with disease modifying anti-rheumatic drugs increases risk of serious infection. Further, some patients do not respond to drugs whilst others develop resistance leading to loss of efficacy. Hence, we aim to develop a gene therapy that modulates RA disease without the immunosuppressive effects. Our approach involves developing receptor-targeted nanoparticles (RTN) to selectively deliver therapeutic genes to the inflamed synovium, thus improving efficacy and reducing systemic side-effects. The RTN comprises a cationic lipid that self-assembles with the anionic DNA backbone and a neutral lipid DOPE to aid DNA endosomal escape. RTNs also include a peptide containing a cationic, 16-lysine domain for electrostatic DNA packaging and a synoviocyte targeting ligand, separated by a cleavable (RVRR) or hydrophobic (XSX) linker to alter RTN stability (Table 1). Further, we aim to develop a DNA vector that provides high and sustained transgene expression, while minimising inflammatory responses, based on mini-intronic plasmids (MIPs). MIPs lack a bacterial backbone, making them safer and potentially less inflammatory, and have been shown to provide greater and more prolonged gene expression than conventional plasmids. Rabbit synovial fibroblasts (HIG-82) were transfected with luciferase or GFP reporter plasmids with RTNs containing different peptides. Transfections to assess cell type specificity were performed with chondrocytes (C28/I2) or hepatocytes (HepG2). Luciferase or GFP expression was measured after 24 or 48-hours incubation, respectively. MIPs contained a luciferase reporter gene with either a CMV or EF1α promoter and luciferase activity following HIG-82 RTN transfection was compared to that of conventional plasmids over time at equimolar ratios. RTNs with synoviocyte peptides demonstrated superior transfection efficiency in HIG-82 cells than positive control peptides, regardless of linker. In comparison, synovial-targeted RTNs yielded poorer transfection efficiency in hepatocytes and chondrocytes compared to RTNs with positive control peptides, which transfected all cell types indicating a degree of targeting specificity for synoviocytes. RTNs with cleavable peptides gave more efficient transfection than hydrophobic in all cell types, presumably due to enhanced RTN disassembly following uptake. Initial experiments showed similar or greater luciferase expression following RTN transfection of HIG-82s with MIPs than conventional plasmids, with EF1α promoters providing more sustained expression than CMV. These results, together with the other benefits of MIPs, e.g. lack of CpG-rich bacterial sequences, warrant their further investigation. Additionally, MIPs are smaller than conventional plasmids, allowing a lower amount of DNA to achieve the same gene copy number, and hence, other RTN components, which may result in reduced toxicity. This work provides the basis for a targeted RA gene therapy approach that specifically delivers DNA to synoviocytes in the inflamed joint.

Table 1: Peptide sequences, where X = epsilon amino hexanoic acid.

Peptide Name	Description	Peptide Sequence (linker underlined, targeting sequence italic)
KG-32	Positive control, cleavable	K_{16}RVRRGA*CYGLPHKFCG*
KG-31	Positive control, hydrophobic	K_{16}XSXGA*CYGLPHKFCG*
KS-34	Synoviocyte targeting, cleavable	K_{16}RVRRGA*SFHQFARATLAS*
KS-33	Synoviocyte targeting, hydrophobic	K_{16}XSXGA*SFHQFARATLAS*
KT-34	Negative control, cleavable	K_{16}RVRRGA*ARPLEHGSDKAT*
KT-33	Negative control, hydrophobic	K_{16}XSXGA*ARPLEHGSDKAT*

1088. Development of Multi-Functionalized Gemini Nanoplexes for Non-Viral Neurotrophic Factor Gene Delivery to the Retina

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Background and Purpose: Current glaucoma management relies on reducing and maintaining a low intraocular pressure (IOP), an important risk factor for the progression of visual field loss, by pharmacological and invasive surgical treatments. Alternatively, gene therapy with neurotrophic factors (NF) has the potential to provide neuroprotective and neuroregenerative functions for retinal ganglion cells (RGCs) to prevent or restore visual deterioration. The main objective was the development of a new generation of nanoplex (NPX) system based on cell adhesion peptide (CAP)- from modified dicationic gemini surfactants (m-s(p)-m) for targeted delivery of neurotrophic factor genes to the retina. **Methods**: Two integrin-binding (p_{1-2}) and three immunoglobulin superfamily (IgSF)-peptide (p_{3-5}) modified m-s(p_{1-5})-m gemini surfactants (*m-alkyl tail [12-18C], s-spacer 7C with imino-peptide substitution*) were synthesized and purified using prep-HPLC and analyzed by ESI-MS, ^{1}H- NMR and HPLC. NPXs constructed from m-7N(p_{1-5})-m and gWIZ-GFP plasmid were optimized at various ratios and assessed for size, zeta potential, transfection efficiency (TE) and toxicity in A7 astrocytes using flow cytometry and confocal microscopy. Penetration studies

were performed in a MatTek EpiCorneal® tissue equivalent model. m-7N($p_{1,3}$)-m compounds, Lipofectamine (LF) 3000 and normal saline control carrying gWIZ-GFP were tested in retinal neurospheres to assess TE in a 3D environment. *In vivo* studies were performed in 6-week old CD1 mice (n=4 eyes/group); m-7N(p_1 and p_3)-m-NPXs and 18-7NH-18 NPXs were injected intravitreally containing 0.5 µg tdTomato plasmid-BDNF plasmid/2µL dose, LF3000 and normal saline as controls. Whole retinas were isolated and gene expression was evaluated by CLSM. BDNF-ELISA assay was carried out to determine total BDNF in the eyes after treatment. **Results**: m-7N(p_1 and p_3)-m-NPXs with 5:1 charge ratio, 220.96±2.5nm and 257.4±3.2 nm size and +30 ± 0.2 mV and +37.13± 0.5 mV zeta potential, respectively, were selected for *in vivo* studies. m-7N(p_3)-m NPXs showed improved TE compared to m-7NH-m NPXs and LF3000, 15.36±1.5%, 10.97±0.4% and 12.07±1% (n=6), respectively, and >90% viability. NP penetration studies in the EpiCorneal® model showed 90±20µM penetration of the m-7N(p_1 and p_3)-m-NPXs and 100±20µM for m-7NH-m NPXs. Retinal neurosphere TE studies showed higher TE for m-s(p_3)-m NPXs and mature RGCs presence in the retinal neurosphere. Intravitreal m-s(p_3)-m NPX treated mice showed higher reporter gene expression and BDNF production (422.60±42.60 pg/eye) compared to m-s(p_1)-m NPX, 230.62±24.47, 18-7NH-18 NPX, 245.90±39.72 and control, 131.33±20.30 pg/eye. The results show that novel IgSF m-s(p_3)-m NPXs as potential candidates for targeted non-viral gene delivery to the retina to treat glaucoma and potentially other ocular neurodegenerative conditions.

1089. Bioengineering Bacterial Outer Membrane Vesicles as Delivery Systems for RNA Therapeutics Targeted to Lung Epithelial Cytosols

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Intact epithelia lining the airways and alveoli in the lung are essential to maintain lung function. Structural or functional damage of epithelial cells leads in severe diseases, including COPD/emphysema, fibrosis or ALI/ARDS. This central role of epithelia in pulmonary diseases identifies these cells as primary candidates for targeted therapy. With the exception of surface-expressed molecules, however, targeting intracellular components is severely restricted due to poor delivery. We aim to overcome this obstacle using topically administered, bioengineered, biocompatible bacterial outer membrane vesicles (OMVs) as recombinant drug delivery systems for novel biopharmaceuticals. Engineering recombinant surface expression of eukaryotic receptor ligands in ClearColi®, a commercial *E.coli* BL21 (DE3) strain deficient in lipopolysaccharide production, we have used red fluorescent protein reporters to track OMV loading, transgene expression, and eukaryotic cell trafficking. We demonstrate statistically significant differences in the levels of over 700 proteins between differentially engineered and purified OMV preps with additional differences in transcriptome and lipidome consistency. We also characterised visual and particle size differences observed by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Here we report early bioadhesion and cellular uptake results in A549 culture as well as air-liquid interface culture of re-differentiated lung epithelia. This project aims to bridge the biotechnological gap in the intracellular biopharmaceutics drug delivery challenge for respiratory epithelia through highly controlled, and scalable bio-nanotechnology process. If successful, our work will unlock intracellular imaging and therapeutics research for respiratory diseases with a significant epithelial component, paving the way for other targeting ligands and potentially non-respiratory indications.

1090. Delivery of an Endosomolytic CRISPR-Cas9 RNP Enzyme via Receptor-Mediated Endocytosis for Targeted Genome Editing of T Cells

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The RNA-guided genome-editing enzyme CRISPR-Cas9 has been successfully employed to target different genetic disorders in model systems and is a powerful tool for generating cell-based therapies. However, selective delivery to specific cells or organs for genome editing *in vivo* is a substantial barrier to therapeutic translation. To address this unmet need, we have developed a platform for receptor-mediated uptake of the Cas9 endonuclease and guide RNA ribonucleoprotein (RNP) complex as an alternative to currently available methods of *in vivo* delivery. This non-viral platform represents a novel approach for therapeutic delivery that combines the appealing safety profile of Cas9 RNP with the ability to use molecular targeting for cell/tissue specificity. Furthermore, engineered RNP enzymes can be much more readily manufactured than viral vectors, and are more robust than nanoparticles. We have demonstrated that tethering the Cas9 RNP enzyme to a T cell-targeting antibody can facilitate cell-specific endocytosis into T cells. Endosomal escape, a necessary step for cytosolic delivery and subsequent nuclear localization, can be facilitated by endosomolytic peptides recruited to the RNP surface in a programmable manner. Using various embodiments of our platform, we have enabled T cell-targeted uptake in a mixed white blood cell context, as well as gene knockout. We have thus established proof-of-concept for this system's utility in the cell-targeted genome editing of immune cells, with an aim towards *in vivo* therapeutics.

1091. Microfluidic Device for Intracellular Delivery of Nucleic Acids Into Human CD34+Hematopoietic Stem and Progenitor Cells

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Human CD34+ hematopoietic and progenitor cells (HSPCs) constitute a keystone cell carrier in the advent of ex vivo gene therapy, illustrated by successful regulatory approval and commercialization of such therapies in the United States and Europe. However, significant challenges remain in the genetic engineering process of these cells, preventing HSPC gene therapy products from becoming more inexpensive and accessible to patients. In the present work, we demonstrate for the first time the capacity of our microfluidic platform

to deliver nucleic acids (i.e. mRNA) into human HSPCs with high efficiency. This microfluidic-enabled mechanism of delivery, previously characterized in cell lines, relies on transient cell volume exchange to convectively drive payload into the cells. We also show that, when compared to electroporation, our process results in better cell viability, cell recovery, and maintenance of stemness; as indicated by trypan blue cell counts, and analysis of cell surface markers of stemness and differentiation by flow cytometry. We conclude that, when all these processing aspects are factored in, our device results in a higher yield of transfected cells than electroporation, and thus it constitutes a new competitive platform for HSPC transfection.

1092. VEsiCas: A Traceless Delivery Tool for Efficient Genome Editing

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CRISPR-mediated genome editing is a rapidly evolving technology which opens interesting perspectives both for gene therapy and precision medicine. Expanding the panel of available delivery tools to introduce CRISPR components into target cells is an urgent need in order to support the advancements made by the field, helping the translation to effective therapeutic applications. VEsiCas is a DNA-free vesicle-based tool to deliver pre-assembled complexes of SpCas9-gRNA into target cells. Its distinctive feature is the T7 RNA polymerase-mediated gRNA transcription in the cytoplasm of the producer cell line which enhances gRNA accumulation in budding vesicles, increasing the potency of the delivered nuclease. Thanks to the presence of the VSV-G envelope glycoprotein on vesicles surface, VEsiCas can efficiently target an heterogenous panel of cells to edit different endogenous human loci. *In vitro* experiments demonstrated that VEsiCas are particularly suitable for editing approaches in hepatocytes, combining high editing efficiency with low cytotoxicity. We are currently optimizing VEsiCas production to enhance vesicle yield and standardization in terms of particle quality and concentration to guarantee scalability and reproducibility of the editing performance for *in vivo* use. Combining a DNA-free traceless delivery of CRISPR RNP complexes with the efficiency of VSV-G mediated cellular entry, we believe that VEsiCas will provide a flexible as well as powerful delivery tool to boost genome editing performance *in vivo*.

1093. Inhibition of Caspase-3 Improves Electrotransfer Efficiency and Cell Viability

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Cell therapy often requires genetic engineering of cells with viral vectors[1]. However, viral vector production is currently labor-intensive and expensive[2]. To solve the problem, electrotransfer of molecular cargos has been implemented in various cell engineering processes including the production of chimeric antigen receptor (CAR)-T cells in a clinical trial[3]. This non-viral method for gene delivery has been used routinely in basic research and preclinical studies although relatively low cell survival rate and electrotransfer efficiency (eTE) are impeding its applications in the clinic[4]. One dilemma faced by investigators is that a high eTE often requires to compromise cell viability[5]. To address the issue, we investigated inhibitors of potential target genes that may affect cell apoptosis, intracellular vesicle transport, cytoskeleton remodeling, and DNA degradation pathways with an immortalized human T cell line, Jurkat clone E6-1. Small molecule inhibitors being tested included pan-caspase inhibitor (z-vad-fmk), ATM inhibitor (KU55933), ATR inhibitor (VE-821), cGAS inhibitor[6] (Aspirin), actin filament inhibitor (cytochalasin B), apoptosome inhibitor (Apoptosis Inhibitor II). The flow cytometry and the cell live/dead assays were used to determine cell viability and eTE, respectively, and the Annexin V apoptosis assay was used to measure the percent of apoptotic cells. To confirm the results from apoptosis inhibitor study, we also used small interfering RNA (siRNA) to knock down caspases in Jurkat cells prior to electrotransfer. The gene knockdown was determined by using the western blot analysis. Data from the study showed that the eTE and cell viability could be enhanced by >50% and >25%, respectively, after the caspases were inhibited through treatment with small molecule inhibitors or siRNA. Similar experiments were repeated with NIH/3T3 fibroblasts and primary human T cells. The data indicated that the caspase-dependent apoptosis was a main pathway for electrotransfer-induced cell death, suggesting that inhibition of apoptosis, especially the activation of caspase-3, could be a new approach to improving electrotransfer in cell engineering applications such as non-viral CAR-T cell production.

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1094. Towards Targeted Repression and Excision of HIV-1 in the Brain

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Recent advances into HIV-1 treatment and cure strategies have produced novel designs and technologies. One such advancement is the direct evolution of a CRE recombinase, known as BREC1. BREC1 recognizes and binds to LoxBTR sequences present in the 5' and 3' LTR of HIV-1 and can excise HIV-1 provirus from the host genome. While BREC1 appears to be a promising approach to excising HIV from infected cells, delivery to HIV infected cells remains enigmatic. Targeting HIV systemically and in microglial cells in the brain remains a challenge due to the blood brain barrier (BBB), which impedes most substances from entering the brain. To address these issues, we modified BREC1 to contain a cell penetrating peptide (TAT) and several nuclear localization signals. We find that our TAT-BREC1 constructs are able to functionally modulate HIV-1 in multiple cell lines and are able to recombine the integrated HIV-1 out in a reporter cell line. Further, we show by chromatin immunoprecipitation that the BREC1 constructs are enriched at the LoxBTR loci in the LTR of HIV-1. In addition, we find that these BREC1 constructs show greater excision frequency in cell lines in which HIV-1 is transcriptionally active. Next, to explore delivery of the enhanced BREC1 constructs through the BBB to the brain, we developed BREC1 packaged macrophage derived exosomes. We find that polarizing monocyte-derived macrophages (MDMs) into a broadly type II phenotype with MCSF, results in a large

quantity of exosomes, that can be harvested at multiple time points in serum-free media. Further, we are able to reproducibly transfect MDMs with the exoTIC device, allowing for packaging of the anti-HIV BREC1 therapeutic RNA. We characterized these modified exosomes as well as the exoTIC expressing MDMs, in *vitro* and *in vivo* for their bio-distribution profiles and their ability to traverse the blood-brain-barrier. Collectively, these preliminary data suggest that the next generation BREC1 modalities can functionally target and modulate HIV-1 in infected cells, and that exosome-based delivery strategies may prove useful in systemic targeted excision of HIV from infected individuals and result in a functional cure.

1095. Uncovering the Unique Properties of Cell Derived Vesicles, a Novel Carrier with Therapeutic Promises

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Introduction: Extracellular vesicles (EVs), or more frequently known as exosomes, have recently emerged as novel therapeutics in various clinical applications. EVs are nanosized vesicles which are produced in most cells. These lipid vesicles shutter genetic materials and proteins between cells and thus have been proposed as a novel delivery vehicle. Despite the growing number of evidences demonstrating that EVs are a crucial mediator of intercellular communication, challenges to commercialize EVs in clinical applications remain largely unresolved. Here, we developed a scalable manufacturing process using extrusion technology to produce cell derived vesicles (CDVs), with superior yield compared to other EVs. Moreover, characterization of CDVs at physical, biochemical, and molecular levels provides substantial evidence to further support the therapeutic potentials of CDVs. **Methods**: Two different types of cells, mesenchymal stem cells (MSCs) and natural killer (NK) cells were used in this study. We first developed a manufacturing-scale extruder to process a large volume of cell mixture in a serial extrusion. Then, cellular impurities were removed using the tangential flow filtration method. The purified CDVs have a size of 150-200 nm in diameter. Then, several methods were used to characterize the physical and biochemical properties of CDVs such as DLS, NTA, Cryo-EM, and FACS analysis. Additionally, multi-omics profiling was carried out to comprehend the fundamental understanding of the molecular contents of CDVs. **Results**: We demonstrate that a manufacturing-scale extrusion process was developed to produce a large quantity of CDVs with consistent quality (Figure 1). Surface marker and membrane composition analyses show that the CDVs are enriched with cellular markers on their surface which are primarily derived from the plasma membrane of source cells. Comprehensive profiling of molecular components reveals unique protein and RNA effectors inside CDVs, providing a better insight into the mechanistic understanding of the formation of CDVs and their therapeutic potentials.

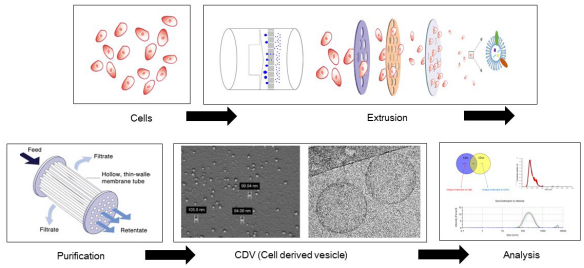

Figure 1. Overall manufacturing process of CDV.

Summary/Conclusion: In this study, we have successfully established a manufacturing process to enable clinical applications of CDVs. We also highlight key molecular features of CDVs that can be harnessed to offer a powerful tool for regenerative and anticancer medicine.

1096. Lipid Nanoparticle Pre-Treatment Improves rAAV Diffusion in the Primate Liver and Enables an Increase of Therapeutic Transgene Expression

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Recombinant adeno-associated virus (rAAV)-vectors have been successfully used in preclinical studies and in clinical trials to express therapeutic proteins in the liver. Transduction of a limited percentage of hepatocytes can be enough for expression of secreted proteins. However, most hepatic metabolic monogenic disorders would require a high percentage of hepatocytes to be transduced to achieve a therapeutic effect. This can be routinely achieved in rodents but has proven to be a significant hurdle in large animals hampering the feasibility of such liver directed AAV based gene therapies. The liver has a very high capacity to remove particles from the circulation. The cells from the reticuloendothelial system (RES) play a central role in this clearance process. The saturation of the hepatic reticuloendothelial cells by nanoparticles or lipids has been shown to block uptake of particles from the circulation. We hypothesized that saturation of the RES could increase AAV-vector transduction and distribution in the liver, subsequently improving transgene expression, in large animals. Therefore, we explored the potential of pre-treatment with Intralipid, an FDA approved emulsion of soy-bean oil, egg phospholipids and glycerin in non-human primates. Non-human primates (NHPs, n=5) negative for the presence of anti-AAV5 neutralizing antibodies were injected intravenously with Intralipid (2g/kg) one hour before intravenous administration of AAV5-hFIX ($1x10^{13}$ gc/kg). A control group (n=5) was injected with AAV5-hFIX ($1x10^{13}$ gc/kg). The animals were sacrificed 8 hours (n=2) or 8 weeks (n=8) after AAV5-hFIX administration. Plasma levels of hFIX transgene were analyzed by ELISA and AAV-vector DNA and transgene RNA copy numbers in liver were determined by Q(RT)PCR. Presence and tissue distribution of AAV-vector DNA and transgene RNA in liver samples was determined by fluorescent in situ hybridization (FISH).

Image analysis was performed with HALO-AI software (Indica Labs). Cells positive for AAV-vector DNA and transgene RNA were scored between low (1+) to strong positive (4+) based on intensity and area of probe positive signal. The distribution of cells positive for AAV-vector DNA and transgene RNA through the hepatic units was based on central vein and hepatocytes membrane staining (IHC) coupled with staining of AAV-vector DNA and transgene RNA (FISH). Following Intralipid pre-treatment, an increase in hFIX protein was observed in the animals injected with AAV5-hFIX compared to the control group (average 3-fold). A similar increase was observed at the hFIX mRNA levels. Accordingly, at 8 weeks sacrifice, vector DNA copy numbers were higher in liver tissues of animals treated with Intralipid than in control group (average 1.7-fold). Intralipid pre-treatment resulted in an increased percentage of +2 (1.3-fold), +3 (2.7-fold) and +4 (2.2-fold) positive cells on the overall positive cell population. Furthermore, an enhanced diffusion through the hepatic unit from portal to central vein was observed after Intralipid pre-treatment. At 8 hours sacrifice, the AAV-vector DNA was found in control animals to be localized predominantly in the sinusoidal space (53.4%) when compare to the hepatic cords (46.6%). Remarkably, when Intralipid was used an increase of AAV-vector DNA in the cords of hepatocytes was observed with 71.8 % of the cells positive for 28.2 % in the sinusoidal area. In summary, our data demonstrate that a pre-treatment with a lipid emulsion prior to AAV5 administration improves significantly the efficacy of AAV vector delivery to the liver and enables a broader hepatic cell targeting throughout the tissue. This approach represents a valuable tool for liver-targeted gene therapies application.

1097. Flexible Bio-Adhesive Polymeric Thin Film for Controlled Adeno-Associated Viral Vector Delivery Carrier

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Recently, adeno-associated virus (AAV), which has been commercially approved by the Food and Drug Administration (USA), and European Medicines Agency (EU), has been widely researched for its efficiency and safety in clinical trials for various gene therapy applications. As next-generation gene delivery vehicles, the convergence of functional biomaterials and AAV vectors have been developed for controlling AAV delivery modes (e.g. sequential and sustained gene delivery). Herein, we developed the flexible and bio-adhesive polymeric thin film for *in vitro/in vivo* controlled AAV delivery. The adhesive property induced by the marine mussel adhesive moiety could be utilized for sustained release of therapeutic biomolecules (i.e. AAV) *in vitro* and *in vivo* under water-rich condition. Furthermore, the flexible and stretchable property of AAV-film system showed enhanced transduction efficiency, and patterned transgene expression of released AAV vectors. We expect that this AAV-film could be optimized for maximizing gene delivery efficiency and minimizing off-target risks (i.e. side-effect). As a result, this AAV-film delivery system could be expected to cover a variety of biomedical applications (e.g. residual cancer cell treatment, targeted organ specific AAV administration, and natural tissue pattern mimics for tissue engineering).

1098. Novel Microfluidic Platform for Scalable Transfection of mRNA and CRISPR/Cas9 RNP in Human T Cells

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Genetically engineered human T-cells present a promising platform to advance treatments of refractory cancers and solid tumors. With the advent of chimeric antigen receptor (CAR) and T-cell receptor (TCR) therapies, a promising future for oncology is on the horizon. However, the process of genetically engineering cells suffers from setbacks when converting these therapies to a clinical scale. Currently, the manufacturing of genetically engineered T-cells relies heavily on the production of lentiviral vectors. The supply of lentiviral vectors to develop T cell therapeutics is costly due to their complex manufacturing process, and thorough qualification requirements. On the other hand, the most prominent non-viral alternative for the transfection of T cells is electroporation, which is encumbered by cell loss and disruption of normal cell function. We have developed a novel microfluidic based platform that relies on the process of transient volume exchange to transfect cells in a high-throughput manner. Our results show a high efficacy transfecting T-cells with mRNA, while conserving viability and overall cell number recovered from the device. We also demonstrate we can successfully delivery CRISPR/Cas9 ribonucleoprotein complexes targeting the human T-cell Receptor Alpha Constant (*TRAC*) gene, maintaining similar viability and recovery rates. Finally, we show that this microfluidic approach to transfection is readily scalable to match research demands, with a current throughput of least 50 million cells processed in under 10 minutes.

1099. Novel Lipid Nanoparticle Technology for Effective Non-Viral Engineering of Primary Human T Cells Towards T Cell Therapy Applications

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The recent approval and clinical adoption of the first cell-based immunotherapies, and the incredible promise of future cell therapies, mark the evolution of drug development from molecular to genetic-based intervention. However, major barriers still exist, including translational barriers, manufacturing bottlenecks, and safety profiles. For example, the currently approved therapies employ viral gene transfer, where manufacturing of GMP grade viral products and the potential immunogenicity and long-term effects of permanent, random gene insertion events are of particular concern. Furthermore, non-viral gene delivery methods, such as electroporation, also suffer from issues of scalable manufacturing, and can result in significant cellular toxicity. Lipid nanoparticles (LNPs) offer an attractive alternative to conventional viral and non-viral delivery, and can eliminate or reduce the issues of manufacturing, immunogenicity and toxicity. LNPs encapsulate nucleic acids and deliver them to the cytoplasm of cells via

receptor-mediated endocytosis. They are chemically-defined, rapidly manufactured at any scale, and easily administered to cells in a single step. Here, we present a novel LNP reagent optimized for mRNA delivery to primary T cells. LNPs encapsulating different mRNA constructs were manufactured using microfluidics technology. The LNPs were produced in less than 5 minutes including prep time, and the mRNA was encapsulated at greater than 80% efficiency. Donor-derived human T cells were isolated from whole blood and activated following standard protocols. The T cells were dosed with a single addition of the LNPs in complete media, resulting in efficient and homogenous mRNA-based gene transfer (up to 90% of cells), with little impact on cellular viability (greater than 90%) and normal proliferation. Testing of the LNPs across multiple donors with a range of ages showed consistent results regardless of donor age. Transfection efficiencies were consistent between different T cell subtypes as well as between cells isolated and activated with various research and GMP grade reagents, ensuring compatibility with a wide range of pre-existing workflows. The therapeutic utility of the LNP reagent is demonstrated by delivery and expression of a CAR construct in primary T cells. LNPs encapsulating a CD19 CAR mRNA construct resulted in CD19 CAR transgene expression in 40% of cells, similar to that of viral counterparts. This novel LNP reagent is suitable for applications such as mRNA-based expression of therapeutic genes, and screening of novel CAR, TCR and constructs that target neoantigens in primary human T cells without the need to generate viral libraries. The LNPs are chemically-defined, and manufacturing is highly-scalable, allowing for rapid translation to GMP-grade and scale-up for clinical trials. This LNP reagent combined with microfluidics manufacturing allows those without an extensive formulation chemistry backgrounds to easily access the transformative capabilities of nanoparticle-mediated nucleic acid delivery. This technology will enable the development and clinical translation of the next generation of immunotherapies and other cell-based therapies.

1100. Folate-tagged Gold Nanoparticles in Gene Delivery for Cancer Therapy

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The use of nanotechnology in the field of gene therapy has emerged as a promising form of treatment against cancer. Gold nanoparticles (AuNPs) are considered favourable gene delivery vehicles due to their low toxicity, ease of synthesis and their ability to be functionalized. This study aimed to prepare functionalized AuNPs and evaluate their folate targeted and non-targeted gene delivery in breast cancer cells *in vitro*. AuNPs were synthesized through citrate reduction and functionalized with chitosan and histidine (Au-CS-His) to confer positive charges and enhance endosomal escape. Folate targeting was introduced by the conjugation of folic acid to the chitosan and histidine functionalised AuNPs (Au-CS-FA-His). Characterization via electron microscopy revealed both nanoparticles to be spherical, while nanoparticle tracking analysis (NTA) results showed high colloidal stability (zeta potential) with hydrodynamic sizes of 121.5 nm and 122.2 nm for the non-targeted and targeted nanoparticles, respectively. The presence of gold and successful functionalizations were confirmed by UV-vis spectroscopy and Fourier-transform infrared spectroscopy (FTIR). DNA binding studies revealed that both nanoparticles were able to form nanocomplexes with the pCMV-*luc* plasmid DNA, with the non-targeted nanoparticle displaying stronger compaction ability. The nanoparticles also afforded partial protection against nuclease degradation. Evaluation of the cytotoxicity of the nanocomplexes utilizing the MTT assay in the HEK-293, MCF-7 and SKBR-3 cell lines, revealed minimal cytotoxicity with the targeted nanoparticles displaying greater cell viability with some of cell proliferation noted. The luciferase reporter gene assay was used to assess the transfection efficiency, with the non-targeted nanoparticles producing greater gene expression in the HEK-293 and SKBR-3 cells, while the targeted nanoparticles displayed enhanced transgene activity in the MCF-7 cells. A receptor competition binding assay confirmed folate receptor-mediated uptake in the MCF-7 cells. The advantageous physicochemical properties in addition to the low cytotoxicity and favourable transgene expression, suggest that both targeted and non-targeted nanoparticles may be suitable delivery vehicles for therapeutic molecules. Further optimization and modifications may be required for future *in vivo* gene therapy studies.

1101. Nanosomes as Drug Carrier for Lung Cancer Treatment

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Introduction: Targeted drug delivery has become an essential requirement for avoiding treatment-related adverse effects observed with conventional cancer treatment modalities. To achieve this goal, several drug delivery approaches including nanotechnology have been undertaken and few advanced for clinical testing. Gold nanoparticle (GNP) is one among several delivery vehicle that has gained attention and tested for its theranostic properties. However, use of GNP as drug carrier has limitations. In an attempt to override some of the GNP limitations, we have combined Nanotechnology with Exosome technology to produce hybrid particles labeled "Nanosomes". Exosomes (Exo) which are nano-sized cellular vesicles (30-120 nm) have been actively explored as drug delivery vehicles as they exhibit small size, ability to easily move across barriers, and avoid eliciting an immune response. Herein, we designed a tumor-targeted nanosome that consists of a chemotherapeutic, Cisplatin (CDDP) conjugated to GNP via a pH-sensitive linker (GNP-CDDP) and loaded into exosome. The efficacy of the tumor-targeted nanosome was tested against human lung cancer cells. **Methods:** The tumor-targeted nanosome was formulated by physically loading exosomes derived from normal lung fibroblast with GNPs conjugated to CDDP by a pH-cleavable ester bond (GNP-CDDP) and decorating on the surface with transferrin (Tf) ligand. Transferrin conjugation and gold content in the nanosomes were determined by mass-spectrometry, and Inductively Coupled Mass Spectrometry (ICP-MS) respectively. The specificity, cell uptake, and therapeutic efficacy of the nanosomes was assessed by conducting receptor blocking studies, fluorescence microscopy, cell viability, Western-blotting, and apoptotic analysis in non-small cell lung cancer cells (A549), normal human lung fibroblasts (MRC9) and Human Embryonic Kidney (HEK293) cells. **Results**: Successful loading of GNP-CDDP onto the exosomes was confirmed by transmission electron microscopy

(TEM). Drug release kinetic studies demonstrated nanosomes exhibited enhanced drug release under acidic conditions (~33% drug release at pH 5.5) compared to release rate at physiological conditions (~18% drug release at pH 7.7). Further, enhanced cell uptake of the tumor-targeted nanosomes was observed in transferrin receptor (TfR) positive A549 cells compared to uptake of non-targeted nanosomes. Further, the tumor-targeted nanosome uptake was significantly reduced in MRC9 cells that expressed low levels of TfR. Cell viability studies revealed that the tumor-targeted nanosomes exhibited greater cytotoxicity (~55% inhibitory activity) compared to cytotoxicity by non-targeted nanosomes (~37% inhibitory activity) towards cancer cells. Molecular studies revealed activation of the apoptotic (caspase 9 and PARP) cascade and DNA damage (H2AX) markers in tumor-targeted nanosome-treated A549 cells. Cytotoxicity in tumor-targeted nanosome-treated normal MRC9 cells was significantly reduced (23% inhibitory activity) compared to free CDDP-treatment (44% inhibitory activity). Additionally, CDDP-mediated nephrotoxicity was greatly reduced in tumor-targeted nanosome-treated HEK293 cells that correlated with reduced expression of the apoptotic and DNA damage markers when compared to free CDDP treatment. **Conclusions**: Our study results mark the establishment of "Nanosome" as an amenable tumor-targeted drug delivery vehicle for cancer treatment with reduced off-target cytotoxicity to normal cells. In addition, the nanosomes have the ability to carry different types of anti-cancer drugs (chemotherapy, miRNA, siRNA).

1102. Suicide Gene Therapy with Exosome Associated AAV6 Vectors Loaded with an Inducible Caspase 9 Gene is Therapeutic in Hepatocellular Carcinoma Models In Vivo

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Exosome associated Adeno-associated virus (exo-AAV) has gained attention as a novel gene delivery platform due to ease of their isolation and an improved gene transfer potential. The efficiency of such exo-AAV vectors during suicide gene therapy in general, and for highly fatal cancers affecting the liver such as hepatocellular carcinoma (HCC), in particular, is not known. We reasoned that development of an exo-AAV based vector which is naturally hepatotropic and containing an inducible caspase 9 (iCasp9) gene, that activates terminal effectors of cellular apoptosis may be beneficial for this condition. Moreover, the feasibility of harvesting exo- AAV vectors from spent media which otherwise is routinely discarded during vector production, motivated us to test the utility of this system for suicide gene delivery. In our preliminary studies, we identified that AAV6 serotype can efficiently target human hepatic cells. Based on these observations, we generated exosomal fractions during conventional AAV6-iCasp9 vector packaging, by an differential ultracentrifugation based protocol. Our culture conditions here, mimicked the routine packaging conditions for AAV production, without the incorporation of specialized reagents such as exosome-specific media. The exo-AAV vectors thus harvested, were further validated by suitable morphological and biochemical analysis. Transmission electron microscopy of the exo-AAV fraction revealed particles of ~50nm size (range: 30-130 nm). A colorimetric quantification of this fraction (EXOCET Exosome Quantitation Kit, System Bioscience) showed the presence of ~5 x 10e7 exo-AAV particles per microliter of the harvested sample. We further characterized the exo-AAV6 vectors by immunoblotting and confirmed the presence of specific tetraspanin markers (CD9, CD63) and AAV capsid proteins (VP1-3). In our next set of experiments, we tested the cytotoxic potential of these exo-AAV6-iCasp9 vectors, in a human hepatocellular carcinoma model *in vitro* and in a xenotransplantation model of HCC *in vivo*. Huh7 cells infected with the exo-AAV6-iCasp9 vectors and a pro-drug (AP20187) demonstrated a significant loss in cell viability (57 vs. 100 %, $p < 0.001$) in comparison to mock-treated cells. To further validate the therapeutic utility of exo-AAV vectors, we utilised a xenotransplantation model of HCC. Athymic nude mice (8-10 weeks old) was challenged with 5x10e6 Huh7 cells. When the animals developed palpable tumors (n=7-9 mice per group), they either received PBS (mock), AAV6-iCasp9 or exo-AAV6-iCasp9 vectors (~2x10e10 vgs, intra-tumorally) and injected thrice with AP20187 (1mg/kg body weight). Our outcome data, showed a significant regression in the tumor volume (2.4 fold) of animals treated with exo-AAV6-iCasp9 vectors, but not in the mock-treated group. AAV6-iCasp9 vectors also demonstrated a similar cytotoxicity profile both *in vitro* and *in vivo* establishing the functional equivalence of exo-AAV6 vectors to naked AAV6 vectors. Further validation of tumor sections from exo-AAV6-iCasp9 vector treated animals by TUNEL assay, revealed a small pyknotic nuclei and a marked presence of pro-apoptotic cells (175 vs 72; 175 vs 7, $p<0.001$) in comparison to either the conventional AAV6 vector administered or mock-treated animals. In conclusion, we have demonstrated the therapeutic utility of exosomal-AAV6 vectors for suicide gene therapy of HCC. This mode of gene delivery is especially attractive, since exo-AAV6-iCasp9 vectors can be harvested from media during conventional AAV packaging which is routinely discarded, the iCasp9 is a self-protein and thus is non-immunogenic and potentially applicable for the treatment of multiple solid malignancies (breast or head/neck cancer).

1103. mRNA Synthesis Reagents and Manufacturing: Research Through Clinical Development

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Advances in messenger RNA (mRNA) technology bring new possibilities for the treatment and prevention of diseases. As a relatively new therapeutic modality, mRNA offers advantages: It can instruct the ribosome to make almost any protein, it functions without entering the cell nucleus, and it degrades without leaving behind unwanted or harmful genetic traces. The clinical use of mRNA was initially limited due to low efficacy and undesirable immunogenicity. These issues have been addressed through optimization and modification of mRNA elements such as the 5' cap, nucleoside structures, poly(A) tail and 5'/3' UTRs. As a result, increasing demand for clinical use is straining manufacturing capacity. To reduce supply chain risk, minimize lead time and ensure the highest quality for our clients, Aldevron manufactures the linear plasmid DNA template and recombinant enzymes used for mRNA

synthesis performed by *in vitro* transcription (IVT). The production of mRNA at Aldevron can begin with the electronic construct sequence or supercoiled plasmid DNA provided by the client. During mRNA synthesis, Cap1 and poly(A) tail structures are added enzymatically or alternatively co-transcriptionally using a cap analog and template-encoded poly(A) tail sequence. Modified bases can also be incorporated dependent on project requirements. Purification is accomplished by column chromatography. A robust process was established by rigorous development. mRNA production is available in a range of quality grades from research grade to GMP and the process is identical between the service grades, allowing for a seamless transition from research applications to the clinic.

1104. Generation of Human CAR-T Cells Using the Solupore Non-Viral Cell Engineering Platform

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Solupore® is a non-viral ex vivo cell engineering platform that enables development and manufacture of cell therapies, using reversible permeabilization to achieve rapid intracellular delivery of cargos. The technology achieves intracellular delivery and subsequent reversal of cell permeabilization by precisely controlling the contact of the target cells with a solution containing the cargo. We have termed the method 'soluporation'. Non-viral methods, such as soluporation, that enable intracellular delivery of various cargo types for clinical applications are attractive candidates for next-generation delivery modalities because of potential benefits for manufacture, safety and regulation compared with viral vectors. While electroporation is currently the most widely-used non-viral method used for cell engineering, it can be damaging to cells. In contrast, we have previously demonstrated that soluporation causes minimal perturbation of human T cells. Here we have further investigated the impact of soluporation and nucleofection on T cells by carrying out gene expression profiling in transfected cells. In nucleofected cells, 263 out of 574 immune related genes were misregulated at 6 h post-transfection. These genes clustered to several immune-related pathways including T cell activation, metabolism and exhaustion. In contrast, 8 genes were misregulated in soluporated cells. This indicates that soluporation maintains cellular function similar to untreated control cells, unlike nucleofection. We also assessed whether Solupore® could be used to engineer T cells to express a chimeric antigen receptor (CAR) and if these cells could effectively kill cancer cells in vitro. CD19 CAR mRNA was delivered to primary human T cells with on average 60% CD19 CAR expression detected at 24 h post-transfection. These CD19-CAR T cells were able to kill target cancer cells in a density-dependent manner. These studies demonstrate that the Solupore® technology is a gentle modality for efficient engineering of immune cells. The technology is highly reproducible, automated, and scalable and has the potential to enable a broad range of T cell engineering applications.

Neurologic Diseases

1105. Crispr-Cas12a Editing of an Usher Syndrome Deep-Intronic Mutation Restores USH2A Splicing

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Usher syndrome is an autosomal recessive disease and represents the most common cause of inherited deaf-blindness. Usher syndrome patients are characterized by vision loss, hearing loss, and sometimes balance problems. Based on the clinical symptoms Usher syndrome is classified into three types, of which type 2 Usher syndrome is the most common one. Among the several genetic loci that have been associated with Usher syndrome type 2, mutations in USH2A, the gene encoding for the Usherin protein, account for 55-90% of type 2 Usher syndrome patients. The c.7595-2144A>G mutation, the second most frequent USH2A mutation, is a deep intronic mutation causing the incorporation of a 152bp-pseudoexon into the mature transcript, finally resulting in the premature termination of translation. Since the correction of the USH2A splicing in affected cell types is expected to revert the disease phenotype in Usher syndrome patients, we developed a strategy to target the c.7595-2144A>G mutation within the USH2A gene in order to permanently restore the correct splicing of the USH2A pre-mRNA, thereby leading to the expression of wild-type Usherin protein. Our approach exploits Cas12a nucleases to target splicing regulatory elements within USH2A intron 40. We first generated minigene models recapitulating the splicing pattern observed in wild-type and c.7595-2144A>G mutated USH2A transcripts and tested our strategy in HEK293 cells expressing the minigenes. Our approach was demonstrated to be highly efficient, with almost complete restoration of the splicing pattern of the mutated allele and no effects on its wild-type counterpart. The best performing gRNA candidates were further tested on type 2 Usher syndrome patient-derived fibroblasts carrying the c.7595-2144A>G mutation. In conclusion, we identified an allele specific approach correcting the splicing defect caused by the USH2A c.7595-2144A>G mutation. Further development of this allele specific editing strategy may result in a safe and effective approach that may be adapted to work as a one-off treatment for Usher syndrome type II patients carrying the targeted mutation.

1106. RNA Editing for Usher Syndrome Using CRISPR-Cas13

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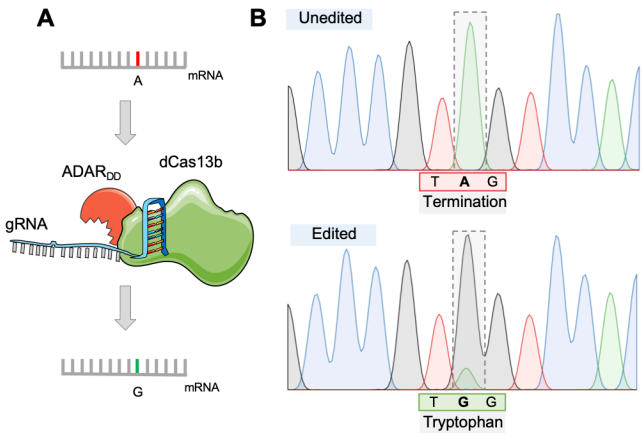
Background: Usher syndrome is the leading cause of deaf-blindness, causing progressive retinal degeneration and sensorineural deafness. Usher syndrome is an autosomal recessive disorder commonly caused by mutations in *USH2A*, which encodes the Usherin protein in photoreceptors and the inner ear. RNA editing is a potential therapeutic

strategy to correct common pathogenic variants. Adenosine deaminase acting on RNA (ADAR) enzymes convert adenosine to inosine, which is read as guanosine by the cell. Deactivated Cas13b enzymes bind RNA as directed by a guide RNA, and when fused to an ADAR deaminase domain, enables targeted correction of G>A mutations in RNA (Fig A). **Aim**: To investigate the use of RNA editing with CRISPR-dCas13b-ADAR to correct a common *USH2A* mutation c.11864G>A (p.W3955X). **Methods**: A dual luciferase reporter assay was developed to contain mutant sequences from human (*hUSH2A*) and mouse (*mUsh2a*) transcripts for guide RNA screening. Editing activity was measured via restoration of a firefly luciferase in HEK293T cells transfected with constructs expressing the reporter assay, dPspCas13b-ADAR variants and guide RNAs, RNA editing rates were analysed by trace decomposition of Sanger sequencing reads of cDNA. **Results**: Guide RNAs targeting hUSH2A and mUsh2a were designed and screened, demonstrating a range of on-target activity (2-55%) determined by the mismatch distance between the target adenosine and the guide RNA scaffold. RNA sequencing demonstrated the restoration of the target premature termination codon (TAG) to the wildtype tryptophan codon (TGG) at a mean rate of 77% (1.5) and 86% (2.1) of hUSH2A and mUsh2a transcripts respectively (n=3, (SD)) (Fig B). Constructs containing ADAR with the hyperactive E488Q mutation demonstrated high on-target editing activity but also off-target editing of adenosines across the transcript. Constructs with the specific E488Q/T375G mutation demonstrated a 50% loss of on-target activity relative to constructs with on the E488Q mutation, but off-target editing was undetectable. Editing was not detectable with non-targeting guide RNAs, or with expression of guide RNAs without dCas13-ADAR. **Conclusion**: These data support RNA editing as a potential therapeutic approach for pathogenic G>A variants in Usher syndrome.



1107. rAAV-Mediated *PEX1* Gene Delivery Improves Retinal Functions and Peroxisome Metabolites in a Mouse Model for Zellweger Spectrum Disorder

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Introduction. Zellweger spectrum disorders (ZSDs) are a group of autosomal recessive disorders caused by mutations in any one of 13 *PEX* genes whose protein products are required for peroxisome assembly and function. More mildly affected individuals harboring the common PEX1-G843D mutation consistently develop a potentially blinding retinopathy. To test whether we could improve vision in these patients, we performed a proof-of-concept trial for *PEX1* retinal gene augmentation therapy using our mouse model homozygous for the equivalent PEX1-G844D mutation. This model exhibits a gradual decline in scotopic full field flash electroretinogram (ffERG) response, a residual photopic ffERG response, diminished visual acuity (optokinetic reflex, OKR), and photoreceptor cell anomalies. **Methods/Results.** AAV8.CMV.*HsPEX1*.HA vector was administered by subretinal injection to 2 mouse cohorts of 5 or 9 weeks of age; AAV8.CMV.GFP vector was used as a control in the contralateral eye. Successful expression of HsPEX1.HA protein in the photoreceptor layer with no gross histologic side effect was observed by immunohistochemistry. *In vitro* studies using *PEX1*-deficient mouse and human cell lines confirmed vector expression and successful recovery of peroxisome import after *HsPEX1* delivery. Preliminary ffERG and OKR analyses on a subset of animals at 8 weeks post injection showed two-fold increase in photopic ffERG and a slight (p=0.054) improvement in visual acuity, respectively, in the *PEX1*-injected eyes. ffERGs were improved by 1.6 to 2.5-fold at 25-weeks of age in both cohorts (16 or 20 weeks post gene delivery). At 32 weeks of age (23 or 27 weeks post gene delivery), the average ffERG response in the *PEX1*-injected eyes was double that of GFP-injected eyes in both cohorts. LC-MS/MS was used to measure peroxisomal metabolites in whole retinas, revealing that in the younger cohort *PEX1* gene augmentation reduced the average C26:0 lysophosphatidylcholine (lyso-PC) in PEX1-G844D mice, which is elevated due to peroxisome dysfunction. Finally, matrix-assisted laser desorption/ionization (MALDI)-imaging MS was used to resolve the spatial distribution of retinal lipids along retinal sections, and we explore the use of this technique for correlating regions of rAAV delivery with biochemical recovery. **Conclusions.** Robust PEX1 protein expression with no gross histologic side effect was observed in homozygous PEX1-G844D mice injected subretinally at 5 or 9-weeks-of age with AAV8.CMV.h*PEX1*.HA. Neither the injection, nor exposure to the AAV8 capsid or the transgenic protein negatively altered the ffERG or OKR response. At 5-6 months after gene delivery, therapeutic

vector-treated eyes showed improved ffERG compared to control eyes, on average, in both the younger and older cohorts, and peroxisome metabolite recovery indicated a broader recovery of retinal peroxisome functions. Imaging MS provided a power new tool for demonstrating metabolic recovery at distinct tissue regions following rAAV gene delivery, without contamination by non-transduced regions. Overall, our results support the clinical potential of retinal gene augmentation to improve vision in patients with ZSD at both earlier and later stages of disease.

1108. Abstract Withdrawn

1109. Abstract Withdrawn

1110. Abstract Withdrawn

1111. CRISPR-Cas9 Gene Editing Strategies to Treat Spinocerebellar Ataxia Type 1

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Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that causes rapidly progressive loss of motor coordination, respiratory issues and eventual death. This disease is adult onset with symptoms typically appearing in the mid-30s, although this is highly variable. Symptoms typically progress for 10-20 years before death; however, in the juvenile onset disease, progression is much faster. SCA1 is caused by a mutation in the *ATXN1* gene leading to an expansion of the polyglutamine repeat region.Normal *ATXN1* alleles contain 6-42 CAG trinucleotide repeats with interspersed CAT nucleotides, while disease alleles have an uninterrupted CAG region with 39-100+ repeats. The mechanism of SCA1 pathogenesis is unknown; however, some features of the disease include neuronal degeneration and formation of toxic mutant ATXN1 (mATXN1) nuclear inclusions. Although mATXN1 is expressed ubiquitously, it affects primarily Purkinje cells (PCs). There are currently no treatment options for SCA1. We hypothesize that CRISPR-Cas9 editing of *ATXN1* will reduce mutant ATXN1 and be therapeutically beneficial. We have designed three different strategies targeted to reduce ATXN1; by using a single guide RNA (gRNA) to target early in the gene near the translational start site, using a single gRNA to target near the exon-exon junction to induce non-sense mediated decay, and a dual guide system to target before and after the CAG repeat and excise it. We screened gRNAs that target *sp*Cas9 to *ATXN1* in HEK293 cells and collected mRNA and protein for assessment. We identified gRNAs from each strategy that significantly reduced the amount of *ATXN1*mRNA and ATXN1 protein levels. We found that the dual guide system targeting the CAG region for excision was the most efficient followed by the exon-exon single guide strategy. This work indicates a potential therapeutic strategy that we plan to test in SCA1 mice to determine if knockout of *ATXN1* is able to prevent disease onset in a mouse model of disease. We have crossed SCA1 mice with mice that endogenously express *sp*Cas9 to create a model to test our guide strategies *in vivo.* We characterized these mice in different behavioral assays for motor coordination and found that they have significant motor defects at 20 and 30 weeks. These results provide a clear path forward to further test different CRISPR/*sp*Cas9 strategies to treat SCA1 in mice and a potential translational therapy for humans with SCA1.

1112. The Efficacy and Safety of Wnt and Notch-Signaling Modulators in the Cochlea

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Research Background & Objectives Sensorineural hearing loss is usually caused by damage to cochlear hair cells, which is irreversible in mammals. *In vivo* regeneration of hair cells is a desired treatment option for this type of hearing loss. Previous studies described the feasibility to generate neonatal and adult mouse cochlear hair cells *in vitro* via manipulation of Wnt and Notch signaling pathways, leading to the differentiation of Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) expressing supporting cells into hair cells. **Objectives**:**(i)** Establish the normal hearing thresholds and characterize the status of mature hair cells and LGR5$^+$ supporting cells in the normal hearing **adult** mouse cochlea (postnatal day 30, P30), **(ii)** Study the ototoxicity effects on the status of LGR5$^+$ supporting cells, **(iii)** Optimize the therapeutic efficacy of growth factor administration *in vivo* to stimulate the (trans-) differentiation of the LGR5$^+$ supporting cells into mature and functional hair cells with minimal or no toxicity. **Methods** Auditory brainstem responses (ABRs) were measured in adult (**P30**) wild type (WT) and Lgr5-EGFP-IRES-creERT2 heterozygous (*LGR5$^{EGFP/\pm}$*) littermates to test hair cell function and their connections with the auditory nerve. Cochlear whole mounts and cryosections were prepared and processed for immunohistochemistry. **Results Hearing thresholds of adult *LGR5$^{EGFP/\pm}$* mice are comparable to wild type:** No differences in average ABR thresholds (in dB peak equivalent (pe) SPL) were observed between the two groups: 45.8 ± 2.8 (WT, *n*= 4) and 41.3 ± 5.5 (GFP, *n*= 5). **LGR5- EGFP expression is detected in specific supporting cells of the organ of Corti:** In the organ of Corti, inner (IHCs) and outer (OHCs) hair cells were reactive to Myo7a and EGFP expression was detected in the supporting cells, including the 3rd row of Deiter (DC3) and, to a lesser extent, in the pillar cells (PCs). **Ototoxicity - Induced Hearing Loss (Figure 1)**

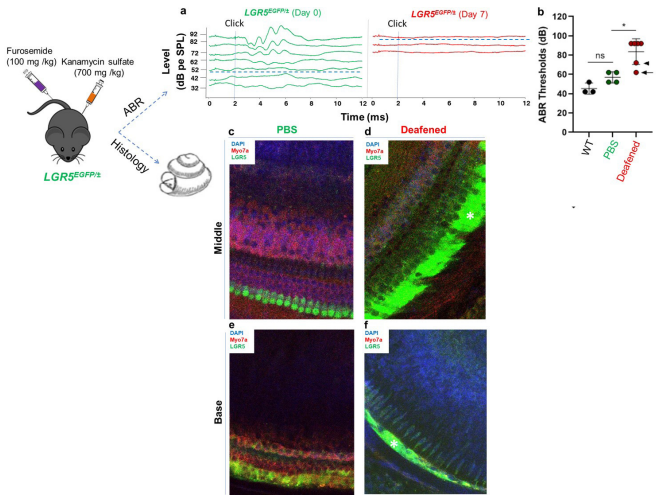

Figure 1: Elevated hearing thresholds, hair cell loss and abnormal morphology of the supporting cells after deafening. a) Representative click-evoked ABR tracings from normal hearing P30 *LGR5*$^{EGFP/\pm}$ and the same mouse seven days after injection of ototoxic drugs. Dotted line represents the ABR thresholds (dB pe SPL). **b)** ABR thresholds of normal hearing WT mice, normal hearing *LGR5*$^{EGFP/\pm}$ recipients of PBS and deafened *LGR5*$^{EGFP/\pm}$ (day 7 after injections). One mouse did not seem to respond to the ototoxic medication (arrow) and another mouse had a minimal threshold shift (arrow-head). Mann Whitney test: *$p< 0.05$; ns, not significant. **c-f)** Z-stack images (40x) of whole mounts. Compared to the PBS recipients (c, e): IHCs, but not OHCs, were detectable in the middle turn of deafened mice (d), IHCs and OHCs were absent in the basal turn (f) and LGR5^{+} supporting cells displayed abnormal morphology (*, d and f). **Conclusions**We report that LGR5+ supporting cells are detectable in the adult LGR5EGFP/± mouse cochlea and hearing thresholds are comparable to wild type mice. Ototoxic drugs induced hair cell loss and altered the integrity of the LGR5+ supporting cells, following a base to mid turn gradient. This is the first study to report cellular changes of the adult mouse cochlea LGR5+ supporting cells in response to ototoxicity. Elucidating the molecular mechanisms underlying these changes in relation to the altered microenvironment is fundamentally important for treating hearing loss.

1113. An *Isogenic* hiPSC-Derived Model System for Establishing Gene Therapy Approaches in Alzheimer's Disease

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The apolipoprotein ε4 (*APOE* ε4) allele is the strongest and most reproducible genetic risk factor for late onset Alzheimer's disease (LOAD). Since the seminal original discovery of the *APOE* gene, its role and the role of its protein isoforms, APOE ε2, APOE ε3, and APOE ε4, have been studied extensively in the context of LOAD. Although the function of the APOE protein in health and disease has yet to be uncovered, numerous studies have pointed to the adverse effect of the ε4 allele compared to the natural role of the ε3 allele. These studies lend support for the unmet need of developed therapeutic approaches specifically targeting the ε4 isoform. Our team's overarching goal is to develop a gene therapy-based approach to lower the expression of the toxic APOE ε4 isoform. The first step toward achieving this goal was to develop a suitable and feasible model system for screening and evaluating the efficacy of the gene therapy approach. Two CRISPR/cas9-edited *isogenic* human-induced pluripotent stem cell (hiPSC) lines were employed. The hiPSC lines were edited to be homozygous for the ε3 and ε4 alleles, but were isogenic on whole genome level, such that the only difference between the lines was SNP rs429358. The two lines were differentiated into cholinergic neurons that are involved in LOAD pathogenesis. The hiPSC-derived neurons were characterized comprehensively for pathological LOAD and aging-related phenotypes. Evaluations of LOAD-related pathological phenotypes include relative levels of secreted Aβ42 to Aβ40, ratios of phospho-tau to total tau, and neurite outgrowth. Compared to the *APOE* ε3/ε3 hiPSC-derived neurons, we observed a significantly greater ratio of secreted Aβ42 to Aβ40 as well as an increased relative level of phospho-tau in the *APOE* ε4/ε4 hiPSC-derived neurons. In addition, we showed a decrease in the number of neurites per cell and in total neurite length in the *APOE* ε4/ε4 hiPSC-derived neurons compared to the ε3/ε3 neurons. Evaluations of age-related perturbations included DNA damage, nuclear circularity and nuclear membrane folding. These examinations revealed higher levels of DNA damage, a reduction in nuclear circularity, and an increased proportion of abnormal, damaged nuclei in the *APOE* ε4/ε4 hiPSC-derived neurons compared to ε3/ε3 neurons. Furthermore, the same *isogenic APOE* hiPSC lines were differentiated to astrocytes, the brain cell-type in which APOE is abundant. Similar to the neuronal line, the astrocytes derived from the ε4/ε4 hiPSCs demonstrated an exacerbation of the age-related phenotypes compared to the ε3/ε3 line. This developed set of thorough phenotypic characterizations is useful and applicable in screening therapeutic approaches precisely targeting the *APOE* ε4 allele and in screening particular gene therapy tools aimed at the specific downregulation of ε4 allele expression. Upon applying the gene therapy tools on the *APOE* ε4/ε4 hiPSC-derived neurons, we will assess the extent of rescue of this set of phenotypic perturbations and identify the gene therapy tool that best corrects the pathologic and aging-related perturbations, such that the rescued phenotypes are comparable to those of the control *APOE* ε3/ε3 neuronal line. Finally, current effort is aimed in the development of a 3D *isogenic* human AD tri-culture model to recapitulate key interactions between neurons, astrocytes, and microglia in the nervous system. These human based model systems are essential for establishing proof of concept for gene therapy tools targeted to mitigate the adverse effect of *APOE* ε4 on LOAD risk and progression.

1114. Bridging Efficacy Data in an ALS Mouse Model with Clinical Route of Administration in Nonhuman Primates to Derive a Meaningful Clinical Dose Range

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Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease caused by degeneration of motor neurons leading to rapidly progressive paralysis. Survival is typically 3 to 5 years. There is no cure for ALS, and FDA-approved treatments only modestly extend survival. About 10% of ALS cases are caused by gain-of-function mutations that are transmitted as dominant traits. A potential therapy for these cases is to suppress the expression of the mutant gene. APB-102 is comprised of an AAVrh10 vector which effectively targets both upper and lower motor neurons, encapsidating an artificial miRNA that silences SOD1 regardless of the specific mutation harbored in the SOD1 gene. This vector demonstrated therapeutic efficacy in an aggressive ALS mouse model, the high-copy SOD1^{G93A} mouse, where it significantly extended survival. To inform clinical dose selection, dose escalation studies were carried out in parallel in mice and cynomolgus monkeys. Consistent with prior proof of concept data, treatment with APB-102 significantly extended survival at all doses tested, in a dose-dependent manner. In mice, vector genomes, mature artificial miRNA, and SOD1 mRNA were quantified by ddPCR in laser-captured motor neurons at cervical and lumbar level. The same dataset was generated in non-human primates that were part of a GLP toxicology/biodistribution study. Considering several differences between studies and particularly transgene copy number (2 in NHP vs 18-25 in mice) and delivery route (intrathecal in NHP vs intracerebroventricular in mice), the comparison of both murine and cynomolgus datasets helps delineate therapeutic efficacy and constitutes a basis for selecting a clinically meaningful dose range. SOD1 silencing was consistently around 90% in NHP at all doses, highlighting the efficacy of the clinical route of administration selected and the potency of APB-102 produced with our proprietary process.

1115. AAV Expressing Retinol Dehydrogenase Can Reduce Retinoid Toxicity in STGD1 Rat Model

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The photoreceptor-specific ATP-binding cassette transporter encoded by the *ABCA4* gene, prevents the build-up of the toxic by-products that result from phototransduction. Upwards of 1000 mutations have been identified in the ABCA4 gene and these mutations can result in Stargardts disease (STGD1). We have analysed two rat models of STGD1, one with a knockout of *Abca4* and the second carrying the most common mutation found in STGD1 - G1961E. This mutation has been demonstrated to result in a compound heterozygous form of STGD1. Moreover, it has a high carrier frequency and has been implicated in increased risk of AMD in certain populations. We investigated the molecular basis of this mutation and its implications for therapy. We show that we can reverse the retinoid toxicity in these rat models by overexpression of human retinol dehydrogenase enzymes. These enzymes actively reduce the reactive all-trans retinal to the less reactive retinol. However, this overexpression is shown to also be disruptive to the delicate metabolic balance of the photoreceptor cell, potentially by depleting reserves of the reducing equivalent NADPH. As such the therapeutic value of such an approach can be limited and still has to be investigated in relevant models.

1116. Effect of Transthyretin Gene Therapy Alzheimer's Disease Progression

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Epidemiological studies and work in transgenic animals suggest that transthyretin (TTR) levels in plasma and/or CSF may modulate Alzheimer's disease (AD) presentation and/or progression. AD patients exhibit lower levels of TTR in CSF compared to age-matched healthy individuals. Similarly, genetic reduction of TTR in mouse models of AD is associated with earlier onset and more severe phenotypes. The current study was designed to determine the potential of somatic AAV mediated TTR expression to modulate disease presentation in AD.We designed an AAV9 vector to express TTR specifically in liver and choroid plexus using a TTR promoter to drive transgene expression. Two months old male and female 5XFAD mice were injected systemically with 2E12 gc AAV9-TTRp-TTR^{T119M} (n=3M+6F), or PBS as controls (n=3M+6F). At nine months of age animals were subjected to neurobehavioral tests to assess cognitive function, and 3D isotropic T2-weighted MRI to evaluate differences in brain volume. In a parallel experiment, we used the same vector to treat APP^{NL-G-F} mice, both systemically at two and seven months of age (pre- and post-symptomatic) and also by direct CSF delivery at a pre-symptomatic stage.Cognitive function was assessed through measure of working and spatial memory using forced and spontaneous alternation tasks in the Y-maze, and novel object recognition tests. In the 5XFAD model, all treated animals showed increased recognition index of novel objects, increased % of alternation, and % time in novel arm, compared to controls. General locomotion and anxiety were evaluated using the open field test, where AAV treated females presented higher locomotor activity and decreased anxiety levels. Interestingly, MRI revealed increased CSF volume in all treated animals compared to controls, with females showing larger differences. However, no differences were observed in the APP^{NL-G-F} model in any of tests, independently of age of treatment or administration route. Our initial results show that systemic delivery of an AAV9 vector that expresses TTR in appropriate tissues improves overall cognitive function, locomotor activity and anxiety symptoms in 5XFAD mice, but this benefit is more noticeable in females. The unexpected increase in CSF volume may be related to increased TTR production by the choroid plexus of AAV treated animals, and additional experiments are ongoing to determine the cause of this outcome. Surprising, our gene therapy approach only

showed behavioral effect in one mouse model of AD. Biochemical and histological studies of the brain are currently ongoing to assess the disease status in all experimental groups.Overall, this new AAV vector represents a therapeutic platform to study biologically relevant questions about the role of TTR in AD.

1117. Modulating Stress Granule Formation for Treatment of ALS via CRISPR-Cas13-Mediated Knockdown of Ataxin-2

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Amyotrophic lateral sclerosis (ALS) is an incurable and fatal neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain and spinal cord. A major pathological hallmark of the disease, present in more than 90% of ALS cases, including familial and sporadic forms of the disorder, is the cytoplasmic mislocalization and aggregation of TAR DNA-binding protein 43 (TDP-43). TDP-43 toxic deposition can be induced by the formation of highly dynamic cellular structures termed stress granules (SGs) that assemble transiently in response to cellular stress. Ataxin-2 (ATXN2) is an RNA-binding protein that has been identified as a crucial SG component and a contributor to neurodegeneration. ATXN2 is abnormally localized in cytoplasmic accumulations in motor neurons of ALS patients and is known to associate with TDP-43 in a complex. Accordingly, antisense oligonucleotide (ASO)-mediated reduction of ATXN2 has been used to decrease stress-induced TDP-43 pathology in human cells and a TDP-43 mouse model of ALS, indicating its potential as a therapeutic target. However, the application of ASOs for clinical care has several limitations, including their transient effect and the resulting need to repeatedly administer an ASO over the lifetime of a patient. RNA-targeting CRISPR-Cas13 systems are an alternative technology for gene silencing that can enable long-term knockdown in motor neurons when delivered via a viral vector, such as an adeno-associated virus (AAV). Thus, we have hypothesized that Cas13-mediated ATXN2 RNA knockdown can be used to reduce the cytoplasmic mislocalization and accumulation of ATXN2 and, consequently, diminish the formation of TDP-43 toxic inclusions in ALS-like backgrounds. To test this hypothesis, we developed a reporter system that links mouse ATXN2 cDNA expression to EGFP fluorescence. Using this reporter, we identified a panel of CRISPR-Cas13 effectors that can knock-down mouse ATXN2 with efficiencies exceeding 90%, which we confirmed by western blot. Owing to homology between the mouse and human ATXN2 cDNAs, we were able to harness one of these systems to modulate the dynamics of stress granule formation in human cells, which we tested by first transfecting cells with an ATXN2-targeting CRISPR-Cas13 system and then inducing stress in these cells using an SG-provoking agent. Using immunofluorescence, we found that cells expressing the ATXN2-targeting guide RNA and Cas13 exhibited an ~40% reduction in ATXN2^{+} inclusion formation and an ~30% reduction in the average size of ATXN2^{+} inclusions. Additionally, compared to control cells, we found that CRISPR-Cas13 decreased the number of TDP-43^{+} inclusions per cell, as well as the average size of G3BPI^{+} SGs by ~56% and ~43%, respectively, indicating that ATXN2 knockdown by CRISPR-Cas13 can inhibit stress-induced assembly of SG cores. We are now evaluating this strategy in mouse models to further characterize its effect on ALS-like pathology. These results suggest that CRISPR-Cas13 technology can be adapted to modulate the dynamics of ALS-linked SGs and, in the future, may help to prevent the aggregation of SG-associated RNA binding proteins that contribute to neurodegeneration.

1118. Enhancing Efficacy of Systemic AAV9 Gene Delivery in Sod1G93Amouse Model of ALS

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Neuronal transduction by AAV vectors is highly efficient upon local delivery. However, after systemic delivery, central nervous system (CNS) transduction requires crossing of physical barriers, such as the blood-brain barrier (BBB). In adults, the BBB is formed by highly regulated tight junctions in the brain endothelia with very low permeability to macromolecules. One approach to manipulate this mechanism and enhance the efficiency of AAV9 CNS gene transfer is to temporarily disrupt the BBB, using for example focused ultrasound. Here we tested a new approach to increase the efficiency of systemic AAV9 CNS gene delivery through the co-administration of vector with a previously described peptide capable of permeabilize the BBB. Previously, we have shown that co-delivery of this peptide with AAV9 results in ~600 fold increase of Firefly luciferase activity in the brain of BALB/cJ and C57BL/6J mice, without any significant change in liver. In order to test the therapeutic potential of this approach, we conducted short-term studies in a mouse model of amyotrophic lateral sclerosis (ALS). Several genetic mutations have been associated with increased risk of ALS, both sporadic and familial cases. A small percentage of cases are associated with a toxic gain of function mutation in the superoxide dismutase (SOD1) gene, leading to the development of therapeutic approaches aiming to silence gene expression in the brain and spinal cord. Multiple studies have been published using AAV vectors to silence SOD1 in the transgenic SOD1^{G93A} mouse by delivering an artificial miRNA against SOD1, with significant increases in lifespan and reduction of SOD1 expression by almost 50% when an AAV9 vector was administered into the CSF of neonate animals by ICV injection.In this study, SOD1^{G93A} mice were treated at 6 weeks of age by systemic administration of 1E12 vg of AAV9-U6-amiR^{hSOD1} with or without BBB-disruptor peptide. At 4 weeks post-injection there was a significant reduction in the gene expression of hSOD1 in the spinal cord of animals treated with AAV9+peptide, when compared to AAV9 alone and PBS control animals, as assessed by qPCR. This effect was more noticeable in males (50%) than in the females (33%). In parallel, we also observed a significant decrease in hSOD1 expression in the motor cortex of males treated with the combinatory therapy. *In situ* hybridization, using RNAscope fluorescent probes, further confirmed these results.In conclusion, the combination of systemic AAV9 CNS gene delivery with a peptide that temporarily disrupts the BBB is a powerful approach to potentiate the efficacy of an already promising gene therapy approach.

1119. Development of Therapeutic Strategies for SCN2A Mutation Related Disorders Using New *In Vitro* Modeling Techniques

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Mutations in the *SCN2A* gene have been identified to cause early onset epilepsy, autism spectrum disorder, intellectual disability and other neurological dysfunctions. *SCN2A* encodes a neuronal voltage gated sodium channel (Nav1.2) that is primarily sought to be expressed in excitatory neurons and at low levels also in astrocytes. There are no hot spot mutations associated with *SCN2A* related disorders making it complicated to mimic the wide disease spectrum phenotypes in mouse models. Our goal was to develop a cell-based assay using astrocytes and/or neurons to evaluate potential therapeutic strategies. This will allow us to test several therapeutic approaches using a variety of *SCN2A* mutations before moving into *in vivo* models and help cluster patients reacting *in vitro* to some therapeutic approaches. Based on results we and others have obtained from other neurological disorders, we hypothesized that SCN2A patient astrocytes would be toxic to other central nervous system cell types. In addition, based on increasing literature of mitochondrial defects in Autism and Epilepsy, we also sought to look at potential mitochondrial defects in astrocytes and check whether SCN2A patient neurons could have lower survival percentage and expression of Nav1.2. Finally, we tested a small molecule known to support mitochondrial health. Since it is difficult to obtain post-mortem brain tissues from SCN2A patients, we reprogrammed skin fibroblast cells from three patient cell lines into neuronal progenitor cells and subsequently differentiated them into induced-astrocytes (i-astrocytes). Using i-astrocytes cells, we assessed SCN2A protein activity by sodium intake assays. Mitochondrial morphology and function were assessed by immunostaining and by directly measuring the oxygen consumption rate (OCR) using seahorse assays. Survival of WT GFP-Motoneurons (MNs) was assessed using co-culture with SCN2A i-astrocytes. We also directly converted SCN2A patient fibroblasts into induced-neurons (i-neurons) to assess percent of neuronal survival, and SCN2A protein expression by immunostaining. i-astrocytes from SCN2A patients demonstrate higher channel activity, varying degrees of toxicity toward MNs and contain abnormal mitochondrial shape. OCR assay showed that all SCN2A i-astrocytes have elevated basal oxygen respiration and adenosine triphosphate (ATP) production, suggesting an increase in mitochondrial activity and differences in astrocyte metabolism. Based on the mitochondrial abnormalities, we tested a compound known to support mitochondrial health. Our preliminary results indicate that this drug leads to (i) a reduction of i-astrocyte mediated toxicity towards MNs, (ii) a normalization of the basal respiration and ATP-linked respiration of SCN2A astrocytes, and (iii) an improvement of neuronal survival in SCN2A patients. In summary, we report for the first time a rapid disease modeling system for SCN2A related disease phenotypes combined with drug candidate testing. The assay we developed in this study is suitable for high-throughput drug screening. Our findings underline the crucial role of astrocytes in SCN2A related disorders and identify mitochondria as a major target for drug development. More importantly, we demonstrate that a tested compound could be a potential therapeutic for the treatment of at least a sub-population of patients with SCN2A mutations.

1120. Optimization of AAV Gene Delivery Vector Achieves Selective, Safe and Strong Müller Glia Targeting in Healthy and Diseased Rodent Retina

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Müller glia-based gene and cell therapies hold promise to alleviate or cure many retinal degenerative disorders including retinitis pigmentosa and age-related macular degeneration. To date, ShH10.Y445F is the only engineered adeno-associated virus (AAV) displaying capsid-driven tropism for Müller glia cells (MGCs), and therefore is used as the standard vector to target glia in the retina. Still, complete cell selectivity, in order to prevent serious off-target effects and also confirm cell rescue in proof-of-concept gene therapy studies, remains a challenge due to large cell-specific promoters limiting DNA carrying capacity. Here, we characterized and compared glial expression specificity, transduction and ocular toxicity levels resulting from intravitreal injection of ShH10.Y445F or 7m8, an engineered variant with strong and pan-retinal tropism for all retinal cells, driving transgene expression under different glial or ubiquitous promoters. We found that 7m8.scGfaABC1D.eGFP was the best vector to selectively and efficiently transduced pan-retinally MGCs and showed significant and consistent visual rescue in rd10 mice injected with 7m8.scGfaABC1D.GDNF. **Methods**: AAV self-complementary plasmids encoding for GFP under the control of CAG (0.780kb), , GfaABC1D (0.681kb), GFAP (2.2kb) and GLAST (2.1kb) promoters were packaged into 7m8 and ShH10. Viruses were injected intravitreally in P30 WT C57BL/6J and rd10 mice. Four weeks post-injection, fundus imaging confirmed high transgene expression. Retinas were flatmounted and cross-sectioned to analyze promoter selectivity. Retinas were stained using GFAP and GS antibodies as markers for astrocytes/glia. Retinas were also dissociated to isolate eGFP+ cells and RNA extracted for gene expression analysis. Electroretinogram recordings (ERGs) were performed pre- and post-injections as well as IBA1 staining on retinas to assess the level of ocular toxicity. Transcription factor binding site (TFBS) motifs in the short and long GFAP and GLAST promoter sequences were identified and analyzed. ERG recordings as well as ELISAs to measure hGDNF secretion levels were performed in rd10 mice injected with either ShH10.Y445F- or 7m8.scGfaABC1.GDNF. **Results**: As expected, ShH10.Y455F displayed capsid-driven specificity for Müller glia, with strong expression along the retinal vasculature. 7m8 transduced Müller glia pan-retinally but not preferentially. The short GfaABC1D promoter sequence, which is enriched in glia missing TFBS, drove strong and selective expression in MGCs, compared to the GLAST promoter (2.2kb), which showed ganglion off-target expression. GfaABC1D displayed a lower ocular toxicity profile compared to the long GFAP promoter. While transcriptional changes in Müller glia during degeneration did not affect the tropism pattern of the different vectors tested, the efficiency of transduction was lower in the degenerated retina, particularly ShH10.Y445F.

The 7m8.scGfABC1.GDNF vector achieved significant hGDNF secretion in rd10 and functional delay of retinal degeneration. **Conclusion:** Selectively targeting glia is critical to more precisely "dissect" the role of glia in the healthy and diseased retina. It represents an attractive approach for novel therapies (e.g. stem cell therapies focused on the dedifferentiation of glia into photoreceptors) requiring no off-target expression. Our study shows that the short GFAP promoter, gfaABC1D, in combination with 7m8 leads to strong, pan-retinal and selective Müller glia expression in degenerating and healthy retinas with a low ocular toxicity profile compared to the long parental promoter, and can be the new standard vector for MGC-targeted gene therapies.

1121. Human Undifferentiated Neural Progenitor Stem Cells (NPC) Alleviate Motor Dysfunction, Repair Morphological Damage and Extend Life Span in a Spastic Han-Wistar Model of Cerebellar Ataxia

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The spastic Han Wistar (sHW) rat represents a model for human cerebellar ataxia characterized by forelimb tremor, hind-leg rigidity, gait abnormality, weight loss, life span about 65 days caused by neurodegeneration of Purkinje cells and CA3 pyramidal neurons. To address these problems, a line of NPCs that are able to tune their proliferation and vector of differentiation in response to micro-environmental cues was manufactured, fully characterized and used in this study. Proteomic analysis of NPC detected secretion of Neuromodulin, 14-3-3 Epsilon Protein, Insulin-like Growth Factor II and other neuroprotective and anti-inflammatory factors that can be a part of mechanism of action of NPC. At 30 days of age, male sHW mutant rats underwent subcutaneous implantation of Alzet osmotic pump that infused cyclosporin (15 mg/kg/day). At 40 days, sHW rats received bilateral injections (500,000 cells in 5 μl media) of live NPCs (experimental) or dead NPCs (control) into interposed nucleus of the cerebellum. Motor activity scores and weights of the animals were recorded weekly. At day 45, the experimental animals began to exhibit improved motor activity and survived past 100 days of age. The motor scores were statistically similar to those of normal animals. Immunohistochemistry revealed few surviving NPCs in the cerebellum of 100 day old experimental animals. Stereological analysis demonstrated significant increase in the numbers of Purkinje neurons compared to controls. These data warrant a clinical trial of NPCs for the treatment of cerebellar ataxias that currently have no recourse

1122. CRISPR/Cas9 Mediated Genetic Correction of Hematopoietic Stem Cells from Patients with Friedreich's Ataxia

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Friedreich's ataxia (FRDA) is a multi-systemic autosomal recessive disorder that is predominantly caused by a homozygous GAA repeat expansion mutation within the first intron of the frataxin gene (*FXN*). This mutation leads to the transcriptional silencing of *FXN* through heterochromatin formation, and an accompanying reduction of frataxin, a mitochondrial protein involved in iron metabolism. FRDA is characterized by neurodegeneration leading to ataxia, areflexia, sensory loss, muscle weakness, and cardiomyopathy. Currently, there is no treatment for FRDA. In 2017, we showed that transplantation of mouse wild-type hematopoietic stem and progenitor cells (HSPCs) in the YG8R mouse model of FRDA, prevents the development of the locomotor deficits, and the neuronal degeneration in the dorsal root ganglia, as well as reduction of the oxidative stress in brain and muscle. We also showed that the mechanism of rescue was mediated by the transfer of frataxin from the HSPC-derived microglia/macrophages to neurons/myocytes. This study builds upon these previous findings through the manufacturing optimization of the future clinical product consisting of autologous gene-corrected HSPCs isolated from the peripheral blood of FRDA patients. Gene correction occurs through CRISPR/Cas9-mediated excision of the intronic repeat expansion mutation. Optimization was performed in lymphoblasts isolated from FRDA patients in which gene editing efficiency reached up to 60%. Corrected cells displayed frataxin expression levels comparable to their carrier parents' cell lines for both transcription and translation levels, and displayed better mitochondrial function. Then, we transitioned to developing the human product first in $CD34^+$ cells isolated from healthy donor peripheral blood; gene editing efficiency ranged from 33.6 to 49.8%. A decrease in proliferative capacity was transiently observed in the edited cells over the 48 hours post electroporation due to double stranded breaks exacerbating p53 expression. *In vitro* Colony Forming Unit assays and *in vivo* transplant into NOD scid gamma immunodeficient mice showed the gene-modified $CD34^+$ cells retained normal hematopoiesis. Gene editing in $CD34^+$ cells isolated from FRDA patients ranged from 12.1 to 55.9% and correlated with a therapeutically relevant increase in frataxin expression. No off-target crRNA indel events were found at computationally predicted sites. This editing methodology is now being tested on FRDA murine HSPCs ($Sca1^+$) which are transplanted into an FRDA mouse model to evaluate their therapeutic capacity *in vivo.* With this study, we are laying the foundation for a future clinical trial using autologous HSPC transplantation for FRDA.

1123. CX3CR1 Haploinsufficiency Improves the Ability of Hematopoietic Stem and Progenitor Cells to Generate a Microglia-Like Progeny Upon Transplantation

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Hematopoietic stem and progenitor cells (HSPCs) contribute to the turnover of brain resident myeloid populations upon transplantation in recipients receiving a proper pre-transplant preparatory regimen. Transplanted HSPCs home to the brain, engraft locally and give rise to a mature progeny that shares transcriptional, morphologic and functional features with microglia when functionally defined microglia progenitors are (partially) ablated by pre-transplant chemotherapy.In the context of metabolic and neurological diseases, engrafted cells can act as vehicles for therapeutics to the brain as well as critical modulators of neuroinflammation towards neuroprotection. However, the impact of this approach is affected by the slow pace of reconstitution of resident microglia by transplanted HSPCs progeny, as compared with the rapid progression of most targeted neurological disorders. In order to foster this process, we identified a key *locus* to be targeted that may enhance the ability of HSPCs to generate a microglia-like progeny upon transplantation.CX3CR1 is a chemokine receptor expressed on microglia that binds Fractalkine (CX3CL1) and regulates microglial recruitment to sites of neuroinflammation. Currently, several reports propose the CX3CL1/CX3CR1 axis as a potential target for therapeutic intervention in the context of neurodegenerative diseases. Here we describe that transplantation of CX3CR1 haploinsufficient HSPCs results in a more robust and faster myeloid engraftment and generation of *bona fide* microglia as compared to wild type HSPC transplant. Interestingly, in a competitive transplantation setting, haploinsufficient cells prevail over wild type HSPCs in the repopulation of hematopoietic organs and brain of recipients. Moreover, branching analysis performed on brain-engrafted cells revealed that haploinsufficient cells rapidly acquire a mature microglia-like morphology and phenotype, suggesting also a qualitative advantage over wild type HSPCs. We are now elucidating the biological mechanism underneath this phenomenon. All together these evidences may set the bases for designing a gene editing approach aimed at editing of (and, possibly, targeted gene addition at) the CX3CR1 locus in human HSPCs in order to translate these results towards clinics.

1124. Characterization of the Retinal Phenotype in a Humanized FD Mouse Model with Defective *ELP1* Splicing

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Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disorder caused by a splice mutation in the gene encoding Elongator complex protein 1 (ELP1, also known as IKBKAP). This mutation results in skipping of exon 20 and tissue specific reduction of ELP1 protein levels, predominantly in the central and peripheral nervous system. Although FD patients exhibit a complex neurological phenotype due to the degeneration of sensory and autonomic neurons, progressive retinal degeneration severely impacts quality of life. Two different mouse models have been previously generated to study the retinal phenotype that results from complete loss of ELP1. However, neither of these models accurately recapitulates the tissue specific reduction of ELP1 observed in FD patients. Therefore, we developed a novel FD phenotypic mouse model by introducing a transgene carrying the human ELP1 gene with the FD splice mutation (TgFD9) into a hypomorphic mouse that expresses low levels of endogenous Elp1 (Elp1Δ20/flox). This mouse model displays both the clinical features of the disease and the tissue specific mis-splicing observed in FD patients therefore, can be used as a model to evaluate in vivo efficacy of ELP1 splicing modulation using modified Exon specific U1 (ExSpeU1). Detailed characterization of the retinal phenotype in the FD mouse model was performed to investigate the pathology associated with the splice mutation in ELP1. Optical Coherence Tomography (OCT) was performed to investigate retinal thickness and H and E staining to study retinal morphology. Retinal ganglion cell (RGC) counting was performed using retinal whole mounts.Optical Coherence Tomography (OCT) analysis revealed significant reduction in the thickness of the retinal nerve fiber layer (RFNL) and Ganglion cell layer (GCL) of FD mice when compared to control littermates. Analysis of retinal morphology revealed progressive loss of RGC's. Results from whole mount analysis also indicated the loss RGC's in the FD mouse model. Our findings suggest that our novel FD mouse model recapitulates optic neuropathy observed in FD patients and will provide a platform to evaluate the in vivo efficacy of ELP1 splicing modulation using ExSpeU1 mediated gene therapy to increase functional ELP1 in the retina.

1125. Abstract Withdrawn

1126. Characterization of 15 rAAV Serotypes for Gene Delivery to Non-Human Primate Retina

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Multiple retinal diseases are caused by specific genetic defects. Gene replacement and gene editing are promising strategies to treat those diseases. Recombinant Adeno-Associated Viruses (rAAVs) have been shown to be the most suitable vectors for delivering therapeutic gene into retina so far. A specific retinal disease is caused by dysfunction of a specific gene, which expresses at certain level in specific retinal cells in physiological condition. For gene replacement therapy, we need to select a rAAV serotype to deliver the therapeutic gene to the target cells with an appropriate expression level. Therefore, it is critical to know the retinal cell tropism and transduction efficiency of different rAAV serotypes. In this study, we first investigated the correlations of pre-existing Neutralizing Antibodies (NAbs) to different rAAV serotypes between serum and vitreous humor (VH) in cynomolgus macaque population to select suitable animals for retinal gene transfer. We then characterized the retinal cell tropism, gene transfer efficiency and immune responses by either subretinal or intravitreal injections of 15 different rAAV serotypes. Briefly, serum and VH samples were collected from 47 naïve cynomolgus macaques for NAb assay in vitro. NAb titers of the samples were calculated as the highest dilution that inhibits 50% of rAAV transduction in Huh.7 cells. Among 15 rAAV serotypes, rAAVrh10 showed overwhelmingly high NAb titers, followed by rAAV9 and rAAVrh8. There was no significant correlation between the titers of NAbs in serum and VH. For a specific rAAV serotype, the animal with negative NAb in VH, and negative or low levels NAb in serum was selected for the retinal gene delivery. 15 rAAV serotypes carrying a transgene cassette with eGFP gene driven by CBA promoter were generated. The same dose and volume of each rAAV serotype was administrated intravitreally in one eye and subretinally in the contralateral eye. The images of fundoscopy revealed different onsets and levels of eGFP signals with 8 out of 15 rAAV serotypes at 15 days post sub-retinal injection, and the signal densities continue to increase to the end point (30 days) of the study. Additional 3 serotypes were only detected at 30 days. Histological evaluation of retinal tissues harvested at 30 days showed that all 15 rAAV serotypes transduced macaque retinas successfully with different cell tropism profiles in rod-, cone- photoreceptors and RPE at variable expression levels, but no transduction was detected after intravitreal injections. Importantly, staining with different immune cell markers revealed no significant immune responses to all 15 different rAAV serotypes. Our results indicate that different rAAV serotypes have different profiles of preexisting NAb in the serum and VH, retinal cell tropism and transduction efficiency, providing a highly informative dataset for developing rAAV gene therapy of retinal diseases.

1127. Restoring Neuronal Cholesterol Metabolism Efficiently Rescue ALS Mouse Model

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Defects in brain cholesterol metabolism contribute to neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD) and Spinocerebellar ataxias (SCAs). Beside contributing to myelin compartment, cholesterol is a critical component of membranes where it plays key structural and functional roles. Cholesterol cannot cross the blood brain barrier (BBB) and is synthesized *in situ*. Cholesterol 24-hydroxylase (CYP46A1) ensures the conversion of cholesterol into 24S-hydroxycholesterol that can freely cross the BBB. This key neuronal enzyme of brain cholesterol metabolism plays a crucial role in maintaining brain cholesterol homeostasis and is a major neuronal stress response in conditions with oxidative stress, like ageing or toxic protein accumulation. We previously demonstrated in the laboratory that overexpression of CYP46A1 through AAV-encoding CYP46A1 delivery, efficiently rescued clinical and neuropathological hallmarks of mouse models of AD; HD and SCA3. CYP46A1 gene therapy is able to restore deficient autophagy process in these diseases and clearance of protein aggregates (amyloid, huntingtin, ataxin 3). This suggest us that CYP46A1 could be a relevant therapeutic target for ALS to improve stress response, in the clearance of toxic aggregated proteins, synaptic transmission, inflammation and finally neuronal survival. In a preliminary step, we demonstrated that AAV-CYP46A1 could improve the severe $SOD1^{G93A}$ ALS mouse model, characterized by a rapid decrease in muscular strength and motor dysfunction leading to progressive paralysis at 3 months. We used AAVi serotype that crosses the BBB following intravenous administration. We demonstrated a significant and prolonged motor rescue of animals treated either pre or post-symptomatically, compared to untreated animals as well as an improved survival. We already demonstrated a preservation of motoneurons in the spinal cord and a significant improvement of muscular fiber structure and preservation of neuromuscular junctions.

1128. CRISPR/Cas9-Mediated Correction of β-Galactocerebrosidase Gene by Stimulation of Homology-Directed Repair for the Treatment of Krabbe Disease

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Globoid Cell Leukodystrophy, also known as Krabbe Disease, is an autosomal recessive lysosomal storage disorder caused by a deficiency in β-Galactocerebrosidase. This deficiency leads to a build-up of the toxic substance psychosine which results in demyelination of neurons, ultimately leading to death before the age of 2. Current treatment methods are largely ineffective with the exception of the adult-onset form of the disease where bone marrow transplantation has demonstrated benefits. Current preclinical research has investigated a wide variety of therapeutic techniques including bone marrow transplant, enzyme replacement, substrate reduction, immunosuppression, and gene therapy. Of these techniques, gene therapy has shown the most

promising results with one study prolonging survival in a mouse model of Krabbe disease from 40 days to 284.5 days. This study used adeno-associated virus to deliver the β-Galactocerebrosidase gene but did not integrate the gene into the host cell genome. This technique is unlikely to produce the long-term gene expression necessary to effectively treat Krabbe disease. To overcome this hurdle, CRISPR/Cas9 technology will be used to stimulate homology-directed repair to correct the mutant form of β-Galactocerebrosidase. Eight single guide RNAs (sgRNAs) targeting the β-Galactocerebrosidase gene were designed using Benchling CRISPR Guide Design Software. Oligos with the sgRNA sequence were annealed together to create double-stranded DNA with sticky ends which was ligated into a plasmid containing staph. aureus derived Cas9 enzyme. Restriction enzyme digestion and Sanger sequencing were used to confirm successful ligation. The eight sgRNAs were next tested for their ability to create double-stranded breaks *in vitro* using the T7E1 mismatch assay. The most efficient sgRNAs from this experiment will be subsequently used to stimulate homology-directed repair *in vitro* and ultimately *in vivo*.

1129. AAV-NRIP as a Potential Treatment for Amyotrophic Lateral Sclerosis

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Nuclear receptor interaction protein (NRIP) is calcium/calmodulin binding protein involved in muscle functions. Both global NRIP knockout mice (gKO) and muscle-restricted NRIP knockout (NRIP cKO) show muscular weakness. The interaction between motor neuron (MN) and muscles is mutual regulation for muscle contraction. Intriguingly, NRIP cKO reveal NRIP of skeletal muscle can retrograde control the development of MN growth. NRIP cKO mice display spinal MN degeneration with abnormal neuromuscular junction (NMJ) integrity and act as a model for muscle pathology contributing to MN degeneration; hence NRIP cKO can be NMJ-weakness mouse disease model. NMJ abnormal is the major cause for MN degeneration. NMJ formation involves in acetylcholine receptors (AChR) clustering. The components of NMJ include AChR, rapsyn, α-actinin 2 (ACTN2) etc. NRIP can interact with ACTN2. Currently we found NRIP involved in NMJ formation. Immunofluorescence assay showed NRIP colocalized AChRs, rapsyn and ACTN2; and α-bungarotoxin (BTX) pull-down immunoprecipitation assay revealed NRIP with rapsyn and ACTN2 in complexes from muscle tissues and muscle cells (C2C12). Conclusively, NRIP is a new one of NMJ structural components. To evaluate whether AAV-NRIP as a drug for motor neuron disease; gene therapy with AAV-NRIP was performed in SOD transgenic mice (a mouse model of amyotrophic lateral sclerosis) in which muscle NRIP expression was lower in SOD transgenic mice than WT. Forced NRIP expression by AAV in SOD mice through intra muscle injection could rescue motor neuron number, increase NMJ formation, enhance axon innervation along with increased compound muscle action potential (CMAP) and motor functions.

1130. Abstract Withdrawn

1131. Establishment of a dCas9/CRISPRa Platform in iPSC-Derived Neurons

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Genetically-validated neuroscience targets with high unmet clinical can require up-regulation which is hard to address. Establishing a platform to enable up-regulation of gene and protein expression for screening for new targets, pre-clinical proof-of-concept studies, and/or therapeutic potential would be valuable. Here, we used dCas9/CRISPRa technology to generate a protocol for up-regulation of gene and protein expression. iPSC carrying a stably integrated, inducible NEURG2 gene which allows for the rapid conversion of iPSC to neurons were transduced with dCas9-VPR lentivirus to generate a stable NGN2/ dCas9-VPR iPSC line. The inducible NGN2/dCas9-VPR iPSCs and NGN2 control iPSCs were differentiated into glutamatergic neurons by treatment of doxycycline for 6 days. The neurons were transfected using Lipofectamine Stem with *GRN* gRNA or *SHANK3* gRNA, as well as positive and negative control gRNAs. Three days after transfection, RT-qPCR and ELISA were performed to detect *GRN* or *SHANK3* RNA expression and/or protein production. RT-qPCR results showed that *GRN* gRNA specifically increased *GRN* RNA expression by 21-fold only in dCas9-VPR neurons, not NGN2 control neurons. *SHANK3* gRNA specifically increased *SHANK3* RNA expression by 2-fold only in dCas9-VPR neurons, not NGN2 control neurons. ELISA data demonstrated that *GRN* gRNA specifically increased GRN protein expression by 3-fold in dCas9-VPR neuron lysate and 6-fold in dCas9-VPR neuron culture media. Our data demonstrates that dCas9/CRISPRa provides a unique tool to upregulate gene expression in iPSC-derived neurons.

1132. Preclinical Development of an *Ex Vivo* Gene Therapy for GM1-Gangliosidosis

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GM1-gangliosidosis (OMIM #230500) is a rare, autosomal recessive, neurodegenerative Lysosomal Storage Disorder. It is caused by mutations in the *GLB1* gene, encoding the lysosomal hydrolase β-galactosidase. Infantile GM1-gangliosidosis is characterized by neurodevelopmental delay, hypotonia, dysphagia, seizures and death by 3 years of life. Due to the rapid progression and severe nature of this disease, which involves storage of undegraded metabolites and secondary mechanisms of cell damage, correction requires a rapid and robust enzyme delivery to the whole central nervous system (CNS), possibly associated to reduction of local inflammation. Here we propose an *ex vivo* gene therapy (GT) strategy aimed at preventing or ameliorating the symptoms of the disease in the murine model of the disease. Multiple copies of *GLB1*, alone or in association with a neuroprotective factor, will be delivered *ex vivo* to hematopoietic stem/progenitor cells (HSPCs) by lentiviral gene transfer to determine a sustained and robust expression of the therapeutic enzyme in the CNS of transplanted

mice. Enzymatic-competent microglial cells, derived from the LV-transduced HSPCs, will reconstitute the myeloid brain compartment of transplanted mice, releasing the therapeutic β-galactosidase directly into the CNS. The re-establishment of therapeutic enzyme availability in the CNS will possibly induce cross-correction of resident enzymatic-deficient cells, as neurons and other brain cells. Different versions of human GLB1 gene have been *ad hoc* designed to enhance the protein secretion in human cells, including a codon-optimized and a chimeric version containing an improved secretion-signal. cDNAs encoding these GLB1 have been cloned into lentiviral vectors intended for clinic, under the transcriptional control of a human constitutive promoter that has been demonstrated to safely drive a high level of transgene expression in several GT clinical trials. Therapeutic LVs have been produced with a high physical and infectious titer, and have been currently tested for their ability to transfer multiple copies of the transgene in human target cells, and to produce and secrete a critical amount of functional therapeutic β-galactosidase. In addition, the level of cellular re-uptake of these engineered therapeutic β-galactosidase will be carefully tested, as a critical factor to achieve a high-level of enzymatic cross-correction *in vivo*. Based on these results, the candidate therapeutic LV will be tested in a preclinical study of *ex vivo* GT in *Glb*$^{-/-}$ mice. HSPCs derived from donor *Glb*$^{-/-}$ mice will be transduced at a critical MOI with the therapeutic LV, and transplanted by different routes of administration in previously fully-myeloablated *Glb*$^{-/-}$ recipient mice. GT-treated mice will be followed for approximately one year to test biochemical and functional improvements compared to control mice. Our working hypothesis is that this optimized GT strategy could successfully control disease manifestations in the animal model of the disease, and if successful, this study will generate a solid proof-of-concept for a rapid clinical translation into an efficacious *ex vivo* GT for infantile GM1-gangliosidosis.

1133. Study of Intercellular Transmission of Frataxin in Friedreich's Ataxia Patient Derived iPSCs Based Model Systems

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Friedreich ataxia (FA) is an autosomal recessive neurodegenerative disease. Most patients carry homozygous GAA expansions in the first intron of the frataxin gene causing reduction in frataxin (*FXN*) expression. FXN is a mitochondrial protein and its deficiency leads to mitochondrial iron overload, defective energy supply and generation of reactive oxygen species. Our group previously showed that wild-type hematopoietic stem and progenitor cell (HSPC) transplantation prevents development of the disease phenotype in a mouse model of FA, providing an evidenced-based approach for treatment of FA patients. We also observed transfer of frataxin-eGFP from HSPC-derived HSPC-derived microglia to diseased neurons in brain, spinal cord and dorsal root ganglia. However, the mechanism of frataxin transfer and their functional implications are still matter of debate. In this study our aim is to explore the transfer mechanisms of FXN from microglia cells to neuronal progenitor cells and in neurons by utilizing the iPSC-based 2D and 3D neuronal model system and to elucidate the role of microglia in transfer of mitochondrial protein. Our results suggest that frataxin could be mobilized through long actin-based membranous extensions called tunneling nanotubes (TNTs), which directly connects the cytoplasm of the neuronal cells. However, frataxin is also present in small extracellular nano-size vesicles with multivesicular bodies origin known as exosomes. Whether or not there is a preferential mechanism of transfer, TNTs *versus* exosomes, remains to be determined. Furthermore, we are currently examining whether frataxin is transferred alone or associated to mitochondria. Overall, the results of this study have the potential to mechanistically explain how the mitochondrial protein, FXN, is transferred and support our previous findings using cell therapy in a mouse model of FA.

Musculo-skeletal Diseases

1134. AAV1.NT-3 Gene Therapy Attenuates Age-Related Musculoskeletal Changes and Improves Function in the Wild Type-Aged C57BL/6J Mice

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Frailty is a clinical geriatric syndrome that is an important public health problem affecting a large proportion of older adults. When it accompanies sarcopenia, age-related muscle loss is seen. In a recent study using AAV1.NT-3 gene therapy, we showed that NT-3 increases muscle fiber diameter through direct activation of mTOR pathway. This suggests a potential application of NT-3 for muscle wasting conditions including aging. For proof of concept, we assessed the therapeutic efficacy of AAV1.NT-3 in wild type-aged C57BL/6J mice representing a model for natural aging and sarcopenia. Twelve C57BL/6 mice of both gender, 76-80 weeks of age received AAV1.tMCK.NT-3 at 1E+11 vg dose, via intramuscular injection into the gastroc muscle. Twelve age and sex matched C57BL/6 mice were injected with Ringer's lactate as controls. The mice were sacrificed 24 weeks post-injection. Two untreated mice died at the age of 84 and 100 weeks and one treated mouse died at the age of 99 weeks before reaching to the end point of the experiment. Animals were tested with run to exhaustion at two, four, and six months post-injection. Additional endpoint tests included rotarod and *in vivo* muscle contractility assays and detection of blood serum NT-3 levels with ELISA. Histological analyses were performed on skeletal muscles to analyze metabolic fiber type distribution and myofiber size changes in the aging muscle. Functional analyses indicated an improvement in the NT-3-treated mice, corresponding to 68%, 74% and 63% longer running distances compared to untreated cohort at 2, 4, and 6 months, respectively ($p= 0.02$, 0.0002, 0.001). Functional improvement was further supported with rotarod test that showed 39% increase ($p= 0.038$) and 23% higher maximum twitch response ($p= 0.014$) from the in vivo muscle contractility assay in the AAV1.NT-3 injected mice. Treated females performed significantly

better in treadmill run to exhaustion test at 4- and 6-months post-injection compared to treated males (p= 0.0476, 0.02243). Serum NT-3 levels did not differ significantly in females compared to males (p=0.487). Age-related musculoskeletal and skin changes including kyphosis, dermatitis, and alopecia were attenuated in treated mice which were semi-quantitively documented. Varying degrees of kyphosis was present in 83% of untreated mice and 1/2 of those were severe while only 50% of the AAV1.NT-3 injected mice had kyphosis and 1/3 of them were severe. Dermatitis observed in 33% of untreated mice was not seen in the treated group. Moderate to severe alopecia around ears were present in 67% of the untreated mice whereas this ratio decreased to 33% percent in treated mice. Histological analyses of tibialis anterior muscle following succinic dehydrogenase stain showed an increase in fiber size in all fiber types, namely slow twitch oxidative (STO, p= 0.0054), fast twitch oxidative (FTO, p=0.02) and fast twitch glycolytic (FTG, p=0.002). The percent of fatigue resistant fiber type (STO) increased in all four muscles analyzed (gastrocnemius, quadriceps, tibialis anterior, triceps) from the treated mice. This study shows that AAV1.NT-3 gene therapy in the wild type C57BL/6J mice at 2 years of age unequivocally improved the function of sarcopenic muscle and attenuated age-related musculoskeletal and skin changes. Considering the cost and quality of life to the individual, as well as the financial burden to health care system, we believe our study has important implications for management of age-related sarcopenia.

1135. Biopotency and Biodistribution/ Toxicology Studies Following Systemic Gene Therapy with AAVrh74.tMCK.hCAPN3 in the Mouse Model for LGMD2A

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Studies from our laboratory revealed a robust functional improvement in the calpain 3 (CAPN3) null mice (CAPN3 KO) following a systemic administration of AAVrh74.tMCK.hCAPN3 vector at low (3E+12 vg) and high doses (6E+12 vg), 20 weeks after gene delivery. In an attempt to explore the possible correlations with functional improvements, we carried out a comparative assessment of systemic AAVrh74.tMCK.hCAPN3 gene delivery at the mRNA and protein levels in four representative muscles, tibialis anterior, gastrocnemius, quadriceps, and triceps from hind limbs and forelimbs. We also assessed gene transfer efficacy by qPCR, calculating the vector genome copies in the tissue samples of low and high dose CAPN3 KO mouse cohorts. In addition, in-house toxicology studies were carried out for histopathological evaluation of multiple organ tissue specimens from the high dose cohort and untreated controls. The vector genome load of the organs and muscles was determined in both cohorts. Genomic DNA was isolated from frozen tissue samples. The qPCR assay was performed with the primer pair exclusively amplifying a product from the 5' region of the hCAPN3 ORF, and region downstream unique to the expression vector, including portions of an intronic element. The results were reported as the mean copy number of AAVrh74 vector per microgram of genomic DNA. We found that the highest vector genome copy number was present in the liver as expected following systemic vector delivery. Vector genome distribution was variable in organs and muscles in a dose-dependent manner. Overall the values were higher in the quadriceps, diaphragm and heart tissues compared to other muscles. In both cohorts, improvements in both functional and histological features were observed following systemically treated CAPN3 KO mice. As observed in the vector copy numbers, CAPN3 expression levels showed variability in tibialis anterior, gastrocnemius, quadriceps and triceps muscles in a dose-dependent manner. In western blots from high dose cohort, full-length 94 kDa CAPN3 protein bands in these muscles were also variable and did not correlate with mRNA levels. In the low dose cohort, overall CAPN3 mRNA levels were lower compared to high dose and the full-length 94 kDa protein was below the limit of detection in all except a gastrocnemius sample with the highest mRNA expression suggesting a threshold for mRNA level for the detection of 94 kDa CAPN3 protein band in muscles with CAPN3-null background. However, the total mRNA levels from all 4 muscles correlated with the run to exhaustion test in the low dose cohort (R^2=0.5192, p=0.0082) while in the high dose cohort, no correlation was seen between mRNA levels and the run to exhaustion test performance. These results favor an mRNA threshold effect required to improve performance. In-house toxicology studies revealed no organ tissue abnormalities; specifically, there was no histopathological evidence of cardiotoxicity. Despite a high vector genome load and mRNA levels observed in heart tissues, full-length 94 kDa CAPN3 protein was below the limit of detection in the tested Western blot analyses. These studies provide evidence for the safety and efficacy of AAVrh74.tMCK.CAPN3 vector in the CAPN3 KO mouse model, which should guide us in the design of a future clinical trial for LGMD2A.

1136. Evolutionary Insights into the Structural Nature of the Dystrophin Rod-Domain Informs Next-Generation Transgene Design for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a degenerative, X-linked muscle disease with an incidence of approximately 1 in 4,000 male births. DMD is caused by mutations that abrogate protein expression of the rod-like protein dystrophin that protects striated myocytes from contraction-induced injury. Dystrophin-related protein (or utrophin) retains most of the structural and protein binding elements of dystrophin. Our prior work demonstrated that a codon-optimized, AAV9-delivered synthetic transgene encoding a miniaturized utrophin (μUtro) prevented skeletal muscle pathology in the *mdx* mouse and dystrophin-deficient golden retriever (GRMD) canine models of DMD. Furthermore, normal thymic expression of utrophin provides central immunological tolerance to utrophin-based gene therapy constructs in the German shorthaired pointer (GSHP) canine model of DMD, whereas dystrophin was recognized as a foreign antigen and target by T-cell mediated immunity. Here, we used comparative phylogenomics to reconstruct the deep evolutionary history of dystrophin in order to

gain insight into heretofore unrecognized structural constraints on dystrophin/utrophin for the purpose of designing second-generation miniaturized utrophin transgenes. Surprisingly, gene structures provide compelling evidence that the extraordinary size of dystrophin's rod domain reflects the historical legacy of a parologous class of microtubule-binding proteins in which selection for increasing length occurred before the evolution of striated muscle. These evolutionary insights lead us to the hypothesis that adjacent spectrin repeats within the dystrophin/utrophin rod-domain have extensive inter-domain interactions, thereby forming a structurally interlocking rod-domain. This model of the dystrophin/utrophin rod-domain was then leveraged to generate a second generation of codon-optimized, miniaturized utrophins (nano-Trophins). Following systemic administration of either AAV9-μUtro or the AAV9-nano-Trophins to neonatal *mdx* mice, we demonstrate robust skeletal and cardiac transgene expression in adult mice with restored sarcolemmal localization of the dystrophin-associated protein complex. The findings of our ongoing functional studies have critical implications for the mechanobiology of dystrophin and the design of miniaturized, AAV-deliverable proteins for therapeutic use in Duchenne and Becker muscular dystrophy.

1137. Treatment of Aged Mice and Long-Term Durability of AAV-Mediated Gene Therapy in Two Mouse Models of LGMD

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Background: The limb-girdle muscular dystrophies (LGMD) are a group of monogenic disorders caused by mutations in specific genes encoding proteins involved in muscle structure and function. The sarcoglycanopathies are a subset of LGMD resulting from loss of function mutations in the sarcoglycans (α, β, γ, and δ-SG). These are structural proteins localized at the cell membrane of muscle fibers that together with dystrophin and other proteins make up the dystrophin-associated protein complex (DAPC). Loss of one sarcoglycan results in concomitant loss of other sarcoglycans along with components of the DAPC, leading to increased membrane permeability, myofiber degeneration, chronic inflammation, and elevated creatine kinase levels, among other events. The sarcoglycanopathies present as progressive muscular dystrophies starting in the girdle muscles before extending to lower and upper extremity muscles, and can also present in the diaphragm and heart, resulting in respiratory and cardiac failure in specific patient subtypes. Adeno-associated virus (AAV)-mediated gene replacement therapy has shown early signs of potential to treat these devastating diseases. However, two questions that still remain about the efficacy of AAV gene therapy is the ability to treat older more severely affected muscle, and the long-term durability of the AAV viral vector. Here, we used a mouse model of LGMD2D (α-sarcoglycan) treated at older age with an AAV.hSGCA vector and a model of LGMD2E (β-sarcoglycan) treated with an AAV.hSGCB vector following a long-term endpoint to investigate these two outstanding questions. The goal of this study is to demonstrate long-term durability of AAV-mediated gene replacement therapy and provide evidence for therapeutic efficacy when delivered to aged diseased muscle. **Methods:** To address treatment of aged diseased muscle, we treated 12-month-old *sgca-/-* mice presenting with severe muscle histopathology with systemic delivery of an rAAVrh74.tMCK.hSGCA vector. At an endpoint of 6 months post-treatment, we then assessed the muscle from these mice for protein expression, histological rescue, and functional improvement. Additionally, to investigate the long-term durability of gene therapy, we treated *sgcb-/-* mice at 4 weeks of age by systemic delivery with an rAAVrh74.MHCK7.hSGCB vector and evaluated these mice more than 24 months post-treatment for vector durability and biomarker protein expression. **Results:** IV administration of rAAVrh74.tMCK.hSGCA to 12-month-old *sgca-/-* mice resulted in widespread high-level protein expression in muscles throughout the lower limb, upper limb, and proximal torso muscles, including the diaphragm and heart. We also noted a reduction in levels of fibrosis compared to untreated controls. Finally, we were able to demonstrate functional improvement as evidenced by improved force output in the tibialis anterior (TA) and diaphragm (DIA) muscle and increased resistance to contraction-induced injury in the TA muscle. Durability of AAV gene therapy was established in *sgcb-/-* mice treated systemically with rAAVrh74.MHCK7.hSGCB. Using qPCR, we detected high-level vector genome copy numbers across all transduced muscles more than 24 months post-treatment. Finally, at this long-term timepoint, immunofluorescence staining of treated muscle showed no decrease of protein expression levels in all muscles (>95%) compared to earlier timepoints, with hSGCB protein remaining correctly localized at the membrane. **Conclusions:** Collectively, these data demonstrate the ability for AAV gene therapy to be efficacious and provide therapeutic benefit when delivered at an older age to more severely diseased muscle. Furthermore, these data demonstrate durability of AAV gene therapy.

1138. Antisense Oligonucleotides Restore Muscle Stem Cell Density in a Mouse Model of Myotonic Dystrophy

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Introduction: Satellite cells are resident muscle stem cells (MuSCs) that are responsible for muscle repair due to injury. MuSCs express the transcription factor Pax7, an important regulator of muscle development. In muscle biopsies of patients with myotonic dystrophy type 1 (dystrophia myotonica; DM1), the number of MuSCs and total myonuclei are elevated, suggesting an increase in mitotic activity that may be adaptive or a primary disease feature. However, unlike necrotizing myopathies such as Duchenne or limb girdle muscular dystrophies, the myopathy in DM1 is non-necrotizing and shows little evidence of muscle regeneration, despite the increased MuSCs. In wild-type mice, chronic moderate intensity exercise increases the MuSC pool and function. Therapeutic antisense oligonucleotides (ASOs) are short nucleic acid chains designed to bind and modify the activity of target RNA. **Objective**: To determine the therapeutic effect of ASOs on MuSCs in DM1 mice. **Methods**: We used the HSA^{LR} transgenic mouse model of DM1, which expresses an expanded CUG repeat in skeletal muscle and features myotonia and mis-regulated alternative splicing similar to DM1. Age-matched wild-type mice served as controls. Young (2 - 3 month-old) and older (14 month-old) HSA^{LR} mice were

treated with an ASO that targets the CUG^{exp}transcripts through the RNase H pathway and corrects alternative splicing (Wheeler, et al., 2012; Hu, et al., 2018). ASO dose was 25 mg/kg by subcutaneous injection twice weekly for four weeks followed by the same dose once every two weeks for three months. Some older groups also received a moderate intensity exercise-training regimen that consisted of treadmill running on a flat surface for 30 minutes six days per week for three months. Using fluorescence microscopy, we identified MuSCs in cryosections of gastrocnemius muscles by immunolabeling with combined Pax7 and laminin antibodies, recently regenerated muscle fibers with a developmental myosin antibody, and localized CUG^{exp}RNA by fluorescence in situ hybridization (FISH). **Results**: In young saline-treated HSA^{LR}mice, MuSC density (Pax7+ cells per 100 muscle fibers) was more than three-fold increased over age-matched wild-type controls (8 vs 2.5; *P*less than 0.0001), while the density in young ASO-treated mice (5) was approximately double that in wild-type. In older- saline-treated mice, MuSC density was approximately five-fold elevated over wild-type controls (6 vs 1.2 Pax7+/100 fibers; *P* value less than 0.0001), while ASO treatment reduced MuSC density by 50% (*P* value less than 0.0001). Older mice treated with a moderate intensity exercise training regimen showed a trend for further reduction in MuSC density. CUG^{exp} RNA was absent in all cells expressing Pax7. Regenerating fibers were rare (less than 0.5%) in young HSA^{LR} and absent in older HSA^{LR} mice. **Conclusions**: MuSCs are increased in HSA^{LR} mice even in the absence of muscle regeneration, suggesting that a trans-dominant effect of the CUG^{exp} RNA in MuSCs stimulates their proliferation. ASOs partially rescue MuSC proliferation in HSA^{LR} mice. **Support**: The Elaine and Richard Slye Fund; Muscular Dystrophy Association.

1139. Abstract Withdrawn

1140. A Novel Capsid for Smooth Muscle Pathology in Pompe Disease

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Pompe disease is a fatal glycogen storage disease caused by mutations within the gene encoding acid alpha-glucosidase (GAA), which is responsible for the breakdown of lysosomal glycogen. Without GAA, glycogen accumulates in the lysosomes of cardiac, skeletal, and smooth muscle, as well as in motor neurons. Children with Pompe disease present with cardiorespiratory distress as glycogen amasses. The only FDA approved treatment for Pompe disease is an enzyme replacement therapy (ERT) of recombinant human GAA (rhGAA), which effectively treats cardiac muscle, but is inefficiently taken up by skeletal and smooth muscle. When skeletal and smooth muscle remains untreated, autophagy - a major degradation pathway in the cell - becomes impaired. As ERT prolongs survival in children with Pompe disease, life threatening smooth muscle pathology has been unmasked. Recombinant adeno-associated virus (rAAV) gene therapy is a promising therapy for Pompe disease. Numerous preclinical and clinical trials with rAAV vectors delivering the therapeutic gene, *GAA*, have demonstrated safety and efficacy in many tissues. However, AAV serotype 9 (AAV9), which is currently under clinical investigation, was not effective in clearing glycogen in smooth muscle. The goal of this study is to characterize a rationally designed AAV capsid (AAV8g9) for its potential to transduce skeletal and smooth muscle in the Pompe mouse model ($Gaa^{-/-}$) compared to AAV9. AAV8g9 is a chimeric capsid with the galactose binding domain of AAV9 engrafted into AAV8. $2x10^{12}$ total vector genomes were injected at equal ratios into each adult $Gaa^{-/-}$ mouse. One month later, tissues were harvested for vector genome and protein expression analyses. AAV8g9 has improved transduction in vascular and genitourinary smooth muscle, as well as skeletal muscle, compared to AAV9. We will next complete histology analysis on these tissues to correlate vector genome distribution with protein expression. Based on these results, AAV8g9-*GAA* may be a potential therapy to treat smooth and skeletal muscle pathology in Pompe disease.

1141. Abstract Withdrawn

1142. Blockade of IGF2R Improves Regeneration of Dystrophic Muscle Tissues

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Duchenne muscular dystrophy (DMD) is a debilitating fatal X-linked muscle disorder. Recent findings indicate that IGFs play a central role in skeletal muscle regeneration and development. Among IGFs, insulin-like growth factor-II (IGF2) is a key regulator of cell growth, survival, migration and differentiation. The type 2 IGF receptor (IGF2R) modulates circulating and tissue levels of IGF2 by targeting it to lysosomes for degradation. We found that IGF2R and the store-operated Ca^{2+} channel CD20 share a common hydrophobic binding motif that stabilizes their association. Silencing CD20 decreased myoblast differentiation, whereas blockade of IGF2R increased proliferation and differentiation in myoblasts via the calmodulin/calcineurin/NFAT pathway. Remarkably, anti-IGF2R induced CD20 phosphorylation, leading to the activation of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and removal of intracellular Ca^{2+}. Interestingly, we found that IGF2R expression was increased in dystrophic skeletal muscle of human DMD patients and *mdx* mice. Blockade of IGF2R by neutralizing antibodies stimulated muscle regeneration, induced force recovery and normalized capillary architecture in dystrophic *mdx* mice representing an encouraging starting point for the development of new biological therapies for DMD.

1143. Abstract Withdrawn

1144. A Single AAV Micro-Dystrophin Therapy in 3-M-Old Mdx Mice Results in Persistent Improvement in Whole Body Performance and Heart Function for 22-Months

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Loss of dystrophin protein leads to Duchenne muscular dystrophy (DMD). Micro-dystrophin is a highly truncated version of dystrophin. Systemic AAV micro-dystrophin gene therapy is currently in clinical trials. The ultimate goal of these trials is to test whether a single intravenous injection of the AAV micro-dystrophin vector in young patients can lead to long-term muscle protection. Systemic AAV micro-dystrophin therapy has been extensively studied in rodent and canine models. Unfortunately, none of these studies evaluated long-term outcomes. To address this issue, we injected 1e13 vg particles of the AAV micro-dystrophin vector to 3-m-old mdx mice via the tail vein and evaluated therapeutic outcomes at 12 and 22 months of age. Immunostaining and western blot showed robust micro-dystrophin expression in heart and skeletal muscle till the end of the study. The bodywide performance was evaluated by forelimb grip strength, treadmill running and serum CK levels. Treated mice significantly outperformed untreated mice in grip force and running distance at both time points. Treatment also significantly reduced the CK level. ECG and cardiac catheter assays were used to evaluate cardiac outcomes. Significant improvements were observed in both ECG and cardiac hemodynamics. Surprisingly, several ECG parameters (e.g. heart rate, PR interval, QRS duration, and QTc interval) and hemodynamic parameters (e.g. end-diastolic/systolic volume, dP/dt max and min, max pressure, and ejection fraction) were completely normalized in treated mice at 22 months of age. Our results have provided direct evidence that a single systemic AAV micro-dystrophin therapy has the potential to provide long-lasting benefits (Supported by NIH, Parent Project Muscular Dystrophy, and Jackson Freel DMD Research Fund).

1145. Abstract Withdrawn

1146. Genetic Tool Choice in the Elucidation of Disease Mechanisms and Therapeutic Targets

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Skeletal muscle has a remarkable regenerative capacity thanks to muscle stem cells. However, the malfunctioning of these cells can lead to degenerative muscle disorders. To develop effective gene and cell therapy for these diseases, it is fundamental to elucidate the factors and pathways that ensure the correct function of the muscle stem cells. The Cre-loxP genetic tool is used for this purpose, since it allows time- and cell-specific deletion of factors of interest. As all muscle stem cells express the transcription factor Pax7, CreERT has been inserted into this locus by different groups. Here we compare the two Pax7-CreERT lines that are most commonly used to monitor muscle regeneration and development of pathological features upon deletion of specific factors or pathways. Using different commonly used schemes of Cre induction, we show that in all these regimes one of the studied Pax7-CreERT alleles has an inherent defect in regeneration. This phenotype manifests in different ways, including general muscle architecture, and the size of the muscle stem cell population. In genetic ablation studies of specific factors using Pax7-CreERT, the inherent regeneration defect of the Pax7-CreERT could be misinterpreted as being provoked by the deletion of the factor of interest. To counteract this problem, we propose the use of the alternative Pax7-CreERT allele and the inclusion of appropriate controls (i.e. individuals carrying only the Pax7-CreERT allele), in order to obtain insights into the pathways that should be targeted in the context of gene and cell therapies.

1147. Therapeutic Correction of LGMD2G Using CRISPR-Cas9 Nucleases

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Limb girdle muscular dystrophy 2G (LGMD2G) is a subtype of autosomal recessive muscular dystrophy is caused by mutations in *TCAP* gene. *TCAP* encodes Telethonin which is a 19 kDa cardiac and striated muscle structural protein located in the Z-disc of sarcomeres that links titin proteins stabilizing the contractile apparatus. Homozygous or compound heterozygous inactivating mutations in *TCAP* manifest as severe muscle atrophy and cardiomyopathy that typically develops during late adolescence into early adulthood. A subset of limb girdle muscular dystrophy (LGMD2G) patients harbor a pathogenic 8 bp microduplication in exon 1 of *TCAP*, which is present at a carrier frequency of ~1 in 1000 alleles in the East Asian population. Achieving nuclease-mediated gene correction (wild-type sequence restoration) typically requires the co-delivery of a DNA repair template and the presence of homology dependent repair pathway (HDR) components that are absent from post-mitotic cells such as muscles. In contrast to the HDR based approach, introduction of a double-strand break (DSB) within the microduplication drives collapse through the microhomology mediated end-joining (MMEJ) repair pathway. We leveraged the MMEJ pathway to develop CRISPR/Cas9 based therapy to correct the 8 bp microduplication associated with LGMD2G. As a first step toward this goal, we first evaluated the efficacy of gene correction in iPSC based *in vitro* models. We show that in LGMD2G iPSCs and iPSC-derived myoblasts (iSMs) homozygous for the 8 bp microduplication, SpCas9-induced DSB at the 8 bp microduplication sequence could revert ~75% of the cells to the wild-type sequence on at least one allele. In iPSC-derived cardiomyocytes, introduction of a DSB using SpyCas9 resulted in robust genome editing (~66%) where 43% of total alleles were reverted to the wild-type sequence. We further observe restoration of TCAP expression in SpCas9 treated LGMD2G iPSC-derived myotubes. We are currently testing and optimizing the specificity of Cas9-based nuclease reagents for the efficient collapse of the TCAP 8 bp microduplication to the wild-type sequence while

limiting unintended modifications to the genome in LGMD2G disease relevant cell types. Furthermore, we are validating AAV based approaches for delivery of Cas9 nucleases to muscle tissues and mouse models that harbor human *TCAP* microduplication sequence as a prelude toward development of nuclease-based gene therapy approach for correction of pathogenic microduplication. Advances made in this study will pave the way for development of nuclease based therapeutics for other monogenic diseases caused by pathogenic microduplications.

1148. Fatigue and Myopathy Are Resistant to Therapeutic Antisense Oligonucleotides in A Mouse Model of Myotonic Dystrophy Type 1

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Introduction: Myotonic dystrophy type 1 (dystrophia myotonica; DM1) is the most common muscular dystrophy in adults with a prevalence of about 1/8000 worldwide. Inheritance is autosomal dominant. DM1 is caused by an expanded CTG repeat (CTG^{exp}) in the 3'untranslated region of the *DMPK* gene. In DM1 patients, fatigue and progressive weakness are the most debilitating clinical manifestations. A characteristic molecular phenotype in DM1 is mis-regulated alternative splicing of pre-mRNA that results from expression of pathogenic *DMPK*-CUG^{exp} transcripts. Splice events serve as molecular biomarkers of muscle weakness in DM1 patients and sensitive indicators of therapeutic antisense oligonucleotide (ASO) drug response in DM1 mice. However, the relationship between fatigue and alternative splicing is unknown. **Objective**: To determine the therapeutic effect of ASOs on fatigue and myopathy in a transgenic mouse model of DM1. **Methods**: We used the HSA^{LR} transgenic mouse model of DM1, which expresses an expanded CUG repeat in skeletal muscle and features myotonia and mis-regulated alternative splicing similar to DM1. Age-matched wild-type mice served as controls. To measure fatigue, we used an exercise-activity assay that consisted of treadmill running uphill at a 15-degree angle for ten minutes followed by tracking of spontaneous activity in an acrylic cage for 30 minutes using infrared lasers; data were recorded in one minute intervals. We treated 14 month-old HSA^{LR} mice with an RNase H-active ASO that targets the CUG^{exp} transcripts and corrects alternative splicing at a dose of 25 mg/kg by subcutaneous injection twice weekly for four weeks (Wheeler, et al., 2012; Hu, et al., 2018) followed by the same dose once every two weeks for three months. Some cohorts also received a moderate intensity exercise-training regimen that consisted of treadmill running on a flat surface for 30 minutes six days per week for three months. Measurements of myopathy included capillary density (Hu et al., 2018), the percentage of muscle fibers that contain internal nuclei, and minimum Feret's diameter. We examined ASO drug activity by droplet digital PCR quantification of CUG^{exp}-containing transcripts and RT-PCR analysis of alternative splicing. **Results**: HSA^{LR} mice demonstrated reduced vertical activity, shorter distance traveled, fewer ambulatory counts, and increased rest time as compared to age-matched wild-type controls. All measures of fatigue appeared similar in mice treated with saline or ASOs alone. By contrast, treatment of mice with ASOs combined with an exercise-training regimen improved fatigue by all measures. A group treated with an exercise-training regimen alone, without ASOs, showed a non-significant trend for reduced fatigue. Capillary density was reduced in both ASO-treated groups as compared to the non-ASO-treated groups, but remained higher than wild-type. In all treatment groups, approximately 40% of muscle fibers contained internal nuclei and fiber hypertrophy persisted. In both ASO-treated groups, CUG^{exp} transcripts were reduced by 75 - 80% and alternative splicing patterns appeared similar to wild-type. **Conclusions**: Fatigue and myopathy that result from pathogenic CUG^{exp} RNA are resistant to ASO monotherapy. The relationship between splice events in muscle tissue and fatigue or myopathy is indirect. To achieve optimal therapeutic benefit for DM1 may require the development of treatment strategies that are complementary to RNA-targeting ASOs. **Support**: The Elaine and Richard Slye Fund; Muscular Dystrophy Association.

Cancer - Immunotherapy, Cancer Vaccines

1149. Engineered red-Cell Therapeutics (RCT) as Artificial Antigen Presenting Cells Promote *In Vitro* Expansion of Both Naïve and Exhausted $CD4^+$ T Cells

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T cell mediated immunotherapies are efficacious in a small subset of cancers. Many therapies are designed to promote tumor-specific T cell responses by $CD8^+$ cytotoxic T lymphocytes which recognize tumor specific antigens presented by MHC class I molecules. Accumulating evidence supports a critical role for tumor specific $CD4^+$ T cells to achieve durable and efficacious responses. However, further development on how best to expand/differentiate antigen specific $CD4^+$ T cells for cell therapy as well as manufacturing these therapies at scale are necessary. To address this limitation, we genetically engineered allogeneic artificial antigen presenting cells (RCT-aAPCs) that express a tumor specific antigen bound to MHC II (signal 1), a costimulatory molecule (signal 2), and a cytokine (signal 3) that mimic and amplify normal APC-T cell interactions to drive both the quantity and quality of antigen-specific CD4 T cell responses. RCT-aAPCs are produced using $CD34^+$ hematopoietic progenitor cells from blood type O, Rh-negative, Kell-negative donors. Cells are expanded and transduced with lentivirus, followed by further expansion and differentiation into enucleated red blood cells expressing exogenous proteins on the cell surface. These allogeneic RCT-aAPC cells are designed to expand and activate endogenous tumor-specific T cells, thus eliminating the need to individually manufacture patient-derived T cells. As an *in vitro* proof of principle, red cells were engineered to express murine MHC class II $I\text{-}A^b$ loaded with the OVA 323-339 peptide (this MHC/peptide is recognized by TCR-transgenic OT2 T cells) and CD80 (RCT-MHCII-CD80). We observed >80-fold expansion of OT2 cells 3-6 days post co-incubation as measured by cell trace violet dye dilution. In contrast, red cells expressing only MHC II or CD80 did not induce detectable OT2 proliferation. RCT-MHCII-CD80 expanded OT2 cells had an

activated phenotype characterized by CD44 and CD25 expression. Supplementing RCT-MHCII-CD80 with the RCT expressing IL-12 induced Th1 commitment of OT2 cells, with enhanced proliferation and secretion of IFNγ, TNFα, IL-6 and IL-2. Furthermore, the RCT-MHCII-CD80 increased the percentage of OT2 cells with a memory-like phenotype including stem (Tscm), central (Tcm) and effector (Tem) memory T subsets. Based on these data, we also examined whether CD4 directed RCT-aAPCs can rescue exhausted T cells that have previously experienced multiple rounds of bead-based T cell priming. To this end, exhausted OT2 cells were treated with the RCT-aAPC expressing MHC II and CD80, supplemented with cytokine expressing RCTs. The RCT-MHC II-CD80 in combination with RCT-IL-7 induced significant OT2 proliferation, particularly of the Tem population, whereas RCTs expressing only MHC II and CD80 failed to maintain the OT2 population. Altogether, these results demonstrate that RCT-aAPCs expressing signals 1, 2, and 3 can significantly expand both naïve and exhausted CD4 T cells, leading to T cell activation, Th1 commitment and memory cell differentiation *in vitro*.

1150. Enhancers and Repressors of Immunotherapy: Translational Perspectives on Gene-Mediated Cytotoxic Immunotherapy

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RATIONALE: Gene-mediated cytotoxic immunotherapy (GMCI) is a local tumor immunotherapy that uses aglatimagene besadenovec (a non-replicating serotype 5 adenovirus, expressing HSV1 thymidine kinase) with the prodrug ganciclovir to induce DNA double strand breaks (DSB), leading to immunogenic tumor cell death and intratumoral immune cell invasion. Here we investigate potential repressors and enhancers of GMCI's effectiveness. GMCI is currently in clinical trials in combination with immune checkpoint blockade in glioblastoma. Thus we set out to identify potential areas to improve this approach for future application. Dexamethasone is used in symptomatic treatment of glioma patients, although it is known to cause immune suppression. However, the influence of dexamethasone on the efficacy of GMCI has not been explored. In contrast, DNA damage response inhibitors like the ATR inhibitor (ATRi) AZD6738 might not only amend the cytotoxic but also the immunogenic profile of GMCI, rendering it an attractive combination partner. **METHODS:** We investigated the effects of ATR-inhibition and dexamethasone on GMCI *in vitro* using cytotoxicity, flow cytometry and T-cell-killing assays in glioblastoma cell lines. The impact of dexamethasone and ATRi *in vivo* was assessed in an orthotopic syngeneic murine glioblastoma model. Tumor immune infiltrates were analyzed with flow cytometry. **RESULTS:** Cytotoxicity assays showed that dexamethasone has a slight impact on GMCI in vitro. In T-cell-functional assays, we observed a significantly impaired tumor cell killing. Immune cell response assays revealed a reduced immune cell proliferation after co-culture with supernatant from dexamethasone or combination treated glioblastoma cells. In vivo, while treatment with GMCI alone resulted in longer median symptom-free survival (39.5d) versus no treatment (25d), the combination of GMCI and dexamethasone resulted in the significant reduction of this effect (30d vs 39.5d ; $p = 0.0184$). The combination of ATRi with GMCI proved to be synergistic in cytotoxicity assays. Flow cytometry revealed a significant increase in DSB-associated H2AX foci as well as an improved immune profile by downregulation of GMCI-induced PD-L1 expression. *In vivo*, the combination with ATRi led to an increase in long-term surviving animals (66.7%) compared to GMCI (50%) and proved to be highly significant compared to the untreated control ($p=0.0022$). **CONCLUSION:** Our data suggest that dexamethasone may decrease the efficacy of immunotherapy for glioma through impaired T cell function: this emphasizes the need in identifying alternatives to dexamethasone to prevent attenuated responses in immunotherapies. The combination of GMCI with ATRi however points to additional therapeutic benefit through enhanced cytotoxic efficacy, improved immunogenicity *in vitro* and increased long-term survival *in vivo*, making it a promising future approach for the treatment of glioblastoma.

1151. Multiplexing of Up to 10 Gene Edits Using Crispr/Cas9 to Generate Car-T Cells with Improved Function

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Autologous chimeric antigen receptor (CAR) T cell therapies are currently approved for use in humans with relapsed/refractory B-cell malignancies. These products contain a mixture of T cell subtypes in differing cell states and have the CAR expression construct inserted randomly via viral vectors. Despite remarkable responses in many patients, significant variability in both CAR-T product quality and the responses in different hematological malignancies has been observed. In addition, there are few reports of clinical activity of CAR-T cells in solid tumors. CAR-T products may benefit from gene editing, such as gene knockouts and knock-ins, to enable more efficacious CAR-T cells with enhanced abilities to proliferate, survive, persist, and evade immune-suppressive environments. Here we show that CRISPR/Cas9 can be used to generate CAR-T cells with up to 10 edits. These edits can improve many of the desirable CAR-T properties noted above, as evidenced by CAR-T activity *in vitro* and *in vivo* in mouse models. Furthermore, we have generated highly edited CAR-T cells using starting material from both healthy donors and patients with cancer. Characterization of the resulting CAR-T cells using single-cell RNA sequencing revealed numerous T cell subtypes and heterogeneity across donors. Taken together, this work shows the possibilities of using CRISPR/Cas9 to improve the potency of CAR-T products and demonstrates that the impact of these changes can be further understood using single-cell sequencing methods.

1152. oNKord® Natural Killer Cells are Recognized as a Powerful, Versatile and Universal "Off the Shelf" Treatment to Develop Novel Genetically Modified Anti-Cancer Products

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Cancer immunotherapy focuses on harnessing the power of the host's immune system against malignant cells. In the last years, it has gained a pre-eminent role in the treatment of a variety of solid malignancies, mostly through to the discovery of checkpoint inhibitors (*e.g.* PD-1 in melanoma), and of hematological tumors, via Chimeric Antigen Receptor (CAR)-T cells directed against CD19. Although many tumors are recognized and killed by adaptive immunity, the response can be inhibited by immunosuppressive mechanisms or, as for CAR-T cells, broad applicability is hampered by the autologous nature of the treatment and the severe toxicity effects of the Cytokine Release Syndrome (CRS). Innate immune Natural Killer (NK) cells are gaining a pivotal role in cancer immunotherapy. NK cells can eliminate a variety of neoplastic, infected or stressed cells without prior sensitization via the tight regulation of activating and inhibitory signals transmitted through endogenous surface receptors. As normal, "self" cells are not attacked, only abnormal, "non-self" cells, are recognized and killed. Such mechanism is extremely powerful for the treatment of cancer for two main reasons: 1. NK cells recognize only malignant cells and do not affect healthy tissues, avoiding cytokine storms; 2. there is no need for the challenging and costly development of autologous products, as allogeneic NK cells can be used "off the shelf" as universal treatment. Glycostem Therapeutics (Oss, Netherlands) is a leading pioneer in the development of NK cell-based therapy for the treatment of hematological and solid tumors. Glycostem 's first therapeutic product, oNKord®, are NK cells differentiated *ex vivo* from hematopoietic stem cells from umbilical cord blood. oNKord® has successfully passed a Phase I clinical trial in n=11 elderly Acute Myeloid Leukemia (AML) patients, showing no toxicity, homing to bone marrow and to peripheral blood, *in vivo* maturation and patient response on Minimal Residual Disease (MRD, n=2, from 6-7% to 0.05% after 90 days), Progression-Free Survival (PFS) and Overall Survival (OS, 90% after 1 year). Based on such promising evidence, a new Phase I-II trial on AML and Multiple Myeloma (MM) patients will start in July 2020 using a novel cryopreserved product. Beyond naked oNKord®, Glycostem (together with partners) is developing innovative strategies to overcome known limitations in the field of cell immunotherapy: 1. the poor ability of immune cells to reach solid tumors and to infiltrate the tumor microenvironment; 2. the limited persistence of NK cells *in vivo*; 3. the change in NK-cell receptor repertoire *in vivo*, leading to inhibited therapeutic response. Ongoing programs include the equipment of NK cells with CARs directed against specific tumor markers and the use of RNA-interference to control NK surface receptor expression. Furthermore, Glycostem is developing a bioinformatics and data mining platform to investigate the biological variability between oNKord® products, comparing their *ex vivo* and *in vitro* characteristics (cell expansion, differentiation into NK cells, cytotoxicity) with their transcriptomic profile. This will provide statistic rationales to define the criteria dividing "excellent" vs "average" products and to link them to therapeutic success.

1153. Imaging Cancer Immunology: Magnetic Particle Imaging of Immunotherapies in a Murine Breast Cancer Model

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The rapid growth of research into immuno-oncology research has fueled a need to evaluate the existence, amount and phenotype of a variety of immune cells systemically. Tumor associated macrophages (TAM) plays a pivotal role in cancer immunotherapy. However, existing methods for qualitative and quantitative evaluation of TAM accumulation have generally been insufficient. Magnetic Particle Imaging (MPI) is a novel tomographic molecular imaging technique that can be used to non-invasively track phagocytotic cells, primarily macrophages, in 3D *in vivo*, with contrast similar to nuclear medicine but without the complex workflow, safety, and half-life limitations. A murine breast tumor model was established by injecting 3 x 10^5 4T1 cells into the 5^{th} mammary fat pad of 8-10-week female Blab/c mice. Immunotherapy was initiated by the combination of CD47 mAb and PD-L1 mAb once the tumors become palpable. Single mAb treated and isotype matched mAb treated groups were used as controls. All mice were injected with an iron-oxide MPI tracer (Ferumoxytol, 30mg/kg) and then 3D MPI and MRI (T2-weighted multi-slice, multi-echo (MSME) sequences) images were acquired at 1, 3, 7 and 14 days after tracer injection. MPI and MRI images were co-registered and quantitated. Tumors, liver, spleen and draining lymph nodes were then harvested, imaged, fixed, and stained with Perls Prussian blue for analysis of iron content. Tumour-associated macrophage (TAM) accumulation has been shown qualitatively to increase following CD47 treatment. With the combination of antiPDL1 treatment, more Ferumoxytol accumulated at the tumor site as demonstrated by *in vivo* MPI studies. All mice showed an accumulation of Ferumoxytol in the tumor and liver following injection. For both groups, nanoparticles were predominately detected in the expanding margins of the tumour. There was a statistically significant difference in the accumulation of TAMs between the CD47-treated and untreated groups, with the combination treated group having the most increase in TAM accumulation. The sensitivity, specificity and quantitation of MPI provides valuable non-invasive information on the development of a preclinical model that monitors the efficacy of the combination of CD47 mAb and PDL1 mAb cancer immunotherapies. When combined with the high spatial resolution provided by MRI, co-registered MRI-MPI can be utilized to evaluate the response of TAMs to the combination immunotherapy.

1154. EGFRvIII-Targeting DNA-Encoded Immune Cell Engager (DICE) Generates *In Vivo* Expression of Bispecific Antibody that Induces T Cell-Mediated Cytolytic Activities Against EGFRvIII-Positive Tumors and Controls Tumor Growth in a GBM Mouse Model

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Development of bispecific antibodies targeting T cells and tumor-associated antigens has exponentially expanded in both preclinical and clinical settings in recent years. In 2014, one bispecific antibody was approved to treat acute lymphoblastic leukemia. However, due to its low molecular weight, the serum half-life of the antibody is only about 4 hours. As a result, the treatment requires a continuous IV injection of the antibody over several days, which can extend to several weeks. Poor pharmacokinetic profile has presented a big challenge in development of bispecific antibodies, along with other difficulties associated with manufacturing and molecule stability. To address these problems, we developed an optimized synthetic DNA-encoded immune cell engagers (DICE) which are designed to express bispecific antibodies *in vivo*. In our previous studies, we demonstrated that mice given a single administration of HER2-DICE exhibit long-term *in vivo* expression of the bispecific antibody and T cell-mediated cytolytic activities against a HER2-expressing ovarian cell line for over 120 days. In the same study, HER2-DICE not only controlled tumor progression but promoted tumor clearance in many of animals in an ovarian cancer mouse model. With a similar strategy, we developed DICE targeting EGFRvIII, a tumor-specific antigen which is expressed in 30-50% of glioblastoma multiform (GBM) patients. Supernatant samples from cells transfected with EGFRvIII-DICE *in vitro* showed potent target-specific binding affinity to both EGFRvIII and CD3, and induced T cell-mediated cytolytic activity against a GBM cell line overexpressing EGFRvIII. Co-culturing target cells and primary human T cells in the presence of EGFRvIII-DICE supernatant stimulated robust T cell responses and displayed significant levels of IFNγ, TNFα, and CD107a in cytotoxic T cell population. Finally, in a GBM mouse challenge model, treatment of EGFRvIII-DICE to NSG mice repopulated with human T cells resulted in control of tumor growth, which was not observed in the empty vector control group. These studies support that synthetic DNA delivery of bispecific antibody generates potent and functional antibodies that are capable of invigorating cytotoxic T cell function and could be studied as an alternative approach to development of bispecific antibodies for cancer immunotherapy.

1155. Highly Purified and Concentrated Lentiviral Vectors for Safely Engineering Human Nk Cells to Treat Acute Myelogenous Leukemia

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<Immunomodulation offers great promises for a wide range of therapeutic applications, in particular for blood cancers. Nevertheless, many obstacles exist especially for clinical development which requires efficient and safe delivery technologies, as well as gene expression level and duration tailoring. The emergence of targeted immunotherapy, especially chimeric antigen receptor (CAR) T-cell therapy, has opened new possibilities, demonstrating tremendous success for patients with lymphoblastic leukemia. In the clinic, recent focus has been on improving autologous CAR-T cell manufacturing using mainly gamma retrovirus transduction approaches. Despite some improvements, the entire process remains challenging and significantly costly. This is mainly due to the fact that patient T cells are prone to dysfunction, manufacture failures, and disease progression during the process. A major difficulty is also to circumvent genotoxicity, which is associated with the random insertion of the CAR transgene into the host genome, potentially leading to proto-oncogene activation, triggering of the inflammatory response, or clonal T cell expansion. Natural Killer (NK) cells are other key immune effector cells which, contrary to T cells, do not require antigen priming and are at a low risk of Graft-Versus-Host Disease (GVHD), therefore offering the potential of an allogenic "off-the-shelf" therapeutic product. NK cells can trigger rapid anti-tumor responses and lyse target cells particularly through antibody-dependent cellular cytotoxicity. Moreover, NK cells have been shown to be efficient in generating anti-tumor activity against Acute Myeloid Leukemia (AML). Nevertheless, due to mechanisms unknown to date, primary NK cells exhibit a high resistance to lentiviral transduction, hampering transgene expression and consequently the generation of CAR-NK cells. In this collaborative work, we show that the use of highly purified and concentrated self inactivating lentiviral vectors in combination with an optimized transduction protocol, allow for the genetic modification of human cord blood derived NK cells. Also, we show that transduction does not lead to viability nor phenotypic alterations of the transduced NK cells. Our approach not only achieves high transduction efficiency, leading to strong and stable transgene expression, but also preserves the cytotoxic function of the NK cells, in vitro and in vivo. Here we propose a novel method allowing for the generation and production of lentiviral vector engineered primary NK cells, thus circumventing the problem of poor autologous CAR-T cell efficiency and gamma retrovirus associated risks. This work lays the groundwork for novel cellular therapies based on lentivirally transduced primary NK cells. All of these factors, as well as the ability to produce lentiviral vectors using Flash Therapeutics' GMP compliant production platform, offer additional safety considerations for clinical development and human use. Flash Therapeutics offers a vector manufacturing continuum from discovery to therapy, with an integrated control plan, allowing for the development and for the GMP production of custom lentiviral batches dedicated to clinical applications.>

1156. Bortezomib Significantly Enhances Gamma Delta T-Cell Mediated Lysis of AML and T-ALL

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Engagement between the natural killer group 2, member D (NKG2D) receptor and its ligands is critical for immune cell activation and targeting of cancer cells. Tumor cells can escape immune cell surveillance through decreased surface expression of NKG2D ligands, which are known markers of cellular stress. Drugs that increase NKG2D ligand expression offer a potential way to improve immunotherapies for cancer patients. Previous studies have shown bortezomib, a proteasome inhibitor, increases surface expression of NKG2D ligands on cancer cells, enhancing sensitivity to natural killer (NK) and γδ T cell-mediated cytotoxicity. Here, we investigate the combination of bortezomib and γδ T cells as a novel therapy for acute myeloid leukemia (AML) and T-cell acute lymphoblastic leukemia (T-ALL), two hematologic malignancies in which CAR-based cellular immunotherapy has been challenging. We treated two AML cell lines, Nomo-1 and Kasumi-1, and two T-ALL cell lines, Jurkat and Molt-4 with increasing concentrations of bortezomib for 6, 18, 24, and 48 hours and measured changes in NKG2D ligand expression via flow cytometry. Our results showed 24 hour treatment with 5 nM bortezomib significantly increased expression of UL binding protein-2/5/6/ (ULBP2/5/6) in Molt-4 and Jurkat cells ($n = 3$, $p < 0.05$). Twenty-four hour treatment with 5 nM bortezomib significantly increased expression of ULBP1 and ULBP2/5/6 in Kasumi-1 cells ($n = 3$, $p < 0.05$). Twenty-four hour treatment with 10 nM bortezomib significantly increased expression of ULBP2/5/6 in Nomo-1 cells ($n = 3$, $p < 0.05$). Cytotoxic capabilities of healthy donor-derived γδ T cells against leukemia cells was assessed in a flow cytometry-based assay. To ensure cytotoxicity was mediated by γδ T cells only, an αβ T cell depletion was performed on day 6 of our ex vivo expansion, yielding a final cellular product ≥ 92% CD3+/γδ TCR+ by day 12 (average = 96.38 ± 4.33%, n = 6). Day 12 γδ T cells were incubated with target cells at the following effector to target (E:T) cell ratios: 0:1, 1:4, 1:2, 1:1, and 2.5:1. Target cells were treated with either vehicle control or bortezomib 24 hours prior to the cytotoxicity assay. Target cell death was determined via 7-AAD and Annexin V staining. Bortezomib treatment increased target cell death at all E:T ratios for Jurkat, Kasumi-1, and Nomo-1 cells. Jurkat cell death was significantly increased with bortezomib treatment compared to vehicle control at the 1:4 and 1:2 ratios by 24.11% and 18.29%, respectively ($n = 4$, $p < 0.05$). Treating with bortezomib significantly increased cytotoxicity of Kasumi-1 cells at the 1:4, 1:2, and 1:1 ratios by 35.25%, 34.03%, and 29.79% ($n = 3$, $p < 0.05$). Bortezomib treatment significantly increased Nomo-1 cell death at all E:T ratios by 24.91%, 29.6%, 33.41%, and 25.51% ($n = 3$, $p < 0.05$). The total cytotoxicity for bortezomib treated cells at the 1:1 E:T ratio was 89.76%, 56.50%, and 77.83% for Jurkat, Nomo-1, and Kasumi-1, respectively. Preliminary findings show blocking the NKG2D receptor on γδ T cells prior to the cytotoxicity assays decreased γδ T cell specific cytotoxicity of bortezomib treated target cells by 34.2% in Jurkat cells and 18.52% in Kasumi-1 cells. Current studies are underway to investigate the efficacy of combining bortezomib and γδ T cells in AML and T-ALL xenograft models. These results suggest bortezomib enhances γδ T cell-mediated killing of AML and T-ALL cells through NKG2D ligand-receptor interaction, especially at lower E:T ratios. Since these ratios are more clinically relevant, this treatment combination may be an effective approach to overcome tumor cell evasion of immunotherapies in AML and T-ALL patients.

1157. An Off-The-Shelf CAR T-Cell Strategy for Hematologic Malignancies That Protects Against GVHD and Allorejection

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Introduction Despite the success of autologous chimeric antigen receptor (CAR) T-cells in hematologic malignancies, barriers to more widespread use of this potentially curative therapy exist. Manufacture failure, disease progression prior to infusion, and exorbitant cost prove prohibitive for many. There is an urgent need for an immediately available CAR T-cell option. However, development of "off-the-shelf" CAR T-cells has been hindered by two major pitfalls: potential for polyclonally activated CAR T-cells from unrelated donors to cause graft versus host disease (GVHD), and rejection of allogeneic CAR T cells by recipient alloreactive T-cells. To address these issues, we propose a first-in-man CAR-modified virus specific T cell (CAR-VST) approach that eliminates hematologic malignancies without causing GVHD while avoiding allorejection. The rationale for utilizing a CAR-VST platform is two-fold: 1) VSTs have a more restricted TCR repertoire than polyclonal activated T cells (ATCs), thus are less likely to cause GVHD (as shown by the low incidence of GVHD in studies of allogeneic EBV-specific T cells (EBVSTs)). 2) We have shown that alloreactive T-cells overexpress CD30, making them susceptible to elimination by CD30.CAR T-cells. We hypothesize that a VST engineered to express *both* CD30 and CD19. CARs will result in a T-cell that not only targets CD19 + and CD30+ malignancies, but also alloreactive recipient T-cells, without causing GVHD. This "off-the-shelf" approach to CAR T-cell therapy has the potential for long-term persistence with an improved safety profile.
Methods To generate CD30+CD19.CAR-EBVSTs (dualCAR-EBVSTs), we isolated PBMCs from healthy donors using ficoll density gradient. EBVSTs were generated by providing antigen stimulation with overlapping peptides spanning the protein sequence of 3 latent EBV antigens. After 4 days, cells were transduced simultaneously with second generation CD19 and CD30.CAR constructs. DualCAR-EBVSTs underwent second and third antigen stimulations with EBV pepmix-pulsed irradiated autologous ATCs and an HLA-negative costimulatory cell line. We examined proliferative capacity, phenotype, and antitumor function of the dualCAR-EBVSTs compared to single-CAR transduced EBVSTs or ATCs. We also examined whether equipping EBVSTs with a CD30.CAR allowed them to eliminate alloreactive T cells upon exposure to HLA-mismatched PBMCs (containing alloreactive T cells) in a mixed lymphocyte reaction.
Results DualCAR-EBVSTs were successfully manufactured from multiple healthy donors. Proliferative capacity was comparable to single-transduced EBVSTs or ATCs. Importantly, dualCAR-EBVSTs

were able to kill CD19 *and* CD30+ tumor cells *in vitro.* DualCAR-EBVSTs were also able to eliminate (and thus avoid rejection by) HLA-A2+ alloreactive T cells (distinguished by the activation marker CD71) via the CD30.CAR as shown in Figure 1.

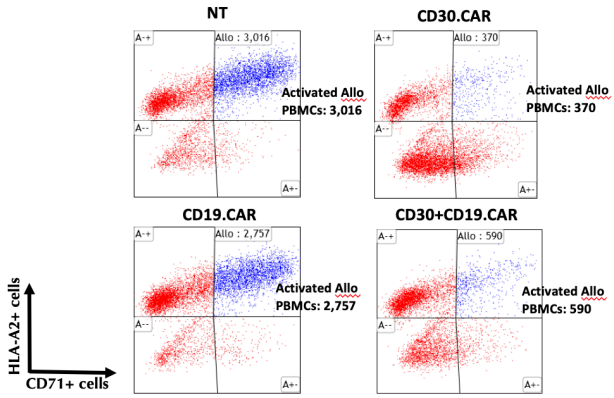

Figure 1. Mixed lymphocyte reaction of CAR-EBVSTs generated from an HLA-A2 negative donor and PBMCs from an HLA-A2 positive donor. Activated alloreactive HLA-A2 positive T-cells are nearly eliminated via CD30.CAR by Day 7

Conclusions We outline a novel approach to generating an "off-the-shelf" CAR T cell using EBVSTs transduced with *both* CD19 and CD30. CARs. *In vitro*, this dualCAR-EBVST eliminates tumor targets *and* alloreactive T cells, suggesting its ability to avoid rejection and persist long-term. This preliminary work serves as an important proof-of-concept, with potential for safely targeting other hematologic or solid malignancies.

1158. Treatment with Iberdomide Enhances Antitumor Function of the Anti-CD19 Chimeric Antigen Receptor (CAR) T Cell Therapy Lisocabtagene Maraleucel (liso-cel)

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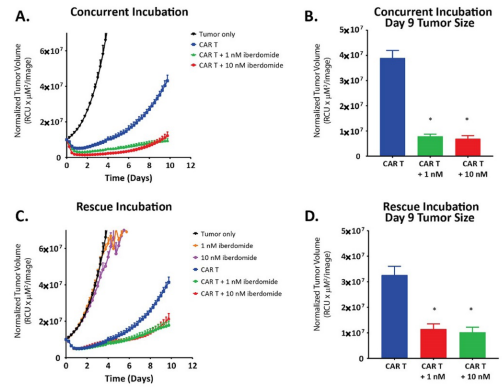
Background. Liso-cel, an investigational, CD19-directed, defined composition, 4-1BB CAR T cell product administered at equal target doses of CD8+ and CD4+ CAR+ T cells, is being evaluated for treatment of B-cell malignancies. Reemergence of disease in patients who achieved a complete response may be due in part to exhaustion of CAR T cells. Thus, combination therapies that promote sustained pharmacological function of CAR T cells may increase the durability of response. Iberdomide is a potent cereblon E3 ligase modulator (CELMoD) compound capable of co-opting cereblon, modulating its activity, and redirecting the protein degradation machinery of the cell toward the elimination of target proteins (Ikaros and Aiolos), resulting in therapeutic effects (enhanced tumoricidal and immune stimulatory activity). Iberdomide is being evaluated in multiple myeloma, non-Hodgkin lymphoma, and lupus. Here, we examined iberdomide in combination with liso-cel in both acute activation and chronic stimulation assays that promote functional exhaustion of CAR T cells to assess how the combination influences liso-cel activation, onset of exhaustion, and rescue from exhaustion. **Methods.** Liso-cel produced from healthy donors was subjected to acute activation (up to 72 hours) or chronic stimulation (6 days) to recapitulate a potential resistance mechanism of CAR T cell exhaustion. Iberdomide was assessed concurrently (during acute activation and chronic stimulation of anti-CD19 CAR T cells) or in a rescue setting (after anti-CD19 CAR T cells achieved a hypofunctional exhausted state). Functional activity measures included proliferation, viability, and cell cycle or cytolysis and effector cytokine production after further culture of liso-cel with CD19+ lymphoma cells or tumor spheroids. **Results.** Iberdomide degraded Ikaros and Aiolos in liso-cel in an acute activation assay after a 24-hour incubation period. In acute stimulation assays, iberdomide incubation uncoupled CAR T cell proliferation from cytokine production, simultaneously increasing interferon gamma levels while slowing proliferation. We speculated that a reduced proliferative rate could preserve antitumor functionality of liso-cel. Indeed, concurrent iberdomide (1 nM or 10 nM) incubation during chronic stimulation limited onset of anti-CD19 CAR T cell exhaustion, increasing both cytokine production and cytolysis against CD19+ lymphoma cells and spheroids (**Fig 1 A, B**). Interestingly, incubation of iberdomide (1 nM or 10 nM) with exhausted liso-cel product resulted in a release of this exhaustion as demonstrated by the significant difference in normalized tumor volume by Day 9 compared with vehicle control ($P<.0001$) (**Fig 1 C, D**). Furthermore, RNA-seq analysis indicated iberdomide incubation partially diminished gene signatures associated with anti-CD19 CAR T cell hypofunctionality. **Conclusions.** At clinically relevant concentrations, iberdomide enhanced aspects of liso-cel antitumor pharmacology across acute activation and chronic stimulation assays, which may prolong the durability of response to liso-cel in the clinic.

Figure 1: Effect of Iberdomide on Cytolytic Activity of Chronically Stimulated liso-cel Against a CD19+ Lymphoma Cell Line Grown as 3-Dimensional Spheroids



Anti-CD19 CAR T cells were subjected to chronic stimulation with 30 µg/mL of anti-CD19 agonistic antibody for 6 days. Anti-CD19 CAR T cells were incubated concurrently during stimulation (**A, B**) with vehicle control or iberdomide 1 nM or 10 nM. For rescue treatment (**C, D**), vehicle control or iberdomide was only added during coculture with tumor cells. Next, anti-CD19 CAR T cells were cocultured with Granta-519 tumor cells expressing CD19 and NucLight Red that were grown as spheroids. Cytolysis was indicated by reduction in spheroid size, measured by loss of red fluorescence over a 9-day period using an IncuCyte® Live-Cell Analysis System. Plots show mean ± standard error of the mean of fluorescence units in the bright-field image (triplicate wells of 3 donor lots of anti-CD19 CAR T cells and 2 independent experiments). Mean values of iberdomide-treated samples were compared with control samples at the end of the experiment using 2-way analysis of variance. RCU, red calibrated unit/total red object integrated intensity. *$P<.0001$.

1159. Bioreducible Amphiphilic Polymers for Nucleic Acid Delivery to Antigen Presenting Cells for Cancer Immunotherapy

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We engineered novel biodegradable cationic polymers for improved nucleic acid delivery to antigen presenting cells (APCs) for a genetic cancer vaccine. We developed poly(beta-amino ester) (PBAE) polymeric nanoparticles (NPs) that achieve efficient and safe delivery of mRNA and nucleic acid-based adjuvants to APCs in vitro and in vivo. This vaccine approach utilizes the body's own defense mechanisms by delivering mRNA encoding tumor-associated antigen and an inflammatory stimulus to APCs, which then endogenously produce the antigen and present it to T cells. Immune cells are conventionally very difficult to transfect, and the most successful platforms to date are viral vectors, which are powerful gene delivery vehicles but have important safety concerns. Non-viral vectors are safer alternatives and have a larger carrying capacity. However, their success in transfecting immune cells has been limited. The novel system developed here addresses the challenges associated with intracellular delivery to APCs to achieve targeted and efficient delivery of nucleic acids using safe, biodegradable materials. PBAEs are cationic polymers that we have designed for non-viral gene delivery utilizing a library of monomers for synthesis so that the structure can be tuned according to particular needs. To optimize transfection, we synthesized a library of novel bioreducible and amphiphilic PBAEs with enhanced properties for mRNA delivery. Acrylate-terminated PBAEs were synthesized by Michael addition reaction of amines to diacrylates (Fig. 1). Bioreducible PBAE structures were synthesized by utilizing a disulfide-containing monomer. For mRNA transfection, bioreducible polymers were found to be preferable as they allow for triggered release of nucleic acid cargo when they move from the extracellular environment to the cytoplasm. Hydrophobic side chain molecules were incorporated into the polymer at varying molar percentages in combination with conventional hydrophilic side chain molecules to increase the lipophilicity of the polymers. PBAEs were then endcapped with an amine-containing molecule. NPs self-assemble electrostatically by incubating mRNA and adjuvants and polymer in 25 mM sodium acetate (pH 5) for 10 minutes. Candidate particles were screened for transfection efficacy and toxicity to dendritic cells (DCs). The PBAEs with the highest rate of transfection and lowest toxicity were selected for further studies. The top PBAE candidates were able to achieve ~95% transfection of murine DCs with minimal toxicity. The top candidates were also screened for transfection in vivo and found to cause high transfection of DCs and macrophages in the spleen, the major site of APCs, after systemic NP administration (Fig. 2). For adjuvant studies, particles were synthesized to co-encapsulate mRNA and nucleic acid adjuvants CpG, Poly(I:C), and/or 2',3'-cGAMP, and were found to effectively mediate intracellular delivery of these adjuvants and lead to DC activation. mRNA encoding ovalbumin (OVA) was used as a model antigen to test the efficacy of this system as a potential genetic vaccine. After delivery of OVA mRNA to DCs using a top PBAE candidate, the cells were found to present the OVA SIINFEKL peptide antigen on MHC I at high levels, comparable to a peptide-pulsed control (Fig. 3). We are currently investigating the therapeutic efficacy of this system as a genetic vaccine for melanoma.

Figure 1

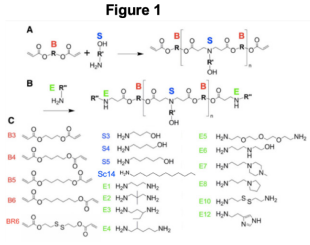


Figure 2

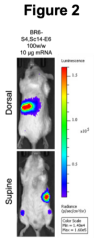
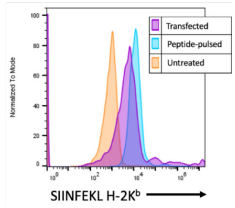


Figure 3



1160. Investigating the Therapeutic Efficacy of Disruption of Cell Intrinsic Checkpoint Regulator CTLA-4 in Chimeric Antigen Receptor T Cells

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Cancer immunotherapy is a rapidly growing field that has led to multiple successful strategies including monoclonal antibodies (MAb), inhibitory receptor (IR) blockade, cancer vaccines and adoptive cell transfer. Adoptive transfer of T cells modified to express chimeric antigen receptors (CAR-Ts) can redirect T cells to tumor-associated antigens and has shown impressive clinical efficacy in patients with B cell malignancies. However, the success of CAR T cells in other types of tumors has been much less encouraging. In some cases, patients relapse post CAR treatment due to poor persistence or immunosuppression in the solid tumor microenvironment. This state is characterized by high co-expression of IRs, including PD-1 and CTLA-4, and deterioration of T cell function. Both CTLA-4 and PD-1 are known to maintain T cell homeostasis. CTLA-4 does this by regulating T cell proliferation in the early stages when T cells are primed in lymph nodes. Systemic administration of MAb against CTLA-4 has shown significant clinical benefit in certain malignancies. However, blocking these T cell-intrinsic inhibitory pathways can activate endogenous T cells throughout the body and has clinically resulted in auto-immunity. Thus, motivating the search for safer ways of inhibiting IR activity and making it imperative to understand the role of these negative regulators in post-thymic human T cells. Disrupting CTLA4 expression in CAR T cells allows antigen-dependent investigation of the role of IRs while potentially minimizing the toxicity of the approach. Thus, the overall goal is to understand the role of this cell-intrinsic checkpoint regulator, CTLA-4, in post thymic T cells and determine if disruption of this receptor promotes anti-tumor efficacy of CAR T cells. Knockout of CTLA4 using CRISPR Cas9 technology in CD19 directed CAR T cells significantly improves tumor clearance in long-term re-stimulation assays against acute lymphoblastic leukemia (ALL) cell lines. CRISPR-edited CTLA4 KO CAR T cells also maintain surface CAR expression for longer durations than unmodified CD19 CAR T cells as shown in Fig. 1A. Xenograft models of ALL also show significantly reduced tumor progression when treated with CTLA4 KO CD19 CART cells in comparison to unmodified CD19 CAR T cells shown in Fig. 1B. Thus, the disruption of CTLA4 endows CD19 CART cells with superior anti-tumor efficacy. These

findings suggest that CTLA4 disruption on CAR T cells expands the therapeutic treatment window, potentially multiplying the types of cancer that can be treated with CAR T cell immunotherapy. begins

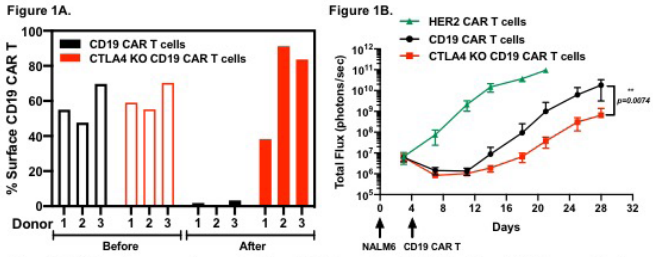

Figure 1. (A) Showing percentages of surface CAR Expression detected using anti-idiotype antibody against CD19 CAR T before and after long-term re-stimulation assay, in three different donors. (B) Showing anti-tumor efficacy of CTLA-4 disrupted CD19 CAR T cells in xenograft models of acute lymphoblastic leukemia (n=8).

1161. Comprehensive Identification of Tumor-Reactive TCRs and Cognate Targets for Novel TCR T-Cell Cancer Therapies

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TCR-engineered T-cell therapy is one of the most promising new approaches to cancer therapy, but is currently limited by a lack of diverse targets and by an inability to de-risk TCRs by identifying problematic off-target interactions. These two limitations can both be addressed by "T-Scan", a genome-wide screening method that enables the unbiased identification of the natural targets and off-targets of T cell receptors (TCRs) of interest. This method is based on a granzyme B-activated fluorescent reporter that measures the functional engagement of T cells with target cells expressing cognate antigen. When combined with a genome-wide library of targets that tiles across the human proteome, it enables the comprehensive assessment of TCR recognition of target peptides that are processed within cells. Here, we demonstrate application of the T-Scan technology to therapeutic development in two ways. First, T-Scan was used to identify all the off-targets of a MAGE-A3-directed TCR. Two previously unknown targets were identified, neither of which were obvious based on sequence similarity. This demonstrates the value of using an experimental approach to de-risk therapeutic TCRs and provides a valuable tool for preclinical safety screening. Second, T-Scan was used to discover a novel TCR target using tumor-infiltrating lymphocytes (TILs) derived from a colorectal cancer tumor. As the screening technology is non-competitive in nature, five tumor-reactive TCRs were screened in parallel, leading to the discovery of a novel gene that is over-expressed in several types of cancer, including colorectal and breast cancers. Notably, the TCR exhibits reactivity to cancer cell lines that correlates with target gene expression levels. Based on these proof-of-concept experiments, a discovery platform was developed to identify tumor-reactive TCRs that recognize novel shared-antigen targets from patient TILs. First, CD8+ T cells are isolated from patient tumors and subjected to single-cell sequencing of paired TCR alpha and beta chains. Second, the genes encoding these TCRs are synthesized as a pool using chip-based oligonucleotide synthesis of CDR sequences. Finally, the resulting TCR library is transduced into primary T cells and co-cultured with a panel of HLA-engineered cancer cell lines. T cells are sorted based on activation markers, revealing tumor-reactive TCRs for novel target identification. Using this platform, we have identified several novel targets, which are currently being evaluated for further development. Collectively, this platform enables construction of a repository of therapeutic TCRs with diverse targets and HLA restrictions, providing a way to develop multiplexed TCR therapy that is tailored for each patient, but draws from a collection of well-characterized tumor-specific TCRs.

1162. Effective Stabilization of Viral vectors in Liquid Formulation Using an Algorithm-Based Development Approach

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In gene therapy and in development of therapeutic as well as preventive vaccines viral vectors are employed. During manufacturing, storage, and distribution the viral vectors are exposed to various kinds of stress, like high temperature, shear stress and freeze/thaw steps. Degradation induced by such stress is a challenge in development and administration of viral vector products based on e.g., adenovirus serotype 5 (Ad5) or adeno-associated virus (AAV). Formulations that stabilize the viral vectors can overcome such development hurdles. We applied an algorithm-based approach comprising Design of Experiment (DoE) in development of formulations that effectively stabilize Ad5 virus vectors. Forty different formulations were designed by this approach and were placed on stability study. The viral vector formulations were stored at three different temperatures, at accelerated conditions at 37°C as well as 25 °C and for long-term storage at 5°C. The results were analysed by non-linear statistics. This evaluation revealed which excipients were stabilizing, de-stabilizing or had no stabilizing effect. Another finding was that the interaction of excipients can lead to even better stabilizing effect. This showed that not only the choice of single excipients is important in formulation development, but a tailored excipient combination with optimized concentrations. Comparison of the data from the three storage conditions demonstrated that results from accelerated storage were predictive for long-term storm storage. This predictive power of accelerated storage condition in stability studies presents an opportunity for a shorter development time of viral vector formulations. Combined with the algorithm-based development strategy including DOE, that enables a wider design space, the approach offers the potential to develop in a short time formulations that stabilize viral vectors in liquid formulations. This approach is currently transferred from Adenovirus to AAV, and the most recent data will be presented at the conference.

1163. Human CAR Monocytes Demonstrate Anti-Tumor Activity and Differentiate into M1-Polarized CAR Macrophages

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Solid tumors remain an intractable challenge for lymphocyte-based adoptive cellular therapy due to antigen heterogeneity, limitations in trafficking, and susceptibility to immunosuppression and exhaustion. Macrophages are the most abundant immune cell in the solid tumor microenvironment (TME) and are actively recruited as monocytes by TME-derived chemokines. Outside of the TME, monocytes and macrophages are potent immune effector cells capable of phagocytosis, cellular cytotoxicity, T cell recruitment, and antigen presentation. We have previously demonstrated that Carisma Therapeutics' proprietary CAR macrophage (CAR-M) adoptive cell therapy platform has potent anti-tumor activity and has the potential to overcome the key barriers to solid tumor efficacy. Currently, CAR-M are generated via *ex vivo* differentiation of peripheral blood monocytes into macrophages prior to genetic manipulation. In this abstract, we introduce the novel concept of CAR monocytes, which are genetically engineered without ex vivo differentiation and with minimal cell culture - shortening the manufacturing process from 7 days to approximately 2 days. Using the chimeric adenoviral vector Ad5f35, we engineered human monocytes with an anti-HER2 CAR. Transduction efficiency and viability both exceeded 90% in numerous human donors. Ad5f35 transduced CAR monocytes maintained CAR expression and viability ex vivo for at least 21 days. CAR monocytes efficiently differentiated into CAR-expressing macrophages when treated with GM-CSF as determined by FACS-based phenotypic characterization and Wright-Giemsa staining. Anti-HER2 CAR monocytes eradicated HER2 expressing tumor cells in a time and dose-dependent manner and had comparable potency to anti-HER2 CAR-M. Additionally, CAR monocytes produced pro-inflammatory cytokines such as IFN-γ and TNF-α. We have previously demonstrated that CAR-M are locked into a pro-inflammatory M1 phenotype after transduction with Ad5f35. Similarly, CAR monocytes demonstrated elevated expression of M1 markers, and maintained an M1 phenotype following differentiation into CAR macrophages. The M1 phenotype was imparted by transduction with Ad5f35. Taken together, this abstract describes the successful development of CAR-monocytes with the potential for a rapid manufacturing process. In addition to direct anti-tumor activity while in the monocyte phase, CAR monocytes have the capacity to differentiate into CAR macrophages in situ, which are in turn capable of phagocytosis, T cell recruitment, TME modulation, and antigen presentation. Given the previously demonstrated pre-clinical efficacy of CT-0508 (an anti-HER2 CAR macrophage), the CAR monocyte platform described herein offers a shortened manufacturing process and a potential advantage in tumor penetration, which will be directly evaluated in upcoming studies.

1164. In Vivo Generation of CAR T Cells Selectively in Human CD4+ Lymphocytes

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T cells modified with CD19-specific chimeric antigen receptors (CARs) result in significant clinical benefit for leukemia patients. Conventional CAR T cell products contain not only CAR expressing cytotoxic CD8+ T cells but also CD4+ T cells. To investigate the role of the CD4 T cell subset in context of the CD19-CAR T cell therapy we generated a CD4-targeted LV encoding the CD19-CAR. Cultivated human PBMC transduced with CD4-LV resulted in CD4+ CAR T cells which proliferated efficiently upon antigen exposure and, notably, were functionally efficient in killing target cells *in vitro*. Administration of CD4-LV into NSG mice transplanted with human PBMC led to 40 - 60% of the human CD4+ lymphocytes expressing the CAR while CD8+ cells remained CAR-negative. CAR+ T cells displayed a Th1/Th2 phenotype, which was accompanied by CD19+ B cell elimination. Administration of CD4-LV into NSG mice reconstituted with human CD34+ cells, induced CAR expression and B cell elimination within two to three weeks post injection. Preclinical analysis in a tumor mouse model revealed that mice administered with CD4-LV exhibited faster and superior tumor cell killing compared to mice injected with CD8-LV alone or in combination with CD4-LV. We hypothesize that CD4+CAR+ cells outperform CD8+CAR+ cells especially at high tumor burden, mainly since CD8 cells are more prone to exhaustion. This study sheds light on the possibility of *in vivo* targeting CD4+ lymphocytes and supports their importance in the field of cellular therapy.

1165. Natural Killer-Optimized CAR Containing NKG2D/2B4/CD3ζ Domains Demonstrate Improved Functionality in Peripheral Blood Natural Killer Cells

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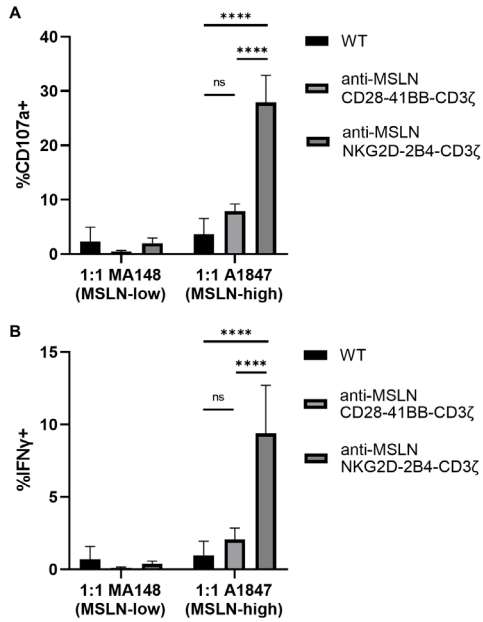
Natural killer (NK) cells are potent effectors of the innate immune system that are able to recognize and eliminate a variety of cancer types. NK cells employ numerous HLA-unrestricted mechanisms to kill their targets, including antibody-dependent cellular cytotoxicity, proinflammatory cytokine production, and the perforin pathway. The effectiveness of NK cells, however, can be greatly suppressed by tumor immune escape mechanisms. The expression of chimeric antigen receptors (CARs) is a promising approach to augment the activation and cytotoxicity of NK cells. While autologous transfer of T cells expressing CARs has shown great success in the treatment of refractory/relapsed B cell malignancies, the need to generate CAR-expressing cells on a per patient basis contributes to the high cost of therapy and makes treatment impractical for patients with rapidly progressing disease. In contrast, NK cells do not cause graft-versus-host disease (GvHD),

and can be deployed as an allogeneic immunotherapy. Furthermore, NK cells have a relatively short lifespan *in vivo*, reducing the chance for long-term adverse events. Combined with CAR-NK cells' ability to retain native non-clonal receptors, CAR-NKs show great potential as an immunotherapy. The majority of studies using CAR-NK cells, however, have used CARs containing CD28 or 4-1BB costimulatory domains, and further optimization of CARs for the activation of NK cells is necessary. Here, we employed a CAR targeted to Mesothelin (MSLN), and containing the NKG2D transmembrane domain, 2B4 costimulatory domain, and CD3ζ signaling domain. Human NK cells were isolated from peripheral blood, activated using artificial antigen presenting cells (aAPCs), then transduced with lentivirus encoding either a 3rd generation CAR (ScFV-CD28-41BB-CD3ζ) or NK-optimized CAR (ScFV-NKG2D-2B4-CD3ζ), each followed by a T2A sequence and an RQR8 molecule. The RQR8 molecule contains a CD34 epitope, allowing facile surface detection and immunomagnetic selection. While NK cells are notoriously resistant to viral gene transfer, immunomagnetic selection using an anti-CD34 antibody achieved RQR8 expression of an average of 83.9% (range 53.8–99.7%). The resultant CAR-NK cells were co-cultured at a 1:1 ratio with MA148 and A1847 ovarian cancer cell lines, which are MSLN low-expressors and high-expressors, respectively. In co-cultures with the MSLN-high cell line, effectors expressing the NK-optimized NKG2D-2B4-CD3ζ CAR showed significant increase in CD107a expression (27.9±4.9 v.s. 7.8±1.3, p=0.0001, Figure A) and IFN-γ expression (9.4± 3.3 v.s. 2.0±0.8, p=0.0001, Figure B) compared to NK cells expressing 3rd generation CD28-41BB-CD3ζ CARs. In contrast, CAR-NK cells showed minimal reactivity to the MSLN-low cell line, indicating that the CAR engaged targets in an antigen-specific manner. These results demonstrate the increased functionality of NK-optimized CAR compared to conventional 3rd generation CARs in peripheral blood NK cells, which can be generated at high efficiency using immunomagnetic selection. Additional cytotoxicity assays, activation assays, and *in vivo* experiments are ongoing, and will be presented at the ASGCT annual meeting.



1166. Abstract Withdrawn

1167. Optimizing CAR-T Cell Therapy Against Glioblastoma

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Background: Adoptive cell therapy with chimeric antigen receptor (CAR)-T cells represents a significant advancement in personalized cancer treatment. While CAR-T cell therapy has demonstrated remarkable response rates in hematological malignancies, significant challenges remain in designing CAR-T cells against solid tumors. This is in part due to the heterogeneous expression of tumor antigens in solid tumors. Glioblastoma (GBM) is a solid tumor with a poor prognosis with a median length of survival of 15 months. Current treatments offer minimal benefit, and there is a dire need for less toxic and more effective therapeutics. Currently, efforts to develop CAR-T cell therapies against GBM are underway. Two antigens, in particular, EGFR variant III (EGFRvIII) and IL13Rα2 are attractive targets as they are both enriched on cancer cells, yet absent on healthy brain tissue. We are investigating overcoming antigen heterogeneity in glioblastoma through the use of tandem CAR-T cells that target both IL13Rα2 and EGFRvIII. These dual-targeting CAR-T cells can increase potency by targeting two different antigens, while also retaining efficacy with the presence of one antigen. **Methods:** We designed 2nd generation CARs targeting IL-13Rα2 (CAR-IL13Rα2) and EGFRvIII (CAR-EGFRvIII) individually and concurrently (tandem CAR). In addition, we generated mixed populations of either IL13Rα2+ or EGFRvIII+ target tumor cell lines. Our *in vitro* model consisted of the glioblastoma cell lines U87 and U251, a glioblastoma-derived cancer stem cell line that forms neurospheres, BT74, and K562, artificial antigen presenting cells, expressing either IL13Rα2+ or EGFRvIII+. The efficacy of the tandem CAR construct against adherent cells was measured through an impedance-based real-time cytotoxicity assay using the xCELLigence RTCA MP instrument. Against GFP+ neurospheres, cytotoxicity was measured through the total average green area using the IncuCyte Live Cell Analysis instrument. To further evaluate the efficacy of the CAR-T cells, we conducted long-term proliferation assays, and CD69-based activation assays. For *in vivo* experiments, we used 3 model systems in NSG mice: intracranial implantation of BT74 neurospheres, intracranial implantation of mixed populations of U87 IL13Rα2+ and U87 EGFRvIII+, and subcutaneous implantation of K562 IL13Rα2+ and K562 EGFRvIII+ on either flank of mice. Each group received either intraventricular or intravenous administration of untransduced T cells, CAR-IL13Rα2, CAR-EGFRvIII, or the tandem CAR. **Results**: Cytotoxicity assays indicated that the tandem CAR demonstrates superior killing capacity in mixed populations of IL13Rα2+ and EGFRvIII+ target cells at effector:target ratios of 3:1, 1:1, and 1:3. The differential cytotoxicities of the tandem CAR versus single antigen CARs were evident as early as several hours after administering CAR-T cells and continued up until 72 hours. Further, when co-cultured for 16 hours with either IL13Rα2- or EGFRvIII-expressing tumors, activation of the tandem CAR (79.4% and 92.2% CD69 positivity, respectively) was comparable to the single antigen CAR-T cells (86.3% and 96% CD69 positivity, respectively). Altogether, this *in vitro* data

indicates that the tandem CAR works just as effectively as its single antigen counterparts. Ongoing *in vivo* experiments are elucidating the efficacy of the tandem CAR compared to CAR-IL13Rα2 and CAR-EGFRvIII in mixed populations of IL13Rα2+ and EGFRvIII+ tumors. **Conclusions**: Our data demonstrate that targeting multiple GBM antigens through a tandem CAR not only has comparable activity to single antigen CARs but may also be superior in terms of cytotoxicity and efficacy in heterogeneous tumor populations.

1168. Oncolytic Adenovirus Ad5/3-D24-GM-CSF Infects, Replicates and Lyses Ovarian Cancer Cells Through Desmoglein-2 Cell Entry Receptor

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Despite the development of new anticancer agents, the mean survival of ovarian cancer women has remained between 41 months to 47 months since 1980s. This cavity-localized cancer lends itself to local administration of modalities, such as the chimeric oncolytic adenovirus (Ad) Ad5/3-D24-GM-CSF (ONCOS-102). ONCOS-102 has three genetic modifications that can contribute to its safety and efficacy against ovarian cancer. Its chimeric adenoviral 5/3 fiber knob changes the binding specificity of the virus: instead of binding to the CAR, this chimeric Ad5/3 adenovirus targets the frequently overexpressed membrane proteins desomoglein-2 (DSG2). The replication of ONCOS-102 is restricted to tumor cells with an altered Rb pathway by its 24 bp deletion in the E1A gene. Its expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) can augment the immunostimulatory milieu in the infected tumor. Previously we have shown in a in phase I clinical study (NCT01598129) that repeated administration of ONCOS-102 to a patient with chemotherapy refractory ovarian cancer induced CD8+ anti-tumor immune responses with overall survival reaching 40. In the current study we assessed the dominant receptor used by ONCOS-102 in four established epithelial ovarian cancer (EOC) cell lines. Adenovirus serotype 3 can use the DSG2. DSG2 was nearly absent in A2780 cells but was expressed in >90% of OAW42, OVCAR3, and OV-90 cells. After 96 hours, ONCOS-102 treatment showed significant oncolytic activity (≧50%) in OAW42, OVCAR3, and OV-90 cells, but minimal activity in A2780 cells, suggesting DSG2 as the dominant receptor for ONCOS-102. Furthermore, retrospective analyses of a phase I clinical trial of ONCOS-102 treatment of 12 patients with varied tumors indicated a correlation between viral genomes in blood and DSG2 RNA expression. These data support the role of DSG2 expression on cancer cells in virus infectivity and the continued development of ONCOS-102 for ovarian cancer treatment (NCT02963831).

1169. CRISPR/Cas9-Based Engineering of Gamma-Delta T Cells for Cancer Immunotherapy

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Adoptive T cell therapies, wherein patient T cells are removed, manipulated, expanded, and re-infused into patients to target tumors, have seen great success for the treatment of cancer. Research has shown that these therapies have anti-tumor activity and are safe to use in patients. However, adoptive T cell therapies face several challenges with regards to solid tumor targeting, including homing to the tumor site and immune evasion by the tumor. Gamma-delta T cells (GDTCs) are a distinct subset of T cells expressing T cell receptors (TCRs) made up of TCR-gamma and TCR-delta chains, which have properties distinct from canonical alpha-beta T cells. GDTCs comprise between 1-5% of circulating lymphocytes and possess a combination of innate and adaptive immune cell characteristics that make them an attractive cell type for cancer immunotherapy. When activated, they can directly kill malignant cells, produce inflammatory cytokines, and form a memory response. Efforts to translate GDTCs to the clinic produced promising initial results, but ultimately face challenges similar to those of traditional T cell therapy. Additionally, under certain conditions GDTCs can develop a pro-tumor, immunosuppressive phenotype through the expression of PD-L1 and IL17A. We proposed that genetic engineering could be used to augment GDTC function, prevent them from becoming pro-tumorigenic, and fully realize GDTC therapeutic potential. We tested methods for activation and expansion of GDTCs, including stimulation with anti-CD3/CD28 beads or zoledronate, and found that either method could be used to facilitate efficient nucleic acid uptake during electroporation, and that this resulted in high rates of gene knockout and targeted integration when combined with donor delivery by recombinant adeno-associated virus (rAAV). We have targeted *PDCD1* and *CISH* for knockout in GDTCs to enhance their antitumor potential. Additionally, we targeted *IL17A* for knockout to prevent a shift toward an immunosuppressive phenotype. Using our optimized methods, we achieved efficient indel formation and protein loss across all targeted genes (Figure 1). We have validated a functional effect of these modifications through activation and killing assays with cancer cell lines. We have also optimized site-specific delivery of chimeric antigen receptors (CARs) targeting mesothelin and have shown antigen-specific killing by GDTCs. Our data support the notion that GDTC-based cancer immunotherapy can realize its full potential through the use of genetic engineering. Future work will focus on preclinical testing of genetically modified GDTCs with the goal of clinical translation.

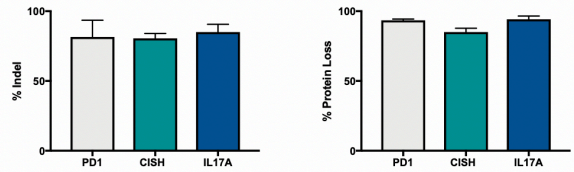

Figure 1. Efficient Gene Knockout in Gamma-Delta T Cells. Gamma-delta T cells (GDTCs) were isolated from healthy human peripheral blood samples (n=3 independent donors). GDTCs were stimulated with anti-CD3/CD28 DynaBeads, and gene editing reagents were delivered using an Amaxa 4D Nucleofector. Indel formation was quantified using the ICE software (Synthego) and protein loss was analyzed by flow cytometry (PD1, IL17A) or Western blot (CISH). Knockout samples were compared to donor-matched control samples.

Cancer - Targeted Gene and Cell Therapy

1170. Towards Local Transplantation of Genetically-Engineered Alveolar Macrophages to Counteract Lung Metastasis

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Macrophages play essential roles in tissue remodeling, inflammation and immunity and constitute an important component of the tumor microenvironment by either promoting or limiting tumor progression. A subset of macrophages, arising from primitive hematopoiesis, selectively resides in tissues and is capable of self-maintenance. Alveolar macrophages (AM) represent the self-renewing tissue resident macrophage population of the lungs. However, their role in lung tumor development and in the establishment and growth of lung metastasis is still poorly understood. By modeling breast cancer-derived metastasis in two AM depletion models, namely wild-type mice treated intra-tracheally with clodronate or GM-CSF receptor knock out mice, we have observed that lack of AM leads to a dramatically reduced metastatic burden in the lungs following intravenous tumor challenge, while masses developed in other sites such as the ovaries. This implies a critical role of AM in the metastatic seeding of breast cancer cells, suggesting that these cells represent a pathogenetically relevant population that could be exploited therapeutically. As their depletion has demonstrated to only partially prevent metastatic spreading, genetic modification of this population to re-direct their function towards anti-tumoral, e.g. by transducing them with immune-stimulatory cytokines, represents an appealing strategy. To better depict this population, we employed local, intratracheal transplantation of bone marrow-derived macrophages and characterized their capacity to engraft and acquire an alveolar-like phenotype in wild type mice conditioned with clodronate. Donor-derived macrophages persisted in the lungs, reaching up to 20% of the bronchoalveolar lavage fluid (BALF) hematopoietic content. Phenotypical characterization showed acquisition of mature alveolar macrophage surface markers such as CD11c and SIGLEC-F and progressive loss of the monocytic marker CD11b. Single cell RNA sequencing of donor cells retrieved at different timepoints after transplantation from the BALF revealed a progressive acquisition of a transcriptomic profile very similar to that of native AM, reaching maximum similarity 14 days after transplantation. Monocle analysis sequentially orders cells from day 3, 7 ,14 post-transplant along the pseudotime trajectory, further supporting an adaptation driven by the local lung environment. Having shown the feasibility to obtain substantial engraftment of locally transplanted bone marrow-derived macrophages, we transduced these cells with a lentiviral vector driving the expression of type-1 or type-2 interferon (IFN) under a strong myeloid promoter and transplanted them into the lungs of mice carrying breast cancer metastases. Animals receiving macrophages expressing beta-IFN or gamma-IFN showed lower tumor burden compared to those receiving control-transduced cells. Further experiments are ongoing to understand how these engineered cells participate to tumor progression and how local expression of cytokines impacts the tumor microenvironment. In conclusion, we have shown a strong dependence of lung metastasis formation on alveolar macrophages, a novel cell target that can be exploited for remodeling of the tumor microenvironment and anti-tumor cell therapy.

1171. Modeling and Simulation Studies to Assess Safety and Efficacy of CAR T Cell Therapy

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Chimeric Antigen Receptor (CAR) T cell therapy is one of the most prominent types of adoptive cell therapies where T cells are harvested either from the patient (for autologous therapy) or healthy donors (for allogeneic therapy) and genetically engineered *ex vivo* to express CAR on their surface to target tumor associated antigens. In 2017, the US Food and Drug Administration (FDA) approved the first two autologous CAR T cell therapies Kymriah™ (tisagenlecleucel) and Yescarta™ (axicabtagene ciloleucel) for treatment of B cell malignancies. CAR T cells are living drugs that can proliferate exponentially and expand rapidly in patients. CAR T cell therapies are often associated with inflammatory toxicities such as cytokine release syndrome (CRS) and neurotoxicity that is mediated in part by inflammatory cytokines such as IL-6, IL-1β, etc. CRS is associated with high fever, headaches, myalgia, hypotension and hypoxia. Tocilizumab (anti-IL6R), anakinra (anti-IL1R) antibodies and corticosteroids are used to treat the symptoms of CRS. However, heterogeneous responses have been observed; patients can develop tocilizumab-refractory CRS and steroids can affect product efficacy. Furthermore, the underlying mechanisms for development of CRS and neurotoxicity are not completely understood. Thus, better characterizing the cellular kinetics of CAR T cells, as well as the factors impacting the kinetics, may provide insight into the mechanisms contributing to CAR T cell-related toxicities and help identify patients who are at higher risk of CRS development and achieve favorable benefit-risk during CAR T cell therapy. Here, we developed a mechanistic mathematical model (Figure 1) based on Ordinary Differential Equations using T cell, B cell, and cytokine concentrations as initial inputs indicated in relapsed or refractory B cell malignancies. This model can quantitatively explore the complex temporal relationships among the tumor burden, CAR T cell dose, and cytokine levels to better understand the determinants of CRS. This model could be a potential tool to help design CAR T cells dosing regimens with a favorable benefit-risk profile.Disclaimer: Our contributions are an informal communication and represent our own best judgment. These comments do not bind or obligate FDA

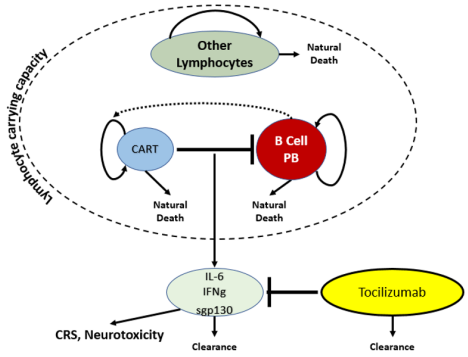

Figure 1. A schematic diagram of the CAR T cell therapy model

1172. Development of Mutation Suppression Therapy in a Swine Model of Neurofibromatosis Type 1

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Over 20% of Neurofibromatosis Type 1 (NF1) patients have a nonsense mutation in the *Neurofibromin 1 (NF1)* gene. A unique class of drugs called nonsense mutation suppressors have therapeutic potential for these patients, as these drugs allow the translational machinery to bypass the premature termination codon (PTC) and continue translation of the full-length protein. To date, the focus of research and clinical trials with this class of drugs has been to treat Cystic Fibrosis and Duchenne muscular dystrophy. However, the immense impact these drugs could have on NF1 patients has yet to be explored. We investigated the therapeutic potential of several nonsense mutation suppression therapies for the treatment of NF1. Our group has had success in preclinical drug testing using a swine model of NF1. We have developed and characterized NF1 minipigs carrying an engineered loss of function mutation in the *NF1* gene which accurately displays the clinical features of human NF1, with Ras hyperactivation, café au lait macules, dermal neurofibromas, and optic pathway glioma. Further, we have used this model for preclinical drug development studies where we have successfully performed dose finding experiments, drug formulation studies, evaluated pharmacokinetic parameters of multiple small molecules, drugs and kinase inhibitors, and performed pharmacodynamic studies. We have now developed a novel swine model of NF1 with a nonsense mutation, suitable for the evaluation of nonsense mutation suppression therapies. We evaluated several drugs known to induce nonsense mutation suppression in several primary cell types isolated from this swine model and show that several drugs have the propensity to induce the production of full length Neurofibromin protein, leading to a subsequent reduction in MAPK signaling. Information acquired from this large animal preclinical model will be leveraged towards initiating a clinical trial in NF1 patients with a nonsense suppression therapy that is safe, tolerable, and effective in restoring *NF1* gene function. By addressing the underlying genetic and biochemical cause of NF1 and restoring the function of *NF1*, nonsense mutation suppression therapies have the potential to ameliorate both the malignant and non-malignant features of NF1. *Funding for this work was provided by the Gilbert Family Foundation through their Gene Therapy Initiative.*

1173. Gene Therapy for Canine Melanoma: Comparative Analysis of *HSV*tk/GCV and CD::UPRT/5-FC Suicide Gene Systems

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Since many cancers occur spontaneously with similar progression patterns in humans and dogs, the development of clinical trials on companion animals is a valuable opportunity for translational medicine. Veterinary clinical trials applying immunogene therapy have been conducted for almost 20 years. Our current protocol consists of surgery adjuvant treatment combining local and systemic gene therapy: intra/peritumoral treatment with herpes simplex virus thymidine kinase/ganciclovir (HSVtk/GCV) suicide gene (SG) plus canine interferon-β gene (cIFNβ) and bleomycin (BLM), complemented with the periodic administration of formolized tumor vaccines potentiated with hIL-2 and hGM-CSF transgenes. These combined treatments drastically controlled spontaneous melanoma tumors while maintaining a good life quiality of the patients. We are currently exploring *in vitro* the use of yeast *S. cerevisiae* cytosine deaminase/uracil phosphoribosyl transferase fusion enzyme (CD::UPRT, CDU) that converts its prodrug 5-fluorocytosine (5-FC), into 5-fluorouracyl (5-FU). Because only CDU-lipofected cells are able to convert 5-FC to 5-FU, the toxicity of 5-FU is limited to the lipofected and surrounding tissue, preventing the toxicity of 5-FU systemic therapy. We hypothesize that the successful outcome of our trials could be explained by the great bystander effect mediated by extracellular vesicles (EVs). During *in vivo* and *in vitro* chemo-gene treatments, all the components secreted by treated cells to the extracellular space could spread antitumor information to surrounding cells. In the present work, we show an extensive comparative analysis of the effect of both suicide genes in canine melanoma cells, as well as the effect of EVs on receptor cells. We performed prodrug concentration-response experiments on lipofected cells cultured as monolayers and spheroids in three canine melanoma cell lines established in our laboratory. We measured cell viability 48h after lipofection by the acid phosphate assay (APH) and observed an expected concentration-dependent cytotoxic effect of both SG systems. With cell survival ranging between 15% (HSVtk/GCV) and 30% (CDU/5-FC), no significant differences were observed in cell viability between both SGs. Clonogenic survival studies of cells lipofected with each SGs displayed a remarkable concentration-dependent reduction in the number of colonies. Acridine orange/ethidium bromide cell staining showed that while apoptosis is the mechanism implicated in cell death, in cells exposed to CDU/5-FC there was an accumulation of

acid vesicles in the cell periphery at 24h, while in HSVtk/GCV exposed cells this effect was visible after 48h. The differential centrifugation (500 xg; 2K xg and 12K xg) of conditioned media (CM) from cells lipofected with each SG, confirmed that the toxicity of the different fractions of CM are accountable for these differences in the cell dynamics. We observed that vesicles between 1400-2200 nm and 500-1000 nm (contained in 500 xg and 2K xg fractions) were the most toxic fractions of the CM from HSVtk/GCV cells, whereas fractions containing vesicles between 30 and 150 nm (enriched in the supernatant after 12K xg centrifugation) were more toxic in CM from CDU/5-FC lipofected cells. Given the effective long-term experience in HSVtk/GCV clinical trials and the new findings on CDU/5-FC *in vitro*, the latest system would have a promising application on the clinic. Our next step will be to identify the content of the EVs responsible for the toxic effect on surrounding/distant cells and address the presence of such content in plasma from canine patients enrolled in clinical protocols.

1174. *In Vivo* Model Development for Genome-Edited T Cell Therapeutics

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The implementation of various mouse models is critical to asses safety, efficacy, and short- and long-term persistence of therapeutic modalities, especially for cell-based therapies. To increase our repertoire of viable humanized murine models, we developed two *in vivo* models by taking advantage of Taconic's immunodeficient mice, one monitoring graft versus host disease (GvHD) and the other addressing human natural killer (NK) cell cytotoxicity. To develop a model of GvHD, we transplanted in NOG mice from Taconic human peripheral blood mononuclear cells (PBMCs) at varying doses and monitored mice for changes in body weight over time. When human natural T regulatory cells (nTregs) were co-injected with the PBMCs in NOG mice, we observed a prolonged survival rate and a less rapid loss of body weight, as compared to PBMCs alone. In addition, we co-injected in NOG mice antigen presenting cells (APCs) and edited human T cells, wherein the endogenous TCR expression was reduced by editing with CRISPR/Cas9 and guide RNA specific to the *TRAC* gene. These mice had an 80% survival rate up to 90 days after co-injection of APCs and edited T cells—by comparison NOG mice co-injected with APCs and wild type T cells were moribund within 3 weeks. To create an NK cell cytotoxicity model, we transplanted human primary NK cells into NOG-hIL15 mice from Taconic, which are NOG mice that constitutively produce human IL-15. We observed successful engraftment and proliferation of NK cells, with peak engraftment occurring 4-5 weeks post injection. We also observed that human primary NK cells were able to persist without signs of xenogeneic GvHD. Utilizing K562 tumor cells that express luciferase, we found by IVIS imaging that the engrafted NK cells have fast and potent cytotoxic activity, resulting in elimination of tumor cells as compared to non-engrafted mice. Our results collectively suggest that the two *in vivo* models developed here will be valuable tools for investigating the clinical benefit of cell therapies.

1175. Elucidation of the Therapeutic Role of Mitochondrial Biogenesis Transducers NRF-1 in Treating Prostate Cancers

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The pathogenesis of prostate cancer cell might be associated with the dysregulation of cellular energy. Nuclear respiratory factor1 (NRF-1) functions as a transcription factor that activates the expression of key metabolic genes regulating cellular growth and nuclear genes required for mitochondrial respiration, and mitochondrial DNA transcription and replication. We hypothesis of this study, under prostate cancer cell line, expression of NRF-1 in prostate cancer cell line is significantly down-regulated cell survival. These observed imply that NRF-1 might have potential to regulate prostate cancer cell; however, there is no related scientific reports so far. **Methods:** The prostate cancer cell regulatory role of NRF-1 will be extensively studied in this project. In vitro cell culture model of PC3 will be used. In vitro transfection of pcDNA-NRF-1 (NRF-1 expression vectors) to prostate cancer cells will be performed. **Results:** pcDNA-NRF-1 reduced the viability of prostate cancer cells by trypan blue exclusion assay. pcDNA-NRF-1 reduced the cell proliferation of prostate cancer cells by MTT assay. More importantly, pcDNA-NRF-1 induced cytotoxicity of prostate cancer cells by LDH assay. The TGF-beta pathway is involved in urinary prostate cancer progression. pcDNA-NRF-1 significantly reduced the expression of TGF-beta1 secretion in PC3 cells. pcDNA-NRF-1 significantly decreased TGF-beta1 signaling, in addition to inducing a marked increase in the down-regulation of Smad7 by western blot assay and immunofluorescence staining. pcDNA-NRF-1 decreased migration in prostate cancer cells. More importantly, pcDNA-NRF-1 decrease the expression of alpha-SMA (alpha-smooth muscle actin) and increase expression of E-cadherin by western blot and immunofluorescence. **Conclusions:** We demonstrated that pcDNA-NRF-1 might act as a novel prostate cancer antagonist by down-regulating TGF-beta1 signaling and modulating epithelial-mesanchymal transition (EMT). The study will be helpful for clinical urologists in treating prostate cancers.

1176. Interfering with lncRNA *MIR22HG* Processing Inhibits Glioblastoma Progression Through Suppression of Wnt/β-catenin Signaling

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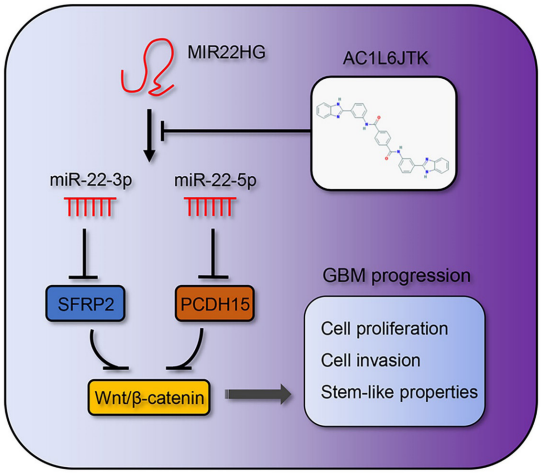
Long noncoding RNAs play critical roles in tumour progression. Through analysis of publicly available genomic datasets, we found that *MIR22HG*, the host gene of microRNAs miR-22-3p and miR-22-5p, is ranked among the most dysregulated lncRNAs in glioblastoma. The

main purpose of this work was to determine the impact of *MIR22HG* on glioblastoma growth and invasion and to elucidate its mechanistic function. The *MIR22HG*/miR-22 axis was highly expressed in glioblastoma as well as in glioma stem-like cells compared to normal neural stem cells. In glioblastoma, increased expression of *MIR22HG* is associated with poor prognosis. Through a number of functional studies, we show that *MIR22HG* silencing inhibits the Wnt/β-catenin signaling pathway through loss of miR-22-3p and -5p. This lead to attenuated cell proliferation, invasion and *in vivo* tumour growth. We further show that two genes, *SFRP2* and *PCDH15*, are direct targets of miR-22-3p and -5p and inhibit Wnt signaling in glioblastoma. Finally, based on the three-dimensional structure of the pre-miR-22, we identified a specific small-molecule inhibitor, AC1L6JTK, that inhibits the enzyme Dicer to block processing of pre-miR-22 into mature miR-22. AC1L6JTK treatment caused an inhibition of tumour growth *in vivo*. Our findings show that *MIR22HG* is a critical inducer of the Wnt/β-catenin signaling pathway, and that its targeting may represent a novel therapeutic strategy in glioblastoma patients.



1177. Bugs as Trojan Horses to Target Necrotic Tumors

Alexandra M. Mowday[1], Natasja G. Lieuwes[1], Rianne Biemans[1], Rachel Gehlen[1], Chris P. Guise[2], Amir Ashoorzadeh[2], Maria Zygouropoulou[2], Aleksandra M. Kubiak[1], Nigel P. Minton[3], Philippe Lambin[1], David F. Ackerley[4], Jeff B. Smaill[2], Ludwig J. Dubois[1], Adam V. Patterson[2], Jan Theys[1]

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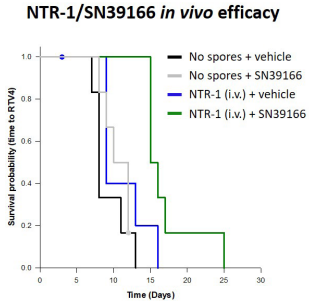
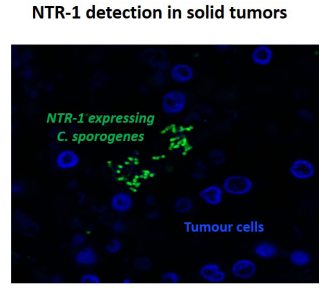
Necrosis is a common histological feature to solid tumours that offers a unique opportunity for targeted cancer therapy as it is strictly absent from normal healthy tissues. Despite this, it has yet to be successfully exploited therapeutically in humans. Tumour necrosis provides an ideal environment for germination of the non-pathogenic anaerobic bacterium *Clostridium* from endospores, resulting in tumour-specific colonisation and modest anti-tumour activity as a single agent. This observation encouraged further development of 'armed' *Clostridium* (Clostridia Directed EnzymeProdrug Therapy, CDEPT), whereby germinated bacteria express a prodrug activating enzyme, resulting in generation of active drug selectively in the tumour microenvironment. We have identified a multi-functional nitroreductase gene (NTR-1) for insertion into *C. sporogenes* that provides the ability to co-metabolise both clinical stage PET imaging agents (for whole body vector visualisation), in addition to clinical and pre-clinical chemotherapy prodrugs (for conditional enhancement of efficacy). The novel enzyme/prodrug combination of NTR-1/SN39166 demonstrated significant therapeutic efficacy in 2D monolayers, 3D cultures, and tumours colonised by NTR-1 expressing *C. sporogenes* (a six-day increase in median survival compared to NTR-1 spores as a single agent, P=0.03). In addition to SN39166, we have demonstrated that NTR-1 can also metabolise positron emission tomography (PET) substrates suitable for non-invasive vector visualisation, and a range of commonly used antibiotics, providing an additional safety feature. Successful pre-clinical validation of this combinatorial approach is a valuable early step towards clinical evaluation, as imaging of therapeutic gene expression and therefore vector spread will be an essential component of patient monitoring in this context. For this reason, development of the appropriate immune-competent *in vivo* models is required to elucidate the important and anticipated role of the immune response in the context of *C. sporogenes* therapy. We have established that *C. sporogenes* endospores are able to cross the blood-brain barrier in rodents following injection of 10^9 colony forming units and have developed a luciferase-expressing orthotopic rat glioma model to test *C. sporogenes*-based therapies in a more clinically relevant and accurate setting with an organ-specific tumour microenvironment.





1178. First-In-Class AAV-BasedGene Therapy for Pancreatic Cancer and Other Tumors Based on the Destruction of Cell-Free DNA with Vector-Delivered DNase I

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Background: In general, there is a balance between the level of serum cell-free DNA (cfDNA) and a plasma nuclease enzyme DNase I. Tumor progression is characterized by increased levels of circulating serum cfDNA that mainly originates from dead cancer cells and active release

from both tumor cells and neutrophils. These neutrophil extracellular traps (NETs), an extracellular DNA released from neutrophils into the tumor microenvironment, as well as tumor-derived cell-free DNA (cfDNA) promote pancreatic cancer progression and metastasis in many ways. For example, by modulating TLR9, triggering cancer transformation or activating metastatic genes and by inducing potent immunosuppressive effects and targeting NETs-cfDNA, the network may significantly increase the efficacy of the first-line therapy. Here we investigate the effect of the combination of adeno-associated virus (AAV) vector-delivered DNase I treatment (as a NET depleting agent) together with the first-line chemotherapeutic drug (nab-paclitaxel) in pancreatic adenocarcinoma mouse model. Methods: We used BxPC-3-luc orthotopic model of BALB/c nude mice, with or without nab-paclitaxel injected systemically with the Anc80L65 AAV capsid with in-house developed hyperactive DNase I transgene cassette under a liver specific promoter(Anc80L65-DNaseI). The mice (n=8 mice/ group) were monitored for tumor-associated bioluminescence, weekly. Tumor size and metastasis were assessed at the end of the study (day 28). Anc80L65's dose-response of transduction efficiency (DNaseI levels) was also assessed. Results: Treatment of mice with Anc80L65encoding-DNase I demonstrated a significant decrease in tumor bioluminescence (by more than 53%) compared to untreated control ($p < 0.05$). The number of ovary, kidney, spleen, and liver metastatic cells were significantly lower in the group treated with Nab-paclitaxel + Anc80L65-DnaseI vector than in both the untreated control group and the group treated only with Nab-paclitaxel ($p<0.05$). Anc80L65-DNaseI enabled a significant increase in serum DNase I levels compared to untreated controls. Conclusion: This study shows a potential in the cancer therapeutic strategy employing a gene-therapy approach allowing long-term expression of DNase I and destruction of NETs and tumor derived cfDNA.

1179. Adoptive Cell Therapy Don't Over Engineer it: Alternatives to Gene Editing to Improve Cell-Based Therapeutics for the Treatment of Cancer

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Following the approval of cell-based gene therapies, Kymriah (tisagenlecleucel) and Yescarta (axicabtagene ciloleucel), in 2017 there has been a significant increase in the number of clinical trials utilizing cell and gene therapies. Both Kymriah and Yescarta consist of genetically modified T cells expressing a chimeric antigen receptor (CAR-T) targeted towards CD19 on B cell leukemias and lymphomas. Treatment of solid tumors has proven to be more difficult, resulting in the development of alternative adoptive cell therapy (ACT) approaches including TCR and NK cell-based therapies. In order to overcome resistance to ACT for the treatment of solid tumors, most developers look to further improve the cells during manufacture. To date, these approaches predominantly include further genetic engineering of the cells. An alternative approach to certain forms of gene editing is gene silencing by RNAi, especially in cases where permanent gene modification is not required or undesirable. To date, use of RNAi has been limited by challenges of delivery to immune cells. Advances in the RNAi field, specifically in the discovery and development of self-delivering RNAi compounds, have shown great promise for the improvement of ACT therapies without the need for gene editing. Here we present the case for utilizing self-delivering RNAi technology as an alternative and/or complement to certain genetic engineering approaches. This overview provides relevant examples of utilizing self-delivering RNAi to reprogram immune cells and their resulting increased activity. Furthermore, we discuss the pros and cons of self-delivering RNAi technology as compared to gene editing, including a comparison of manufacturing logistics, costs, and regulatory and clinical considerations.

1180. SENTI-101, an Allogeneic Cell Product Expressing a Combination of Cytokines, Promotes Anti-Tumor Immunity in a Syngeneic Orthotopic Model of Pancreatic Ductal Adenocarcinoma

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Background: Advanced pancreatic cancer is one of the deadliest cancers and remains recalcitrant to all forms of treatments, with a 5-year overall survival rate below 10%. Unlike other solid tumors, pancreatic ductal adenocarcinoma (PDAC) patients have not substantially benefited from the recent wave of targeted therapies and immune checkpoint inhibitors. Therefore, there is a compelling need to develop alternative therapeutic strategies. A major barrier in PDAC treatment is the frequent harsh tumor microenvironment (TME), which can include high desmoplastic stromal content and immunosuppressive immune cell populations. This type of TME may limit T cell infiltration and lead to T cell exhaustion in pancreatic tumors. A new class of therapeutics, adoptive cell therapy (e.g., CAR-T), has been tested for this indication with limited success thus far, in part due to the immunosuppressive nature of this TME. SENTI-101—allogeneic bone marrow-derived mesenchymal stromal cells (BM-MSCs) genetically modified to express and locally secrete a potent combination of immunomodulatory cytokines (IL12 and IL21)—offers a unique opportunity because these MSCs innately home to solid tumors without being functionally compromised by the immunosuppressive TME. **Methods:** A clinically relevant murine model of PDAC, the KPC tumor cell line (LSL-KrasG12D, LSL-Trp53R172H, Pdx-1-Cre) was used in a syngeneic orthotopic model in the C57BL/6 mouse strain to evaluate SENTI-101 homing and efficacy. Homing and persistence were assessed using whole body and *ex vivo* imaging by comparing, over time, tagged MSCs to a control group. SENTI-101 efficacy has been evaluated by tumor burden and overall survival. This study was divided into 2 arms: [1] PK/PD and mechanism of action were evaluated from an early time point; and [2] and long-term survival was evaluated in a separate cohort. **Results:** Upon administration, engineered BM-MSCs preferentially localized to mouse pancreatic tumors by more than a log relative to

other infra-, retro-, and intraperitoneal organs, and persisted for at least 7 days. More importantly, SENTI-101 led to a dose-dependent prolonged survival relative to controls with a significant benefit in the high SENTI-101 dose (median survival of 22 days and 32 days after randomization in low and high SENTI-101 dose, respectively, vs 14 days in controls, log-rank *$p<0.002$). Furthermore, preliminary data suggest that SENTI-101 induces biological activity consistent with this survival benefit in tumor-bearing mice: SENTI-101 caused increases in both antigen-presenting cells (macrophages and dendritic cells) and intratumoral CD8 T cells in murine PDAC compared to a sham control treatment, which is characterized by poor T cell infiltration to the tumors. **Conclusion:** In a hard-to-treat preclinical model of PDAC, SENTI-101 converts a hostile TME to a more permissive one, resulting in a significant anti-tumor immune response and prolonged survival.

1181. Extracellular Vesicles Induce Mesenchymal Transition and Therapeutic Resistance in Glioblastomas Through NF-kB/STAT3 Signaling

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Glioblastoma (GBM) is the most common primary malignant brain tumor and despite optimal treatment, long-term survival remains uncommon. GBM can be roughly divided into three different molecular subtypes, each varying in aggressiveness and treatment resistance. Interestingly, recent evidence shows plasticity between these subtypes in which the proneural (PN) glioma stem-like cells (GSCs) undergo transition into the more aggressive mesenchymal (MES) subtype leading to therapeutic resistance. Extracellular vesicles (EVs) are cell-derived membranous structures involved in numerous physiological and pathological processes. In the context of cancer, these biological nanoparticles play an important role in intercellular communication allowing cancer cells to exchange information with each other, the tumor microenvironment as well as distant cells. Here, we show that EVs derived from MES cells educate PN cells to increase stemness, cell proliferation, migration potential, tumor aggressiveness, and therapeutic resistance by inducing mesenchymal transition through NF-kB/STAT3 signaling. Our findings could potentially help explore new EV-based treatment strategies for GBM and indicate that EVs may also play a role in mesenchymal transition of different tumor types.

1182. Improving Acute Myeloid Leukemia (AML) NK Cell Immunotherapy via CRISPR Gene Editing and CAR Expression in Freshly Isolated or Primary Expanded NK Cells Using Scalable Electroporation

Diwash Acharya, Rama Shivakumar, Stanislav Khoruzhenko, James Brady

MaxCyte, Gaithersburg, MD

While CAR-T cell therapies have demonstrated clinical efficacy against a number of hematologic malignancies, development of therapies to treat acute myeloid leukemia (AML) have lagged in part due to the lack of targetable tumor-specific antigens. In this poster we highlight the use of a clinically validated electroporation platform for the genetic engineering of primary NK cells via two experimental studies towards the development of an AML-targeted NK cell therapy with improved tumor targeting, reduced therapeutic fratricide and lower inhibitory receptor signaling. In the first study we used CRISPR-mediated knockdown of CD38, a transmembrane glycoprotein and validated target for anti-CD38 therapy in Myeloma. CD38 is expressed on a majority of AML blast cells, however it is also expressed at various levels on NK cells, thus introducing the potential for fratricide. Specifically, our data demonstrate the high efficiency production of CD38-negative ($CD38^-$) primary expanded NK cells using CRISPR engineering via MaxCyte electroporation of gRNA & CRISPR/Cas9 RNP, followed by anti-CD38 CAR expression in the CD38- population using mRNA electroporation. In the second study, we demonstrate high efficiency CRISPR-mediated knockdown of NKG2A, a C-type lectin receptor with two immunoreceptor tyrosine-based inhibitory motifs. NKG2A is upregulated on human cytokine-induced memory-like NK cells (ML), which exhibit enhanced responses against primary human AML blasts. Previous studies have demonstrated that NKG2A blockade enhances memory-like NK cell responses suggesting NKG2A may act as a checkpoint. Our data show that knockdown of NKG2A on freshly isolated NK cells results in significantly decreased NKG2A expression. Additionally, NKG2A-knocked down ML NK cells display enhanced *in vitro* responses against HLA-E+ target cells. Overall, these studies establish that MaxCyte's scalable electroporation platform can result in high efficiency, high viability genetic engineering of primary NK cells with little *ex vivo* manipulation enabling novel approaches for improving NK cell adoptive cell therapies.

1183. Loss of AOC3 Impairs CD4+ T Cells Recruitment in Lung Cancer

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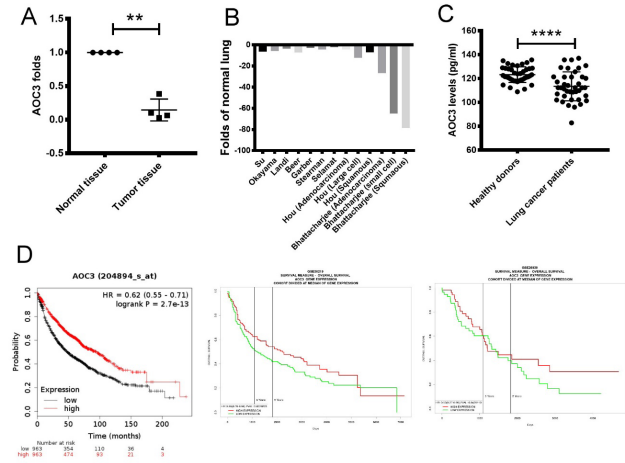
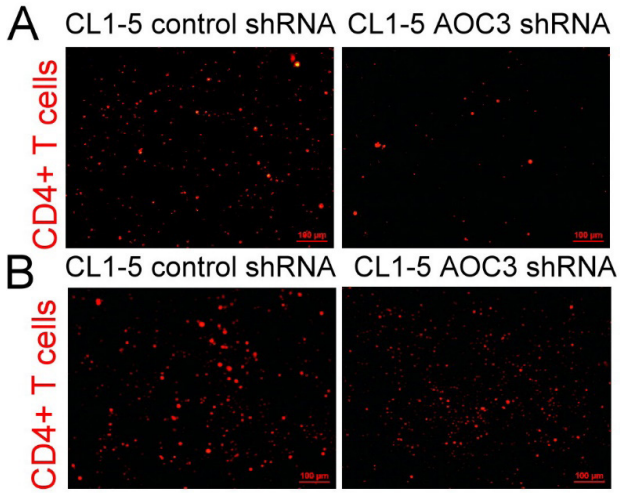
Introduction: Lung cancer still is the most prevalent type of cancer, and despite advanced therapies such as chemotherapy, targeted therapy, and immune checkpoint inhibitors, less than 5% of lung cancer patients survive more than 5 years once metastasis occurs. The investigation of pathological candidate factors that contribute to disease development is still requirement. Methods: Transcriptome of human lung cancer and adjacent normal tissue was assessed by next-generation sequencing (NGS). The levels of ACO3 of sera were determined by ELISA at protein levels. Knockdown of AOC3 in lung cancer cells was perform by shRNA transfection. The overall survival rate was analyzed using KM plotter and ProgeneV2 websites. Results: We profiled transcriptome of 4 matched normal and lung adenocarcinoma by NGS, and it revealed that AOC3 was downregulated in all samples (Figure 1A). Oncomin® database also shows there are 13 studies also supported loss of AOC3 expression in lung cancer tissues compared with normal tissues (Figure 1B). ELISA analysis revealed that the decrease of AOC3 in the sera of 40 lung cancer patients, compared with heathy donors (Figure 1C). KM plotter and ProgogeneV2 also showed the lung cancer patients with higher AOC3 levels had longer overall survival rate (Figure 1D).

Knockdown of AOC3 in human lung cancer cell line CL1-5 showed that reduced the attachment of CD4+ T cells on the lung cancer monolayer (Figure 2A). In addition, the transvascular migration of CD4+ T cells was also decreased in AOC3 silencing lung cancer cells (Figure 2B). Conclusions: In conclusion, our study suggests that the report here not only present a novel mechanism of immunosuppression, but also provides preliminary indication of the potential value of AOC3 as a therapeutic target in fighting lung cancer.





1184. AAV3-miRNA-26a and miRNA-122 Vectors-Mediated Growth Suppression of Human Hepatocellular Carcinoma Cells *In Vitro* and Human Liver Tumors in a Murine Xenograft Model *In Vivo*

Ling Yin[1], Geoffrey D. Keeler[1], Yuanhui Zhang[2], Brad E. Hoffman[1], Chen Ling[3], Keyun Qing[1], Arun Srivastava[1]

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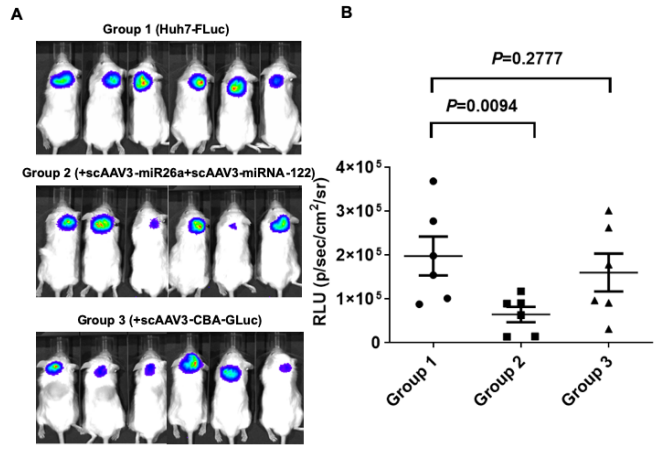
We have previously reported that AAV3 vectors transduce human liver tumors more efficiently in a mouse xenograft model following systemic administration (*Hum Gene Ther*, 25: 1023-1034, 2014). We have also presented evidence that scAAV3 vectors expressing either miRNA-26a or miRNA-122, led to only a modest level of growth inhibition (~12-13%) of a human hepatocellular carcinoma cell line stably expressing the Firefly luciferase reporter gene (Huh7-FLuc) *in vitro*, and that with co-transduction with both vectors, the extent of growth inhibition was additive (~26%). In the present studies, we evaluated the extent of growth inhibition of Huh7-FLuc cell-induced human liver tumors in a mouse xenograft model in vivo. Whereas no significant difference in tumor growth induced by Huh7-FLuc cells or those transduced with scAAV3 vectors expressing a reporter gene, compared with mock-transduced Huh7-FLuc cells, co-transduction with scAAV3-miR-26a and scAAV3-miR-122 vectors led to ~70% inhibition of growth of Huh-FLuc cells-derived human liver tumors (**Figure 1**). To ascertain that the observed growth inhibition of HCC cells mediated by scAAV3-miRNA vectors was specific, it was important to examine the effect of these vectors on non-transformed hepatic cells. HepaRG cells are terminally differentiated hepatic cells, derived from a human hepatic progenitor cell line and retain many of the characteristics of primary human hepatocytes. These cells have been used to study various aspects of liver functions (*Chem-Biol Interact*., 2007;168:66-73, 2007). HepaRG cells were either mock-transduced or transduced with scAAV3-CBA-GLuc vectors or co-transduced with scAAV3-miR-26a-GLuc+scAAV3-miR-122-GLuc vectors as described for Huh7-FLuc cells. GLuc expression levels and total cell numbers were evaluated 72 hrs post-transduction. No significant differences in GLuc expression or total cell numbers were observed. Thus, the combined use of miR-26a and miR-122 delivered by scAAV3 vectors offers a potentially useful approach to target human liver tumors.

Figure 1: Suppression of growth of tumors induced by Huh7-FLuc cells transduced with scAAV3-miRNA vectors in vivo. **A.** Whole-body bioluminescence images of NSG mice 7-days post-injections of Huh7-FLuc cells (Group 1), and those transduced with scAAV3-miRNA-26a+scAAV3-miRNA-122 (Group 2), or scAAV3-CBA-GLuc (Group 3) vectors (n=6 for each Group). **B.** Quantitation of the luminescence signal intensity 7-days post-injections.



1185. Lentiboost Enhances Transduction of T Cells for Clinical Applications

Amairelys Belen Barroeta Seijas, Aleks Guvenel, Alice Giustacchini, Persis J. Amrolia, Claire Booth, Diego Leon-Rico

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Clinical success in gene therapies for the treatment of different types of cancer with Chimeric Antigen Receptor redirected autologous T-cells (CAR-T), as well as for monogenic disorders makes ex-vivo gene therapy a promising option for the treatment of a variety of diseases. Although promising, ex-vivo gene therapy is expensive with clinical grade vectors representing the most costly material used during the manufacturing process. Consequently, enhancing the efficiency of vector transduction becomes a key point in the development and optimisation of protocols used in gene therapy applications. A number of transduction enhancers are available which act by facilitating physical contact between viral particles and target cells. Poloxamers and poloxamines (PLs) are co-polymers capable of self-assembling in micellar structures which have been shown to enhance the transduction efficacy of different viral vectors. Among these compounds LentiBOOST (LB) is the only PL currently in use in ex-vivo hematopoietic stem cell (HSC) gene therapy clinical trials. Whereas in current CAR-T cell protocols other transduction enhancers, such as retronectin are being used, the effect of these PLs has not been described in the literature. Therefore, our aim was to evaluate whether PLs could improve the transduction efficiency of clinically relevant lentiviral vectors (LVs) used for CAR-T and T cell gene therapies to treat primary immunodeficiencies (e.g. X-linked lymphoproliferative disease, XLP). In our study, T cells were expanded from healthy donor PBMCs, activated with anti-CD3/CD28 and cultured in the presence of hIL-2. Cells were transduced using decreasing amounts of LV in the presence or absence of LB. At the end of the culture we evaluated the percentage of transduced cells, vector copy number (VCN), T cell expansion and cell phenotype. Our preliminary results showed that LB-mediated transduction results in equivalent and often higher transduction efficiency at lower multiplicity of infection (MOI) as evidenced by percentage of transduced cells and VCN. Although not statistically significant, a slight decrease in cell number with the highest MOI in combination with LB was observed. LB did not induce significant changes in the phenotype of the cells. Our study shows that the use of LB can reduce the quantity of virus needed in T cell transduction protocols, which may have important economic implications for the development of future gene therapy clinical studies. Maximising vector efficacy and improving transduction during the manufacturing process would hopefully not only increase clinical impact of these therapies, but would also encourage the development of new lentiviral based therapies as the overall costs of the manufacturing process decrease.

1186. Design and Delivery of Tissue Specific Synthetic Gene Circuits into Tumors Using a Baculovirus Vector

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Tissue specificity remains an outstanding challenge in viral gene therapy, as off-target delivery of the genes could induce unwanted death in healthy cells. Most gene therapy payloads use a single gene constitutively expressed under a tissue specific promoter in order to ensure that gene expression happens in on-target tissues. However, these promoters often have low expression levels, and tend to be leaky. Here we propose using a gene circuit designed to classify cells to ensure target specific expression. This circuit will read the microRNA environment of each individual cell in order to determine whether or not to activate transgene expression. Combinations of microRNAs have previously been shown to be able to precisely distinguish between cancer and non-cancer states. Additionally, we have designed and have begun building a novel circuit topology that incorporates negative feedback to create a strong monostable ON switch. This circuit uses eukaryotic parts to avoid unwanted immunogenic responses. Since this gene circuit is larger than the payload capacity of common gene therapy vectors, we propose to use the insect baculovirus, which has been shown to be able to deliver upwards of 100kb of DNA into mammalian cells. Demonstration of this system will be the first reported use of complex therapeutic genetic circuits delivered in tissue, and will signify a generalizable strategy for tissue specificity for future gene therapies.

1187. GDA-201-An "Off-The-Shelf" Nam-Expanded Natural Killer Cell (NK) Therapy

Dorit Aschengrau[1], Ayre Berger[1], Adi Bitansky[1], Guy Brachya[1], Nurit Brycman[1], Sherri Cohen[1], Astar Haylo[1], Aviad Pato[1], Alina Ruiz de Villa[1], Yair Steinhardt[1], Dima Yackoubov[1], Tracey Lodie[2]

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GDA-201 is an investigational, natural killer (NK) cell-based immunotherapy for the treatment of patients with cancer. GDA-201 was previously reported to be generally well-tolerated at multiple doses in combination with monoclonal antibodies and demonstrated early evidence of clinical activity in heavily pre-treated patients with non-Hodgkin lymphoma (NHL) and Multiple Myeloma (MM). [1] GDA-201 is derived from fresh, CD3-depleted donor apheresis units and expanded in culture for 14 days in the presence of IL-15 and nicotinamide (NAM). Fresh cells for the Phase 1 clinical study were derived from either haploidentical or human leukocyte antigen (HLA)-mis-matched donors. Our NAM cell expansion platform is designed to enhance the number and functionality of a variety of allogeneic donor cells and has enabled the generation of multiple clinical development candidates. To further our scientific understanding of the mechanism by which NAM exerts its beneficial effects on NK cells, we performed transcriptome and pathway analysis utilizing the Ingenuity Pathway Analysis (IPA) software to examine NK cells expanded with or without 5mM NAM supplemented with IL-15 and human serum. Gene expression data provided additional insights into the pathways leading to the preservation of NK cell phenotype and increased antibody-dependent cellular cytotoxicity (ADCC) function. NAM increased the expression of CD62L and preserved the expression of CD16 on the cell surface of expanded NK cells, leading to improved *in vivo* homing and retention in lymphoid tissues in NSG mice, and ADCC activity in combination with monoclonal antibodies, respectively. IPA software analysis of transcriptome data predicted

that treatment with NAM also downregulated genes involved in cell senescence. In addition, genes involved in the actin-cytoskeleton signaling pathway were also modulated. We now have developed a novel method for NK cell cryopreservation with the goal of developing a cryopreserved, allogeneic, "off the shelf" NK cell therapy. This unique method of freezing and thawing NK cells yielded very high viability with maintenance of biological activity as measured *in vitro* by cellular cytotoxicity and ADCC assays. Cryopreserved GDA-201 showed ADCC synergy with a variety of monoclonal antibodies such as rituximab, elotuzumab, trastuzumab, and centuximab. Specifically, cryopreserved GDA-201 showed synergy with trastuzumab against the resistant SKOV3 ovarian cancer cell line. In addition, cryopreserved NK cells expanded with NAM demonstrated improved homing to lymphoid organs and reduction of tumor burden *in vivo* in NSG mice. Our data support the early clinical activity observed in the ongoing Phase I trial [2] and suggest that our cryopreserved, allogeneic, "off the shelf", NK cells could have therapeutic potential against solid tumors. [1]Blood (2019) 134 (Supplement_1): 777 [2]ClinicalTrials.gov Identifier: NCT03019666

1188. Sonodelivery of Targeted IL-27 for Treatment of Metastatic Prostate Cancer

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Systemic, long-lasting therapies are required to effectively combat metastatic prostate cancer. Immune-stimulation therapy can be used to treat tumors and their microenvironment by recruiting immune effectors to tumors. Prior studies have shown that gene therapy using the cytokine interleukin 27 (IL-27), delivered to muscle cells via ultrasound-mediated gene delivery (sonodelivery), can reduce tumor growth. In a recent (unpublished) study from our lab, a plasmid was created that encodes for IL-27 fused to a peptide targeting IL-6Rα, which is upregulated on prostate cancer cells. The data suggested that the IL-27-peptide compound exhibited improved antitumorigenic effects compared to IL-27 with a non-targeting peptide. The focus of the present work was to develop new plasmids, each encoding for IL-27 linked to one of three targeting peptides by a flexible linker in a backbone plasmid that is more conducive to *in vivo* expression. The efficacy of the new plasmids was tested *in vitro* by qPCR analysis of genes commonly upregulated by IL-27 (Tbx21, XCL1, and IFNg) on cDNA derived from TC2Ras cells that had been transfected with the new plasmids. An *in vivo* study was conducted in twenty-one C57BL/6 mice. Mice received TC2Ras cells subcutaneously to produce tumors. The treatment group received intramuscular IL-27-peptide plasmid via sonodelivery and the control group received a non-targeted IL-27 plasmid. Preliminary qPCR analysis of the tumors suggested that there was an increase of IL-27 in the treatment group tumors. Current efforts in this project include optimizing the intramuscular sonoporation protocol and the treatment timeframe and frequency, as well as optimizing polymer formulations and vector design for improving gene delivery *in vitro* and *in vivo.* We anticipate decreased tumor growth and enhanced immune effector infiltration in the tumors of mice in the treatment group due to an increase of cytokine expression and improved tumor targeting. Research Grant: 1R01CA196947

1189. Targeting the *DLK1-DIO3* Locus Using miR-122 for the Suppression of Hepatocellular Carcinoma Development

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Hepatocellular carcinoma (HCC) is the third most common form of cancer-related death in the world and few treatment options are currently available to patients. HCC is a highly heterogeneous disease with many risk factors associated with tumor initiation and development. This heterogeneity implies that one treatment modality will not be sufficient and requires personalized medicine approaches. One aggressive HCC subtype is associated with expression of genes restricted during embryo development. These genes direct rapid liver growth and need to be suppressed soon after birth and silenced throughout adult life. If reactivated, cell proliferation is unchecked, though the steps leading to this reactivation are unknown. We performed high throughput sequencing and cluster analysis of human HCC from three independent populations from South Korea, China and The Cancer Genome Atlas (TCGA). In each cohort, we found that embryonic genes were activated including a cluster of ~55 microRNAs and several noncoding RNAs at the *DLK1-DIO3* imprinted locus in 10-15% of samples. Interestingly, many of the tumors with *DLK1-DIO3* locus activation also harbored variants in the long non-coding transcript that hosts miR-122, suggesting that impaired processing of miR-122 or loss of miR-122 could lead to *DLK1-DIO3* locus activation. By querying transcripts within the locus, we found a predicted binding site for miR-122 in the 5'UTR of the *RTL1* gene at the *DLK1-DIO3* locus. Through dual-luciferase reporter assays we found that delivery of miR-122 could suppress *RTL1*, which then prevents increased expression of adjacent transcripts in the cluster. Notably, re-activation of *RTL1* expression is also significantly associated with reduced survival in TCGA HCC samples. The connection between miR-122 loss and *Dlk1-Dio3* locus activation is of relevance to the gene therapy community because locus activation can also contribute to HCC development in mice after rAAV administration. rAAV integration can occur either directly within the locus or at other regions of the genome including at *Tert*, *Ccna2* and *Ccne1* genes. Through small RNA sequencing, we find 30 to 300-fold activation of the mouse *Dlk1-Dio3* locus in tumors from five different mice. Inhibiting the *DLK1-DIO3* locus therefore represents a strategy to reduce or prevent liver damage and tumor development in mice and humans. To test locus suppression, we are administering recombinant adeno-associated virus vectors (rAAVs) that express a miR-122 microRNA sequence to cell lines and mouse models of HCC. These findings will be relevant both for understanding human HCC development associated with embryo gene activation but also in the context of *Dlk1-Dio3* locus activation due to rAAV integration, at least in mice.

1190. Desmoglein-2, an Epithelial Junction Protein and Adenoviral Receptor, as an Ovarian Cancer Biomarker and Prognostic

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Greater than 80% of all cancer cases are carcinomas, formed by the malignant transformation of epithelial cells. One of the key features of epithelial tumors is the presence of intercellular junctions, which link cells to one another and act as barriers to the penetration of molecules with a molecular weight of greater than 400 Daltons (Da). Tumors have been observed to use such tight junctions to subvert the immune system or exclude chemotherapeutics. We explored an epithelial junction protein, desmoglein-2 (DSG2), an epithelial junction protein, as a prognostic and diagnostic biomarker for ovarian cancer. DSG2 is also the main receptor human adenovirus subtype 3, and by extension, for fiber-chimeric Ad5/3 vectors, a vector platform that is used by several groups for oncolytic virotherapy. Ovarian cancer sections were stained for DSG2 and signal intensity was correlated to cancer type and grade. DSG2 immunohistochemistry signals and mRNA levels were analyzed in chemo-resistant and chemo-sensitive cases. Ovarian cancer patient serum levels of shed DSG2 were correlated to disease free and overall survival. Primary ovarian cancer cells were used to study DSG2 levels as they changed in response to cisplatin treatment. DSG2 expression was found to be positively correlated with cancer grade when histopathological ovarian tissue samples were assessed quantitatively. Ovarian cancer patients with high serum levels of shed DSG2 fared significantly worse in both progression-free survival (median survival of 16 months vs. 26 months, $p=0.0023$) and general survival (median survival of 37 months vs. undefined, $p<0.0001$). A subgroup of primary chemotherapy resistant cases had stronger DSG2 IHC/Western signals and higher DSG2 mRNA levels. Furthermore, our *in vitro* studies indicate that non-cytotoxic doses of cisplatin can enhance DSG2 expression, which, in turn, can contribute to chemo-resistance. We suggest that DSG2 can be used in stratifying patients, deciding on where to use aggressive treatment strategies, predicting chemoresistance, and as a companion diagnostic for treatments targeting DSG2. Additionally, the high expression of DSG2 on ovarian cancers could serve as an ideal target for gene therapies or oncolytic therapies using Ad5/3 vectors.

1191. Selective Ablation of Solid Tumors using a P53-Targeted FAST-LNP Gene Therapy

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While chemotherapy is a key treatment strategy for many solid tumors, it is rarely curative as patients will eventually become resistant. In this study, we sought to develop an effective suicide gene therapy approach for solid tumors that specifically exploits their unique transcriptional activation state. The tumor suppressor p53 is frequently mutated or dysregulated in cancer, and as a result the upstream signaling pathways activating p53 transcription are strongly upregulated. RNA-seq analysis has demonstrated that p53 transcription is significantly upregulating in almost all forms of cancer. Additionally, HCT116 cells lacking functional p53 display a 6-fold increase in p53 promoter activity when compared to its wild type p53 parent cell line. To exploit this, we have developed a Fusogenix FAST-LNP formulation to deliver a p53-driven inducible suicide gene, iCasp9, to solid tumors and destroy them upon activation with small molecule dimerizer, Rapamycin. To establish a proof-of-concept, plasmid encoding iCasp9 and luciferase under control of the p53 promoter was constructed and evaluated in a panel of cancer cell lines. While LNPs administered without Rapamycin or Rapamycin administered alone had no impact on cell viability, we observed greater than 90% apoptotic cell death when both were employed in a wide range of cancer cell lines with p53 deletions or mutations, as measured using cell viability assays, imaging assays, as well as Annexin V and TUNEL flow cytometry. Induction of iCasp9 protein expression and caspase-mediated apoptosis was confirmed using Western blot. No cell death was observed in cells with intact p53 such as human umbilical vein endothelial cells or the fibroblast cell line IMR-90. Next, we assessed the efficacy of FAST-LNPs containing p53-iCasp9 in xenograft PC-3 and H1299 models of human prostate cancer and lung cancer, respectively. In PC-3 experiments, tumors were implanted subcutaneously in the flanks of 30 mice and allowed to grow to 500 mm^3 before treatment by four sequential intravenous doses of 100 µg LNP with two daily doses of Rapamycin occurring immediately after LNP treatment. We observed a rapid and dramatic reduction in tumor volume averaging 87% over the following 48 hours, with durable response. Mice enrolled in H1299 study received intravenous injections of 200 µg LNP twice per week with continuous low dosing of Rapamycin. This resulted in a dramatic stabilization of tumor growth. Tumors in control mice from both groups continued to grow exponentially. Overall survival of mice was extended 250% in the PC-3 cohort and 300% in the H1299 cohort. Optimization of number and concentration of LNP doses should allow for long term control of both localized and systemic disease. In conclusion, we describe a novel LNP gene therapy approach for the treatment of cancer with high selectivity for tumors with dysregulated p53 transcriptional activation. This approach has the potential to provide a highly efficacious alternative to current therapies for localized and advanced solid tumors.

1192. *In Vitro* Cell Based Cytotoxicity and T Cell Activation Assays to Assess Safety and Efficacy of Engineered T Cell Therapies

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Chimeric antigen receptor (CAR) and T cell receptor (TCR) engineered T cells are part of a big wave of immunotherapies showing great promise in cancer clinical trials. With the first T cell based therapies targeting hematological malignances now approved, their next challenge is solid tumors. Solid tumors create an additional challenge due to the lack of tumor specific target antigens, posing significant safety risks, i.e. on-target on-tumor, on-target off-tumor and off-target toxicities. Toxic effects previously reported vary from mild-severe cytokine release syndrome (CRS) to neurotoxicity and death. We have developed *in*

vitro assays utilizing primary human cells from healthy tissue and/or differentiated iPCS-derived cells to assess on-target off-tumor and/or off-target cytotoxicity for engineered T cell therapies. The presence of CAR-T cell mediated cytotoxicity was measured through co-culture with healthy human primary cells to assess unwanted CAR-T reactivity as well as a high target antigen expressing control cancer cell line to confirm CAR-T functionality. The human primary cell type was selected based on its potential safety risk by establishing low level protein expression of the target antigen. Readouts included IFNγ production determined by MesoScale Discovery platform as a measure of T cell activation and Hoechst/PI staining of target cells by flow cytometry. Our study generated high quality data of the CAR-T cell, confirming functionality by showing consistent T cell activation and killing against a positive control cell line. Moreover, we were able establish a clear absence of activity against the primary human cells thus providing insight into the safety of the CAR-T therapy. The developed safety assays provide a robust and rapid platform to assess on-target off-tumor and off-target effects within immuno-oncology therapies, either TCR or CAR-T cells, in both early stage development or late stage testing of the therapeutic product. Through inclusion of a wide range of human primary cells, both high risk tissues and major organs at risk of off-target toxicity, a clear safety profile can be generate *in vitro* for these novel T cell therapies. Safety risks associated with cell based IO-therapies is the biggest challenge for the success of these therapies, performing a thorough safety screen on healthy primary human tissues is therefore crucial.

Immunological Aspects of Gene Therapy and Vaccines

1193. Cell-Mediated Inflammatory Response to AAV Gene Therapy in the Retina

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Introduction: Adeno-associated viral (AAV) vector-mediated gene therapies have demonstrated efficacy in the treatment of a range of inherited retinal diseases, including Leber congenital amaurosis, choroideremia and X-linked retinitis pigmentosa. Despite promising results, clinical trials have revealed intraocular inflammatory reactions to AAV as an important determinant of visual outcome. Resident microglia play a central role in the immune surveillance of the retina and can be activated by a wide range of stimuli. Here, we investigate the effects of AAV gene therapy on the retinal microglia and immune cell population. **Methods:** Subretinal injections of $1x10^9$ genome copies of an AAV serotype 8 (Y733F) vector expressing GFP (AAV8.GFP) were performed in three-week old C57BL/6J mice with sham injections of PBS undertaken in the contralateral eyes. Retinae were harvested at baseline and 3, 7 and 14 days post-injection (n=5 per time point) and enzymatically dissociated into single cell suspensions. Cells were stained with antibodies to CD45 (a pan-leukocyte marker) and CD11b (a marker for microglia and macrophages) prior to flow cytometric analysis. **Results:** Following subretinal injection of AAV8.GFP, a mean 3.2 ± 0.4% of retinal cells were GFP^+ on day 3, rising to 42 ± 15.7% by day 7 and 66.6 ± 24% by day 14, indicating successful AAV transduction. In 3 out of 5 AAV-treated retinae, all with the highest transduction efficiencies by day 14, signs of mild vitreous inflammation were detectable by optical coherence tomography as pre-retinal opacities. The retinal $CD45^+$ cell population at baseline was predominantly $CD45^{lo}CD11b^+$ (indicative of resident microglia) with a small proportion of $CD45^{hi}CD11b^+$ cells (likely macrophages). No significant increase in the $CD45^+$ cell population was seen at days 3 and 7 post-injection. However, at day 14, sharp rises in the number of $CD45^+$ cells were observed in the AAV-treated retinae with a mean 8.7-fold increase relative to PBS-injected eyes, positively correlating with the level of AAV transduction (Spearman's rho = 1.0). These $CD45^+$ cells were now predominantly a mixture of $CD45^{hi}CD11b^+$ macrophage-like cells and $CD45^{hi}CD11b^-$ cells, which are indicative of infiltrating lymphocytes (**Figure 1**). Moreover, a mean 8.3% of the $CD45^+$ cells, which were almost exclusively $CD11b^+$, were also GFP^+, suggestive of AAV8-transduced resident microglia. The higher level of CD45 expression among this GFP^+ population raises the possibility of microglial activation. In contrast, these responses were not seen in the PBS-injected eyes. **Conclusion:** Subretinal gene therapy with AAV8.GFP in mice leads to a cell-mediated retinal inflammatory response, despite the use of a dose of vector previously shown to be safe. The inflammatory responses are suggestive of both activation of resident microglia and infiltration of lymphocytes independent of surgical trauma.

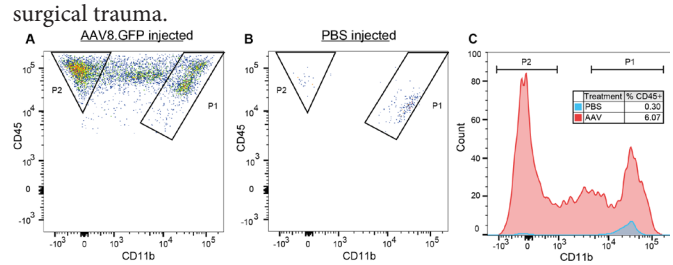

Figure 1. Flow cytometric analysis of the cell-mediated inflammatory response to subretinal gene therapy in mice. Flow cytometry plots, gated for CD45 expression, of (**A**) AAV8.GFP and (**B**) PBS subretinally injected paired eyes from a representative animal 14 days post-injection with additional CD11b+ (P1) and CD11b- (P2) gates. (**C**) Histogram overlay of CD45+ cells from AAV (red) and PBS (blue) injected retinae.

1194. Utilising Apheresis to Remove Neutralising AAV Antibodies in Patients Previously Excluded from Gene Therapy

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Introduction: Owing to their early clinical efficacy and outstanding safety record, AAV vectors have become the gene therapy vector of choice for the treatment of haemophilia and other systemic diseases. It has been suggested that 70% of people will be exposed to AAV during their lives. While infection with wild-type AAV is benign,

the antibodies raised can cross-react with clinical AAV vectors and inhibit transduction of target organs. In our FLT180a phase 1/2 trial we use a transduction inhibition assay (TIA) to screen patients for trial inclusion. As anticipated, healthy and haemophilia populations have varying degrees of neutralizing antibodies (NAb) against our vector, AAVS3. In order to treat as many patients as possible with our transformative therapy, we are evaluating methods that reduce AAVS3 NAb titres in patients so they can benefit from AAVS3 gene therapy. In the current study, we show that double filtration plasmapheresis (DFPP), a routine method for removing antibodies from circulation, can be used to deplete AAVS3 NAb to levels within our trial eligibility criteria. **Methods:** Biobank plasma samples from 36 patients who had undergone up to 5 rounds of DFPP prior to kidney transplant were retrospectively analysed for anti-AAVS3 antibodies by ELISA and, when sample volumes allowed, by TIA. To assess AAVS3 Ab depletion in a controlled setting, studies were conducted using an *in vitro* plasmapheresis circuit to test two commercially available filters, using pooled (fresh frozen) plasma. Coagulation factors were measured in the *in vitro* study and in clinical samples to assess the impact of plasmapheresis on these proteins. **Results:** After each successive round of DFPP in transplant patients, the total and neutralizing titres of AAVS3 Ab decreased. A pattern of antibody re-synthesis and/or redistribution from tissues was observed between cycles, however, subsequent rounds always enabled further reductions in antibody titre compared to baseline. When considering seropositive patients with a starting titre of ≤ 1:1600, 62% of patients that underwent DFPP achieved the titre required for trial inclusion. Our single-cycle *in vitro* study showed that plasmapheresis reduced AAVS3 NAbs by 50% compared to baseline and coagulation factor levels to 40% of baseline. Of the 36 patients assessed for AAVS3 NAb, four patients underwent 3 daily rounds of apheresis. Analysing consecutive rounds of DFPP confirm that the first round is most efficient: 66% of AAVS3 NAbs were removed on the first consecutive day and similar, but lower, percentages were removed on subsequent days. Overall, 3 daily rounds of DFPP decreased AAVS3 NAb titres by 79 ± 9%. Assessment of coagulation FVIII and FIX levels pre- and post-DFPP showed that DFPP was associated with a decrease in FVIII and FIX levels, however, factors rebounded to pre-DFPP levels prior to the subsequent cycle of DFPP. **Conclusion:** This work demonstrates that apheresis can reduce anti-AAV NAb to levels required for clinical trial inclusion. In a subset of patient samples, FVIII and FIX were shown to decrease after DFPP but rebounded prior to the subsequent round of treatment. Further prospective studies are required to better understand the impact of DFPP on coagulation factor levels and bleeding risk in patients that would undergo the procedure. Collectively, the work provides proof-of-concept that plasmapheresis may be used to reduce AAVS3 NAb titres to levels that are acceptable for trial inclusion.

1195. AAV Vectored Immunoprophylaxis Applications for the Prevention of Healthcare-Associated Infections

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The focus of current vectored immunoprophylaxis efforts remain targeted to the treatment of viral and parasitic diseases while overlooking many bacterial infections of major public health concern. Healthcare-associated infections (HAIs) are a cause of mortality in immunocompromised patients and a cause of significant burden on healthcare systems in industrialized countries. In recent decades, healthcare associated *Clostridium difficile* infection (CDI) incidence rates have increased worldwide including the incidence of multiple recurrent CDI. Therefore, we aimed to investigate vectored immunoprophylaxis and subsequent long-term expression of pathogen-specific and toxin-specific monoclonal antibodies (mAbs) as a novel strategy to provide protection against, and treatment of, CDI. Utilizing a rationally designed adeno-associated virus (AAV) triple mutant capsid we can facilitate rapid, robust, and long-term expression of therapeutic antibodies in vivo following a single intramuscular administration. We re-engineered the AAV genome to express mouse/human optimized hIgG1 mAbs with the variable heavy and light domains of Actoxumab and Bezlotoumab to target *Clostridium difficile* toxins TcdA and TcdB. Intramuscular injection of $1x10^{11}$vg of AAV6.2FF-Actoxumab resulted in average serum concentrations of 89.59 μg/ml by day 28 post-injection which was able to extend survival in a high dose TcdA challenge and provided 100% protection from a low dose TcdA challenge (Figure 1). Contrastingly, intramuscular injection of $1x10^{11}$vg of AAV6.2FF-Bezlotoxumab resulted in average serum concentrations, of 195.91 μg/ml by day 28 post-injection that was unable to extend survival at high or low dose TcdB challenge. We were able to detect pathogen-specific antibodies at various mucosal and organ surfaces including in the intestinal surface (136.83ng/ml to 633.09ng/ml) and intraperitoneal cavity (14.54ug/ml to 55.14ug/ml) following IM administration of these AAV vectors. We have recently expanded this platform to express hIgG mAbs with variable heavy and light domains of MEDI3902 to target the type 3 secretion system (PcrV) and exopolysaccharide (Psl) of *Pseudomonas aeruginosa* resulting in high concentrations of antibody in murine serum. In summary, vectored immunoprophylaxis can offer advantages prophylactically over passive immunization as an alternative to antibiotic use for the treatment of healthcare associated infections.

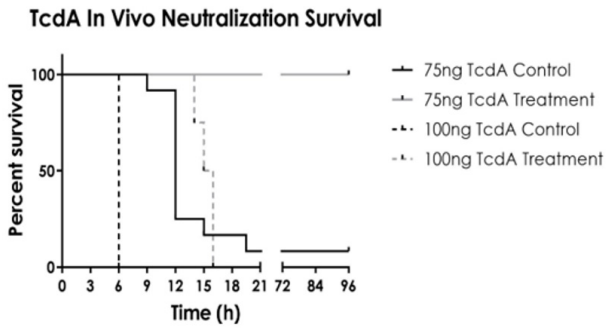

Figure 1. AAV6.2FF-Actoxumab provides complete protection in a TcdA neutralization model. BALB/c mice (n=4 to 12) that received $1.0x10^{11}$vg of AAV6.2FF-Actoxumab IM and were challenged by intraperitoneal injection with a high(100ng) or low(75ng) dose of TcdA 28 days post-injection and monitored for survival.

1197. Smartphone Based Quantitation of Cytokines

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Cytokines are a broad category of proteins secreted by immune cells that function as messengers of the immune system. These proteins regulate the responses of specific cell populations by binding to cell receptors and modulating intracellular signaling pathways. Because cytokines mediate immune responses in both normal and pathological immune states and in response to therapeutic interventions, the ability to accurately measure these proteins is required for the fulfillment of a wide array of basic and translational research objectives, including the development of immune cell therapies such as CAR T. Current methods for measuring cytokines include bead platforms (such as Luminex and CBA), solid phase arrays, and intracellular cytokine staining, but the most ubiquitous means of cytokine measurement is the ELISA. While sandwich-based ELISAs enable accurate, precise, and sensitive measurement of specific cytokines in a range of biological samples, these assays are both labor intensive and time consuming, taking from 90 minutes to 4 hours to complete depending upon the protocol. In addition, cytokine ELISAs are typically provided in formats that incentivize researchers to collect and accumulate samples over days or weeks so that they can save hands-on time and reduce costs by analyzing several samples in parallel using entire ELISA plates at once. In this work we present an iOS and Android-compatible smartphone application that we have developed to analyze accompanying cytokine-specific lateral flow assays and deliver accurate quantitative results in approximately 10 minutes. The simplicity of these assays facilitates quick measurement of cytokine levels to enable real-time monitoring during experiments, and they can also be used for screening applications aimed at minimizing the number of samples analyzed downstream using more traditional immunoassays. The two-step assay consists of adding a small amount of culture supernatant or diluted serum to the lateral flow device followed by imaging and analysis of the results using a smartphone. Densitometric analysis of the resulting assay bands is performed by the intuitive GoStix Plus software application, which compares the results to an automatically downloaded, lot-specific standard curve. The output from the GoStix Plus application is a unit value (pg or ng/ml) that generally falls within the same range as ELISA-based measurements but is generated in a fraction of the time. Analyzing the performance of the assay using recombinant control proteins yielded R^2 values greater than 0.99 and coefficients of variation less than 15%, indicating comparable precision to ELISA-based approaches. Furthermore, we analyzed cell-culture supernatants from CD3/CD28/CD2-activated T cell populations and observed cytokine values in agreement with corresponding ELISA analyses. In summary, our pairing of a highly convenient lateral flow technology with smartphone-based image processing enables accurate and precise quantitation of cytokines in approximately 10 minutes, reducing expenses related to labor and materials and accelerating immunology research and the development of novel therapies.

1198. Prevalence and Avidity Assessment of Pre-Existing Neutralizing Antibodies (NABs) Against Adeno-Associated Virus (AAV) Vector Serotypes 2, 5 and 8 Analyzed in the Serum of 300 Healthy Donors

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It has been reported in preclinical and clinical studies for AAV2 or AAV8 serotypes that pre-existing anti-AAV NABs titers impact negatively transgene expression. Therefore, individuals with pre-existing NABs against AAV above titer of 5 are excluded from most systemic AAV-based clinical trials. We have previously reported that anti-AAV5 NABs titers up to 340 in humans and as high as 1030 in primates did not interfere with the therapeutic efficacy of intravenously administered AAV5 vector. Hence, patients entering the AMT-061 (AAV5-hFIX-Padua) trial are not excluded on the basis of anti-AAV5 NABs levels. Overall, those contradictory observations suggest that the neutralization ability of anti-AAV pre-existing NABs *in vivo* might be different between AAV serotypes. The aim of the present study was to assess the binding characteristics of the pre-existing NABs directed against AAV serotypes 1, 2, 5 and 8. Serum samples of 300 healthy donors were analyzed to determine pre-existing anti-AAV NABs titers and avidity (residual binding) of anti-AAV IgGs to specific AAV serotypes. The results obtained demonstrate that the avidity of pre-existing AAV-specific IgG antibodies in the healthy human population is significantly different between AAV serotypes. Pre-existing anti-AAV2 or anti-AAV8 IgGs form stronger antibody-antigen complexes with respectively AAV2 and AAV8 antigens, while pre-existing anti-AAV5 IgGs generate significantly weaker antibody-antigen complexes with the AAV5 antigen **(Figure 1)**. Additionally, when analyzing donors that were positive for pre-existing anti-AAV NABs against all three tested serotypes (2, 5 and 8), avidity of pre-existing anti-AAV5 IgGs in 80% of those donors was low. However, avidities of pre-existing anti-AAV2 IgGs or anti-AAV8 IgGs in 91% and 83% of those donors respectively, were high **(Figure 2)**. That suggests, in most donors, cross-reactivity of anti-AAV2 antibodies with the AAV5 antigen. These observations support the lack of impact of pre-existing anti-AAV5 NABs measured *in vitro* on the efficacy of AAV5-based transduction *in vivo* when compared to pre-existing anti-AAV NABs against serotypes 2 or 8.

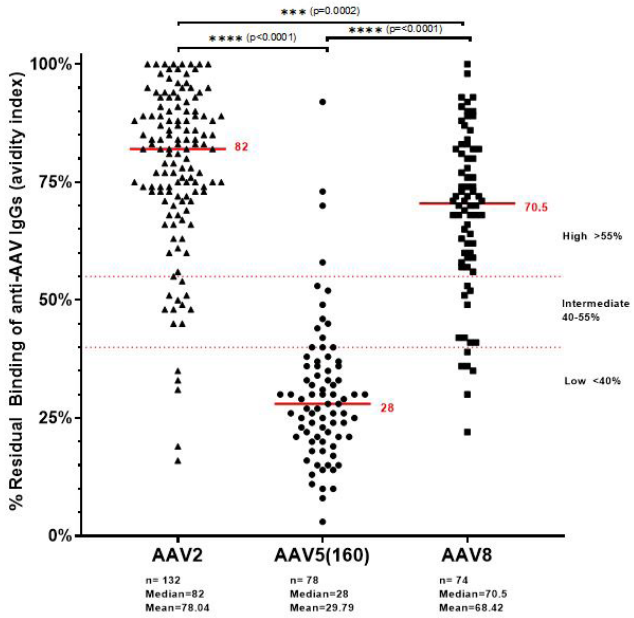
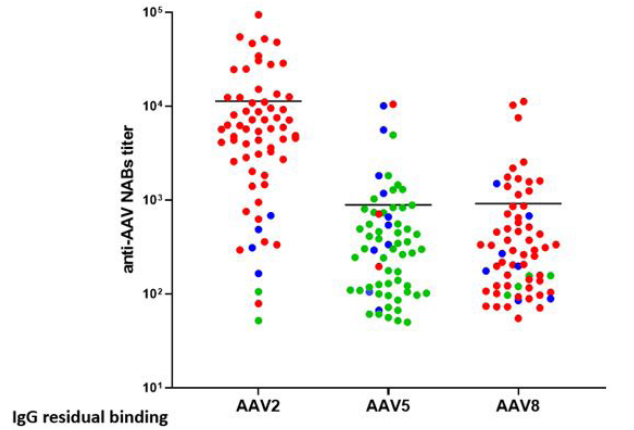


Figure 1. Avidity of AAV-specific IgG antibodies in healthy human population. Significant difference was observed between the different serotypes. One-Way ANOVA followed by Tukey's correction for multiple comparisons test analysis was performed.



IgG residual binding	AAV2	AAV5	AAV8
Low [<40%]	3%	80%	6%
Intermediate [40-55%]	6%	15%	11%
High [>55%]	91%	5%	83%

Figure 2. NABs titers against different AAV serotypes. In this graph all the donors that tested triple positive, for NABs against AAV2, 5 and 8 were plotted (NABs titer >50). In green IgGs that have low avidities (residual binding) to the AAV antigen, in blue intermediate avidities and in red high avidities were plotted.

1199. The Influence of Adenovirus Species C Hexons on Vector Properties In Vitro and In Vivo

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The majority of adenovirus (Ad) vectors are based on human Ad type 5, which is a member of human Ad species C. Species C also includes the closely-related types 1, 2, 6 and 57, but much less is known about the properties of vectors derived from these non-Ad5 types. We evaluated vectors based on these non-Ad5 types both in vitro and in mice, and we also constructed chimeric Ad5 vectors that contain the hexon hypervariable regions from these non-Ad5 types. Our goal was to understand how hexon influences liver transduction by Ad vectors after intravenous injection in mice. In addition, we studied how plasma proteins interact with these vectors, including coagulation factors and natural antibodies (pre-existing antibodies). We found that Ad5 and Ad6 vectors have the greatest ability to transduce mouse liver. Vectors with Ad6 hexon have strikingly different properties from Ad5, showing increased resistance to host natural antibodies and a reduced dependence on coagulation factors for liver transduction. In normal mice, vectors with Ad1 or Ad2 hexon were relatively poor at transducing liver. However, vectors with Ad1 or Ad2 hexon had high liver transduction in antibody-deficient mice, showing that these vectors do not lack liver tropism, but rather that these vectors are very sensitive to inhibition by natural antibodies in vivo. Ad5 hexon is known to bind strongly to coagulation factor X (FX), but binding of coagulation factors to other Ad types has been studied to only a limited extent. We found that all species C vectors had the ability to bind FX, but they also could bind another highly-abundant coagulation factor: prothrombin (FII). Ad5 vectors bound FII relatively poorly, but other vectors had much higher ability to bind FII. We found that vectors based on Ad1, Ad2, Ad6 and Ad57 shared a similar pattern of coagulation factor binding, which was different from the pattern seen with Ad5. In sum, hexons from different human Ad species C viruses confer diverse properties on vectors, including differences in coagulation factor binding and liver transduction.

1200. Using Information from the Microbiome to Improve the Efficiency of Diagnostic-Therapeutic Options for Melanoma: A Cost-Effectiveness Analysis

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OBJECTIVES: This study evaluated the cost-effectiveness of adding a gut microbiome test (GMT) to PD-L1 testing to guide treatment choice between immunotherapy and chemotherapy options in the care of patients with metastatic or unresectable melanoma. **METHODS:** This study applied a decision tree model to study the cost-effectiveness of adding a gut microbiome test (GMT) to routine PD-L1 testing to guide treatment decisions for patients with metastatic

or unresectable. Treatment options included immunotherapy or chemotherapy protocol. The analysis was conducted from the public perspective; the majority of patients were Medicare beneficiaries. The model's time horizon was 32 weeks or 16 weeks depending on whether patients' first-line treatment fails. Costs were calculated in US Dollars (USD, $) and adjusted for inflation to 2019 USD. Quality-adjusted life years (QALYs) were used to measure effectiveness. Both univariate and probabilistic sensitivity analyses were used to assess the reliability of the deterministic results and examine the uncertainties in the model. Microsoft Excel (version 16.29.1) was used for modeling. **RESULTS:** Patients accrued 1.11 more QALYs and cost $32,981 more compared to standard of care of using PD-L1 testing alone. The incremental cost effectiveness ratio of GMT strategy was $75,229 per QALY under base-case assumptions. The 95% credible interval for the ICER was $42,739/QALY to $99,176/QALY. 75.4% of the 10,000-iteration Monte-Carlo probabilistic sensitivity analysis simulations yielded cost-effective results under a $75,000/QALY willingness-to-pay threshold. **CONCLUSION:** Adding microbiome testing to the routine use of using PD-L1 testing is a cost-effective strategy for guiding treatment of metastatic or unresectable melanoma with willingness-to-pay threshold of $100,000 or above. The addition of GMT may provide economic and clinical value in the care treatment paradigm of metastatic or unresectable melanoma.

1201. An Engineered Analogue of IL-2, Fc.Mut24, Prevents the Formation of Factor VIII Inhibitors in Hemophilia Mice Receiving Factor VIII Gene Therapy

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Hemophilia A (HemA) patients are deficient in functional factor VIII protein (FVIII) as a result of a genetic mutation. This deficiency in functional FVIII causes decreased blood clotting function. Currently, HemA patients receive infusions of FVIII protein to treat their disease, of which approximately 30% develop inhibitors to FVIII. Previously, mice receiving IL-2 complexed with α-IL-2 mAbs showed a lack of FVIII inhibitor formation after FVIII treatment, which was associated with selective proliferation and activation of Tregs. We evaluated the capability of a computationally designed analogue of IL-2, named Fc.Mut24 to provide more robust Treg enrichment with direct applicability to the design of human trials. In this study, the Treg population in hemophilia A mice was increased and activated using Fc.Mut24 dosing prior to FVIII gene therapy to prevent the formation of anti-FVIII antibodies. Experimental mice received intraperitoneal injections of 6μg Fc.Mut24 in 200μL PBS, while control mice received 200μL of plain PBS. 4 days later, all mice received hydrodynamic injection of 50μg FVIII plasmid. Experimental mice received a second 3 μg injection of Fc.Mut24 on day 7. Flow cytometry was used to characterize peripheral blood and splenic cell populations, while ELISA and Bethesda assay were used to assess inhibitor concentrations in plasma. Mice receiving Fc.Mut24 showed a dramatic increase of Tregs in peripheral blood compared to control mice on Day 7, from 8% to 20%, while total CD4+ cell population remained consistent. Percentage of Treg activation was also increased, from 29% to 42%. FVIII gene therapy via hydrodynamic injection resulted in high FVIII inhibitor concentration in control mice, while experimental mice treated with Fc.Mut24 produced undetectable inhibitor levels. Functional FVIII levels, measured via APTT assay, remained above 70% at 19 weeks after FVIII plasmid treatment in experimental mice, while control mice showed low or negligible levels of functional FVIII by week 9. At week 20, mice received a secondary hydrodynamic injection of 100μg FVIII plasmid. Differences in FVIII inhibitor levels and functional FVIII between these two groups of mice persisted after the second hydrodynamic plasmid injection. These results show that Fc.Mut24 can significantly increase and activate the Treg population in mice, which prevented formation of high-titer anti-FVIII antibodies when administered in combination with FVIII gene therapy. This tolerance persisted over 6 months, even after a second administration of FVIII gene therapy. We believe these experiments demonstrate a potential method for induction of long-term immune tolerance of FVIII in hemophilia patients.

1202. Multiplexed Neutralizing Antibody Assay Identifies Potential Epitopes on the AAV Capsid

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Neutralizing antibodies pose a significant barrier to successful AAV gene therapy treatments. While many groups have studied humoral AAV immunity, *in vitro* neutralization assays have not been standardized across the field, leading to varied reports of neutralizing antibody seroprevalence in human populations. Additionally, disparities in neutralization assays across research groups have direct implications for potential gene therapy patients, as anti-AAV titer assessments are a key determinant of treatment eligibility. Here, we have developed a multiplexed neutralizing antibody assay that makes use of barcoded AAV libraries and next generation sequencing. Libraries of putative common ancestors of circulating AAV serotypes (AncAAVs) were used to model functional intermediates between natural AAV capsids used in gene therapy and encompass a diversity of capsid sequences that could be assessed for neutralization by monoclonal antibodies or whole serum. Results showed that the multiplexed assay was able to replicate neutralization findings from clonal AAVs, and when the neutralization profiles of individual samples were compared to capsid sequences, candidate epitopes could be predicted computationally. This work has potential implications for a wide range of AAV subjects, from epitope mapping studies to AAV epidemiology and personalized medicine.

1203. Characterization of MHC Class II-Restricted T-Cell Receptors for T-Cell Therapy of HBV Infection

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Hepatitis B virus (HBV) infection remains a severe health problem with current treatment options being unable to achieve viral clearance. Since virus eradication is accompanied by a strong T-cell response in individuals with self-limited infection, restoring the dysfunctional T-cell immunity in patients with chronic HBV infection via adoptive T-cell therapy represents a promising therapeutic approach. We recently demonstrated that $CD8^+$ T cells grafted with T-cell receptors (TCR) restricted by MHC class I have the potential to cure HBV infection *in vitro* and *in vivo*. Nevertheless, also $CD4^+$ T cells are known to be essential in resolving HBV infection and may therefore potentially benefit T-cell therapy. We thus identified, cloned and characterized MHC class II-restricted TCRs from HBV-specific $CD4^+$ T cells. HBV peptides were used to stimulate PBMC from donors with resolved HBV infection. HBV-specific $CD4^+$ T cells secreting TNF-α were sorted by flow cytometry and clonally expanded. Subsequently, TCR α- and β-chain variable sequences were identified, codon-optimized and cloned into a retroviral vector with murine constant domains. After retroviral transduction, all TCRs were stably expressed on primary $CD4^+$ as well as $CD8^+$ T cells and characterized in depth regarding their MHC restriction, specificity, binding affinity and functionality. A total of 23 TCRs specific for seven different epitopes from HBV core, envelope or polymerase protein were generated. Through retroviral titration, TCR integration was limited to under five integrates per cell as determined by qPCR, with transduction rates ranging from 65-85%. Strength of surface expression represented through fluorescence intensity in flow cytometry was found to be characteristic of each TCR rather than the number of integrates. Through co-culture with single HLA-transfected target cells or partially matched lymphoblastoid cell lines, eight different MHC class II restrictions were identified, i.e. HLA-DRB1*0101, DRB1*0701, DRB1*1104, DRB1*1301, DRB3*0202, DPB1*0201, DPB1*1501 and DQB1*0603. One TCR demonstrated highly promiscuous binding properties by specifically recognizing six additional MHC class II molecules. All TCRs specific for core or small envelope protein were able to recognize endocytosed and intracellularly processed viral antigen. 17 TCRs recognized three or four HBV genotype variants and 18 TCRs had a high binding affinity with EC50 values in a low nanomolar range. 20 TCRs were capable of activating both $CD4^+$ and $CD8^+$ T cells independently of their co-receptor, inducing strong granzyme B secretion in 75-100% of transduced $CD8^+$ T cells. Remarkably, 10 TCRs enabled TCR-transduced $CD4^+$ T cells to efficiently kill peptide-pulsed HLA-matched fibroblasts. Taken together, our set of 23 HBV-specific TCRs recognizes epitopes derived from the viral core, envelope and polymerase protein restricted by eight different MHC class II molecules. These TCRs enable both $CD4^+$ and $CD8^+$ T cells to detect antigen on MHC class II in a nanomolar range, resulting in antigen-specific T-cell activation as evidenced by proliferation, cytokine secretion and specific killing of peptide-pulsed fibroblasts.

1204. F(ab')2 Fragments Fail to Completely Neutralize AAV *In Vitro*

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Gene replacement therapy using recombinant Adeno-Associated Viral (AAV) vectors is a promising new therapeutic area that has received regulatory approvals to treat dyslipidemia, spinal muscular atrophy, and retinal blindness. A major challenge in AAV administration is the presence of preexisting anti-AAV neutralizing antibodies (NAbs) from prior—natural--AAV infections that prevent vector transduction. Hence, patients with preexisting neutralizing antibodies are mostly excluded from enrollment in clinical trials. Strategies to circumvent neutralizing antibodies are currently being explored, including immune-modulation and plasmapheresis to allow NAb-positive patient inclusion. IdeS is a *Streptococcus pyogenes* enzyme that specifically cleaves below the hinge of human IgG, removing the Fc portion while leaving the F(ab')2 arms intact. The Fc region of an antibody is required for extending serum half-life and mediating immune effector functions, while the Fab regions are required for antigen binding. Clinical trials using IdeS have resulted in successful HLA-incompatible transplantations by temporarily reducing levels of circulating IgG. In these studies, we used pooled human intravenous immunoglobulin (IVIG) with known preexisting anti-AAV NAbs as an IgG source. We cleaved IVIG with IdeS and evaluated NAb titers post-treatment. IdeS-cleaved F(ab')2 had significantly reduced anti-AAV neutralization when compared to uncleaved controls. Similar results were obtained using IVIG treated with pepsin; another enzyme that generates F(ab')2 fragments. Interestingly, cleaved F(ab')2 fragments efficiently bound vectors yet did not completely neutralize the vector. Similarly, single Fab fragments also failed to neutralize vector. These results suggest that IdeS pretreatment generates F(ab')2 fragments that bind but do not completely neutralize vector. The studies presented here identify a previously unknown aspect of AAV biology where F(ab')2 binding fails to prevent vector uptake to the same extent as intact neutralizing antibodies. Ongoing work will explore the reasons for IgG-mediated vector neutralization and its lack thereof by F(ab')2 fragments.

1205. A Combination Immunosuppression Treatment with Bortezomib and an Anti-CD20 mAb Enables Successful Re-Administration of AAV8 Vector in Mice

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Background: Adeno-associated virus (AAV)-mediated gene therapy has been effective for the treatment of inherited diseases in both preclinical studies and clinical trials. However, human studies suggest that the presence of pre-existing anti-AAV capsids neutralizing antibodies (NAb), even at very low titers, can block viral vector transduction and results in the exclusion of a significant number of

patients from clinical trials. In addition, NAb raised after initial AAV treatment renders re-administration of the same vector ineffective, a strategy that is often required to achieve the durability of gene therapy. In this study, we tested the feasibility of using a combination therapy with two clinically approved immunosuppressive drugs, Bortezomib to target antibody-producing plasma cells and Rituximab (anti-CD20 mAb) to deplete total B cells, to prevent anti-AAV NAb and allow re-administration of AAV vector of the same serotype in a mouse model of Pompe disease (GAA-KO mice). **Methods:** Three-month-old GAA-KO mice were intravenously (i.v.) injected with an AAV8 vector (AAV8-CB-hGAA) that ubiquitously expresses human α-glucosidase at a dose of $5x10^{12}$ vg/kg (Week 0). Group 1 received immune suppression (IS) treatment consisting of monthly intraperitoneal administration of a mouse-specific anti-CD20 mAb and i.v. injection of Bortezomib twice a week (Fig.1A). Group 2 did not receive IS treatment. PBS-treated mice were used as controls (Group 3). At Week 8, 4-6 mice from each group were sacrificed. Plasma samples were used for determination of anti-AAV8 NAb titers. Cells isolated from whole blood, spleen, and bone marrow were analyzed for B-cell depletion by flow cytometry. The remaining mice in each group were re-challenged with of a second AAV8 vector (AAV8-LSP-hSEAP, $5x10^{13}$ vg/kg, i.v.) that contains a liver-specific promoter to express human secreted alkaline phosphatase. The efficiency of AAV8-LSP-hSEAP transduction was evaluated by monitoring the levels of secreted hSEAP in the plasma. **Results:** At 8 weeks post AAV8-CB-hGAA injection, high titers of anti-AAV8 NAb were observed in mice that did not receive IS treatment (Group 2), in contrast, the IS-treated mice (Group 1) had a similar low level of anti-AAV8 NAb to the PBS-treated mice (Group 3) (Fig.1B). CD19+B220+ B cells were almost completely depleted in the blood and spleen in Group 1 (Fig.1C). Bone marrow also had significantly reduced CD19+B220+ B cells but contained a large fraction of B220+CD19- cells. Further investigation of these cells into B cell subsets revealed a loss of the developing B cell population, which was compensated by a higher frequency of PrePro B cells. After re-administration of AAV8-LSP-hSEAP vector, hSEAP activities in plasma kept increasing in both Group 1 and Group 3 for up to 6 weeks, but were not detectable in Group 2 (Fig.1D). The lower activity in Group 1 than Group 3 is likely a result of the incomplete depletion of anti-AAV8 NAb by the IS treatment. **Summary:** Our data demonstrate that this combination IS treatment is effective in reducing neutralizing antibodies against AAV capsid to allow repeated AAV administration in mice. The effectiveness of this treatment strategy needs to be further evaluated in other animal models and clinical trials.

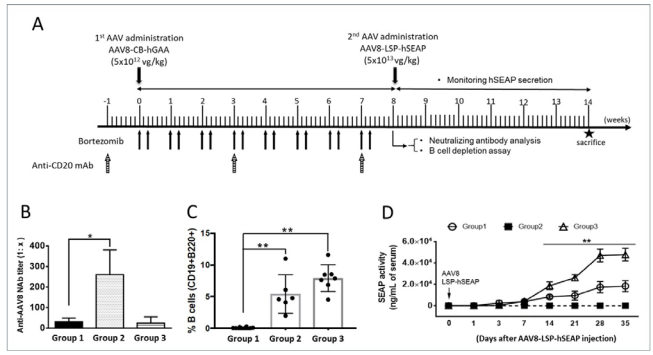

Figure 1. A combination immune suppression (IS) treatment with Bortezomib and an anti-CD20 mAb prevents anti-AAV8 NAb formation, depletes circulating B cells, and enables successful re-administration of AAV8 vector in mice. (A) Outline of AAV injections and IS treatment regimens for Group 1. (B) Inhibition of anti-AAV8 NAb 8 weeks after AAV-CB-hGAA injection. x, dilution folds of plasma samples. N=4 for Group 1, n=4 for Group 2, n=6 for Group 3. (C) Depletion of CD19+B220+ B cells in blood after 8-week IS treatment. N=8 for Group 1, n=6 for Group 2, n=7 for Group 3. (D) Detection of secreted hSEAP in plasma following AAV-LSP-hSEAP injection. Significance at the indicated time points between Group 1 and Group 2 is shown. N=5 for each group. All data are shown as mean±SD. Student t-test. *, $p<0.01$; **, $p<0.0001$.

1206. Developing an In Vitro Assay to Study Innate Immune Responses to AAV Vectors

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Host immune responses against adeno-associated virus (AAV) vector poses a safety risk to patients and can reduce efficacy of gene therapy vectors. Activation of both the innate and the adaptive immune responses against AAV have been observed in animals and humans during AAV vector-mediated gene therapy. Various strategies have been proposed to reduce host immunogenicity against AAV; however, due to a lack of predictive *in vitro* models, the effectiveness of these strategies is often tested in animals, which can be time consuming, costly and results obtained often do not translate to humans. Thus, development of *in vitro* assays to study immune responses against AAV are critical in the development of novel immunomodulatory strategies. AAV-mediated activation of Toll-like receptor 9 (TLR9) signaling has been identified to play a critical role in developing both the innate and adaptive immune response against AAV vector. However, assessment of AAV-mediated TLR9 activation *in vitro* has been challenging. Here we used various human and murine cell lines and primary human cells to assess activation of innate immune response by AAV vector either containing single-strand (ss) or self-complementary (sc) DNA genome. We found that human and murine cell lines responsive to TLR4, TLR7 or TLR9 ligands or type-I interferons (IFNs) did not respond to ssAAV or scAAV. Primary human monocytes were activated by scAAV but not by ssAAV. Activation of primary human monocytes by scAAV was partially rescued by TLR9 inhibitor. Together, these data suggest that human and murine cell lines that respond to TLR ligands or IFNs do not respond to AAV vectors *in vitro.* Primary human monocytes can be used to assess scAAV-mediated innate immune activation; however, inhibition of TLR9 signaling alone may not be sufficient to completely reduce AAV-mediated activation of innate immunity.

1207. Characterizing Immune Responses to AAV9 in a Mouse Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an x-linked disease caused by out-of-frame mutations in the *DMD* gene, which encodes dystrophin. Due to the nature of these mutations, DMD is amenable to gene-editing and gene replacement therapies. Ongoing phase I/II clinical trials for DMD rely on a gene replacement strategy using adeno-associated virus (AAV) to systemically deliver a highly truncated *DMD* gene, referred to as microdystrophin. However, serious adverse events (SAEs) have been reported in patients who received AAV-microdystrophin, which was unforeseen as trial participants are screened for neutralizing antibody (NAb) titers against the AAV capsid and for pre-existing T cell responses against dystrophin. Our goal is to comprehensively and unbiasedly characterize AAV-induced immune responses *in vivo,* to gain insights on AAV-immune interactions. We dosed a dystrophic mouse model with AAV serotype 9 carrying a vector encoding CRISPR/Cas9 and mCherry reporter and performed 10x Genomics single cell RNA-sequencing (scRNA-seq) to analyze immune cell phenotypes that arise after AAV exposure. Three different AAV9-CRISPR/Cas9 doses were tested: 1.2E12vg, 6E12vg, and 1E13vg followed by analysis of PBMCs, pre-AAV administration and 2 weeks post-AAV administration. After a single injection of AAV-CRISPR-mCherry, we observed mCherry positive muscle fibers and a clear shift in phenotype in four main immune cell populations in response to AAV treatment: monocytes, NK cells, B cells, and T cells. Within the T cell population, we detected phenotypic shifts in all subpopulations including CD4+, CD8+, regulatory T cells (Tregs), and gamma delta (γδ)T cells after AAV exposure, regardless of dose. We also tested the effect of two AAV-CRISPR mCherry injections. Upon second exposure to AAV-CRISPR mCherry, mCherry expression was not detected, suggesting that immunological rejection had occurred. Concomitant with rejection was a more profound shift in immune clusters by scRNA-seq (compared to the single injection cohort), and an emergence of new immune cell phenotypes. For example, we detected populations of CD8+ T- and NK-cells with increased CCL5 expression. In addition, we uncovered a phenotypically distinct cluster of activated macrophages and monocytes that emerged after second exposure of AAV, which expressed CXCL2 and C1qa/b/c. Additional analysis and validation studies are needed to identify critical immune cell populations and genes that elicit responses to AAV, and to separate capsid and transgene-specific immune reactions. These studies will enable the identification of new target genes involved in immune responses to AAV.

1208. CpG-Motifs within AAV Vectors Triggers Immune Activation Upon Hepatic Gene Transfer

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Liver-directed gene transfer of coagulation factors (F) VIII or IX offers promising therapies for patients affected with hemophilia. Adeno-associated virus (AAV) vectors are leading candidates for the treatment of hemophilia, and some experimental models have demonstrated safety and efficacy with these vectors. However, the minimal immune responses and persistent transgene expression observed in pre-clinical models have not been representative of clinical trial outcomes. This discrepancy provides a substantial roadblock to further pre-clinical improvement of AAV-mediated hepatic gene transfer. The effect of CpG depletion on induction of innate and adaptive immune responses to a highly immunogenic AAV vector based on serotype AAVrh32.33 was assessed in a mouse model of hepatic gene transfer. CpG-containing AAVrh32.33 vectors were rapidly eliminated from mouse hepatocytes while CpG-depleted AAVrh32.33 vectors achieved sustained transgene product expression. CpG-containing AAVrh32.33 vectors induced maturation of conventional and plasmacytoid dendritic cells in spleens of injected mice, which was linked to stimulation of transgene product-specific T cell responses. Both activation of innate and adaptive immune responses were strongly attenuated upon CpG depletion of the AAV vector. Depletion of mouse CpG motifs from the AAVrh32.33 vector attenuated activation of conventional and plasmacytoid dendritic cells in the spleens of injected mice, significantly reduced the percentage of transgene reactive $CD8^+$ T cells and attendant cytokine secretion, and allowed for sustained expression of the transferred gene. Overall, these results demonstrate that CpG depletion of AAV vector genomes provide an avenue to improve the clinical outcome of AAV-mediated gene transfer by preventing elimination of transgene product expressing hepatocytes by vector-induced T cell responses.

1209. Busulfan Versus TBI Conditioning Regimens Results in Distinct Clonal Patterns Following Rhesus Macaque Autologous Transplantation with Lentivirally-barcoded Hematopoietic Stem and Progenitor Cells

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Conditioning is necessary in preparation for hematopoietic stem cell transplantation (HSCT). In order to have successful engraftment, sufficient myeloablation and immunosuppression is needed to deplete endogenous hematopoietic stem and progenitor cells (HSPCs) in the marrow niche. Historically, total body irradiation (TBI) has been best studied both experimentally and clinically due to potent HSPC ablation and immunosuppressive properties. However, TBI results in significant toxicities, limiting clinical utilization. As an alternative conditioning regimen, the alkylating agent busulfan has been found to be highly effective at clearing HSPC from the marrow for autologous transplantation and HSPC gene therapies. We have previously utilized lentiviral barcoding of HSPCs in a rhesus macaque autologous transplantation model to study clonal dynamics of thousands of HSPC following TBI conditioning (Wu et al, Cell Stem Cell, 2014: Koelle et al Blood, 2017). In the current study, we tracked the hematopoietic reconstitution in this model following busulfan conditioning and compared clonal dynamics to our previous results using TBI, asking

whether the impact of the regimens on the niche changes the pattern of clonal reconstitution. To circumvent potential immuno-rejection, half of the CD34+ cells were transduced with barcoded lentivector library containing no marker transgene, and the other half were labeled with a vector library containing a potentially nonimmunogenic human truncated nerve growth factor receptor (tNGFR) marker. In the first busulfan animal, we observed stable engraftment of tNGFR-transduced granulocytes, B cells and NK cells at levels of 5-8% through 12 months post-transplant. In contrast, T cells had levels of less than 2%, which may due to minimal depletion of endogenous mature T cells with busulfan. In terms of clonal dynamics, we observed initial engraftment with a set of uni-lineage clones, replaced by an initial set of multipotent clones by 2 months post-transplant and a set of additional contributing multipotent clones emerging at 6-9 months. In contrast to our previous results following TBI in a total of over ten animals, with stable contributions from the initial multipotent clones persisting for up to 7 years, many of the first wave of multipotent clones in the busulfan animal disappeared by 9-12 months. In our previous TBI transplants we reported a distinct NK clonal pattern consisting of NK-biased massively expanded clones emerging and waxing and waning over time, suggesting peripheral mature NK self-renewal (Wu et al Sci Imm, 2018). In contrast, the NK clonal pattern following busulfan was polyclonal and correlated with other lineages, without NK-bias. We hypothesize this may be due to limited depletion of endogenous lymphocytes in busulfan conditioning preventing competitive expansion of the newly derived NK clones, and/or lack of CMV reactivation following busulfan versus TBI. We also analyzed the clonal geographic distribution of bone marrow (BM) HSPCs at 3.5- and 6-months post transplantation, which in TBI conditioned monkeys was characterized by asymmetric HSPC distribution in different BM sites for up to two years (Wu et al, JEM, 2018). In the busulfan animal we observed much more rapid clonal mixing, with CD34+ HSPCs clones found on both the left and right as early as 3.5 months. In conclusion, these results suggest busulfan conditioning regimens can variably impact the marrow niche, resulting in marked differences in clonal engraftment patterns, with implications for HSPC gene therapies.

1210. Seroprevalence of Antibodies Against Two AAV Serotype 2 Vectors in Healthy Subjects and Implications for Clinical Gene Therapy Programs

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Adeno-associated virus serotype 2 (AAV2) is the most common vector for gene therapy due to its highly efficient and relatively broad tissue tropism. AAV2 exhibits effective transduction of both smooth and skeletal muscle, neurons, retinal pigment epithelium, hepatocytes, and serotype-specific transduction of kidney cells. A limitation of AAV2 mediated gene therapy is that most humans have been exposed to wild-type AAV2 virus, which frequently generates anti-AAV2 antibodies that may inhibit AAV2 vector transduction. Neutralizing antibodies (NAbs) against AAV2 have been seen as early as within the first three months postnatal. Due to the early and frequent seroprevalence of anti-AAV2 antibodies that may neutralize efficacy of AAV2 mediated gene therapy, it is imperative to have an accurate understanding of pre-existing seroprevalence when designing a preclinical or clinical gene therapy program across demographics. In an effort to develop a clinical assay to detect anti-AAV2 antibodies, 328 healthy human donors were screened using two proprietary AAV2 capsids to generate a seronegative pool for assay validation and sample analysis. An initial reference pool was generated by selecting samples with low assay signal to background noise. Samples from the healthy human donors were then screened by normalizing to the mean signal response of the reference pool used as a negative control on an indirect electrochemiluminescence platform (MSD). An anti-AAV2 antibody was used as a positive control to monitor assay performance. Samples from 260 males and 68 female donors ranging in age from 18 - 82 years were analyzed. Samples with a normalization ratio > 1.5 to a seronegative pool were considered positive for anti-AAV2 antibodies. In total, 78.7% of all samples screened positive and were excluded from the seronegative pool with 78.1% of males and 80.9% of females screening positive. The mean age of positive donors was 40.7 while the mean age of negative donors was 37.7. Males screening positive had a mean age of 39.3 with males screening negative having a mean age of 41.1. Females screening positive had a mean age of 41.8 with females screening negative having a mean age of 29.6. When factoring for self-identified ethnicity, 93.5% of African American females screened positive compared to 83.1% of African American males. Both females and males of Hispanic ethnicity had a 76.5% positive screening rate. Only 55.6% of Caucasian males screened positive. The positive screening rate for Caucasian females was not analyzed due to small sample size in this dataset. Among all samples screening positive, 14.0% exhibited responses > 100-fold of the negative control, 44.2% between 10 and 99.9-fold, and 41.9% between 1.5 and 9.9-fold. A subset of samples were confirmed by using a commercially available AAV2 capsid with > than 95% correlation. This suggests these results accurately reflected general anti AAV2 antibody seroprevalence. These results indicate that the vast majority of adult humans exhibit detectable anti-AAV2 antibodies. Within males, age is a poor indicator of seroprevalence as where females show increasing seroprevalence with age. Ethnicity may be a predictor of seroprevalence. These data do not necessarily predict neutralization capabilities of the antibodies detected, but do demonstrate overall seroprevalence and trends when viewed in context of age, gender, and ethnicity. These factors should be evaluated when designing an AAV2-based gene therapy program to ensure efficacy in the intended demographic.

1211. Effects of Chronic Hepatitis B Virus (HBV) Antigen Exposure on the Epitope-Specificity of CD8+T Cells

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Background: CD8+ T cells are critical for the control of HBV infection. Among patients who fail to clear the virus, the chronic production of HBV antigens causes HBV-specific CD8+ T cells to lose function, become exhausted and eventually die. This exhaustion subsequently hampers the efficacy of HBV-specific therapeutic vaccines in chronically infected patients. **Methods**: We evaluated the epitope specificity of vaccine-induced HBV-specific CD8+ T cells in both control mice and mice intravenously

injected with an AAV8-1.3 HBV vector that produces high and sustained numbers of HBV particles in serum that remain detectable for months. Mice injected with the AAV-1.3HBV vectors, as well as control mice, were immunized with a vaccine expressing a segment of the HBV polymerase gene that was rich in conserved CD8+ T cell epitopes specific for a wide range of human HLA-A haplotypes. The polymerase sequence was genetically fused into herpes simplex virus glycoprotein D which blocks the early T cell checkpoint inhibition triggered by interactions between herpes virus entry mediation (HVEM) on antigen presenting cells and B and T lymphocytes attenuator (BTLA) on T cells thereby increasing and broadening responses to the inserted antigen. **Results**: Vaccination of AAV8-1.3HBV infected mice achieved an ~2 log reduction in circulating HBV genome copy numbers that was sustained for $\geq$ 8 weeks. It also induced a detectable CD8+ T cell response although this response was markedly lower in AAV-1.3HBV-infected than uninfected control mice. Compared to untreated mice, AAV8-1.3 HBV injected animals had a selective loss of CD8+ T cells to immunodominant epitopes accompanied by a preferential expansion of T cells to subdominant epitopes. **Conclusions**: Chronic HBV antigen exposure causes a reduction in T cell responses and a shift in epitopes recognition. These changes should be considered in the design of therapeutic HBV vaccines to ensure optimal immunogenicity in patients since T cell exhaustion will likely result in a lack of memory T cells to the dominant HBV epitopes.

1212. Introduction to Regenerative Macrophages Immunotherapy for Cardiomyopathy with No Treatment Option

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Background: During the past decades, therapeutic immunotherapy has acquired a promising role in regenerative medicine. The application of novel cell therapeutics for the treatment of cardiovascular diseases (CVD) could potentially achieve the ambitious aim of effective cardiac regeneration. The current status of therapy for CVDs is insufficient and the development of safe and efficient alternative treatments is necessary. Nowadays there is an uptrend of immunotherapy as a potential therapeutic method, one of which is called Regenerative Macrophages for the treatment of cardiomyopathy. In RSPAD Gatot Soebroto Presidential Hospital, Jakarta, in the specialized department called the Indonesia Army Cell Cure Center, the authors have started utilizing Regenerative Macrophages vaccines to treat cardiomyopathy that have no other treatment options._The report aims to describe a case in which Regenerative Macrophages vaccine was used to treat cardiomyopathy that had no other option for treatment. **Methods:** Regenerative macrophages of human origin were chosen for developing the Regenerative Macrophages vaccine for this patient. This was done by collecting the blood from the patient, separating the monocytes from other blood component, and treating the monocytes for 4 days. As a result, the monocytes were programmed to be regenerative macrophages. To support the patient's clinical performances, we performed transthoracic echocardiography before immunotherapy and 1 month after immunotherapy. **Results:** The patient did a series of supporting examination such as electrocardiography, chest x-ray and echocardiography. Electrocardiogropahy done [before/ after} treatment showed multiple ventricle extrasystole" Echocardiography prior to treatment showed left ventricular hypertrophy, diastolic dysfunction, left ventricle hypokinesis with low ejection fraction of 44%. After the patient did Regenerative Macrophages vaccines therapy the patient had significant change in their echocardiography left ventricular hypertrophy, norm kinesis, diastolic dysfunction, and the ejection fraction of 56 %. **Conclusions:** The patient with cardiomyopathy that had no treatment options who was treated with immunotherapy by Regenerative Macrophages vaccine showed improved clinical status and echocardiography.

Cell Therapies

1213. Cell-Based Artificial APCs for Efficient Expansion and Lentiviral Transduction of Human T Cells

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Background: Chimeric antigen receptor (CAR)-T cell therapy is an FDA-approved treatment for patients with certain relapsed or refractory B-cell malignancies. Current methods of generating CAR T cells include activation with soluble or bead-bound antibodies followed by transduction with retroviral or lentiviral vectors. Lentiviral vectors are typically pseudotyped with the vesicular stomatitis virus envelope protein (VSV-LV), which confers wide tropism dependent on expression of the low-density lipoprotein receptor (LDLR). We developed an alternative, cell-based aAPC approach for efficient CAR-T manufacturing using K562 cells that have genetically encoded T cell stimulation and are also resistant to lentiviral transduction based on knockout of LDLR. **Methods & Results:** The LDLR was knocked out from the parental K562 cell line using CRISPR/Cas9 gene editing. Two chimeric stimulatory receptors (CSR) were constructed and expressed in the $K562^{LDL\ KO}$ cells: one employing an α-CD3 single-chain variable fragment (scFv) and one with an α-CD28 scFv. Lack of LDLR surface expression, as well as stable expression of α-CD3 and α-CD28 CSR was confirmed by flow cytometry and the newly generated $aAPC^{LDLR\ KO}$ were expanded as single cell clones. Compared to wildtype aAPC ($aAPC^{LDLR\ WT}$), the knockout of LDLR ($aAPC^{LDLR\ KO}$) reduced the APC's susceptibility to infection via VSV-LV significantly (20% vs. 3%). On day 4 of CAR-T expansion the irradiated $aAPC^{LDLR\ KO}$ could no longer be detected in the co-culture. T cell transduction efficiency and total T cell numbers were comparable for $aAPC^{LDLR\ KO}$-expanded and bead-expanded CD19-targeting CAR T cells (CAR-19 T cells). CAR-19 T cells expanded using $aAPC^{LDL\ KO}$ had equivalent *in vitro* effector functions against target cells compared to bead-expanded CAR-19 T cells, including cytotoxic potential, activation and degranulation. In a xenograft mouse model of acute lymphoblastic leukemia, $aAPC^{LDLR\ KO}$-expanded CAR-19 T cells cleared established tumors with kinetics that

were similar to bead-expanded CAR-19 T cells. The level of CAR-19 T cell *in vivo* persistence in the in the bone marrow and spleen of treated mice was similar for both expansion methods. **Conclusion:** Use of aAPC$^{LDL\ KO}$ cells is an attractive approach for CAR-T cell manufacturing that generates CAR-T cells with potent *in vitro* and *in vivo* anti-tumor function. Generation of lentivirally-transduced T cells using aAPC$^{LDL\ KO}$ cells may offer a simpler and more cost-efficient manufacturing procedure than currently available methods.

1214. Generation of a Novel Single Cell-Derived Multi-Engineered Master Pluripotent Cell Line as a Renewable Source of Off-The-Shelf Multi-Antigen-Targeting NK Cell Immunotherapy for Multiple Myeloma

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Adoptive transfer of immune cells expressing chimeric antigen receptor (CAR) has shown great promise in the treatment of various cancers. However, the wide application of current CAR cell therapies is in part hindered by expensive and complex manufacturing and inherent variability in the genetic engineering of patient- and donor-derived immune cells. Induced pluripotent stem cells (iPSCs) are a renewable source for manufacture of homogeneous off-the-shelf immune cell therapies that can be banked and validated in advance. Here, we describe the derivation of FT576, a multi-engineered off-the-shelf CD38- and BCMA-targeting CAR NK cell derived from a clonal master pluripotent cell line for use as an investigational immunotherapy for the treatment of multiple myeloma (MM). FT576 is engineered with four functional modalities: 1) an anti-BCMA CAR for direct MM targeting, 2) IL-15/IL-15 receptor α fusion protein (IL-15RF) for enhanced NK cell persistence, 3) high affinity non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity in combination with monoclonal antibodies (mAbs) including anti-CD38 mAbs, and 4) CD38 deletion for resistance to anti-CD38 mAb induced NK cell depletion and enhanced NK cell function. To generate FT576 master iPSC lines, donor-consented fibroblasts were reprogrammed into iPSCs using a proprietary non-integrating small molecule-driven reprogramming platform. Genetic engineering was performed in two stages. First, cells were engineered with core edits by targeting a gene expression cassette expressing IL-15RF and hnCD16 into the CD38 locus, leading to the uniform expression of inserted genes and a functional CD38 knockout. Single cell-derived clones were characterized for precise CD38 targeting, homogenous expression of IL-15RF and hnCD16, lack of random integration, loss of reprogramming plasmids, and maintenance of a stable pluripotent phenotype. Subsequently, a single, well characterized engineered clonal iPSC line was further transduced with lentivirus expressing the BCMA CAR and subcloned once again. Single cell-derived subclones containing the four engineered edits were evaluated further. Copy number of the BCMA CAR was determined and integration sites are being investigated and mapped. Subclones were evaluated again for pluripotency, genomic stability, differentiation propensity, and anti-tumor functional output. Our data validates the potential of performing sequential engineering and banking of clonal iPSC lines to enable the creation of multiple products that share the same core engineered modalities and the capability to safely and gradually increase the complexity of engineered therapeutic products as needed. The generated master iPSC lines with the four engineered modalities will be used to enable clinical investigation of FT576 as a universal off-the-shelf dual-targeting CAR NK cell immunotherapy to treat MM.

1215. Abstract Withdrawn

1216. Tryptophanyl-tRNA Synthetase (WRS), a Novel Damage-Induced Cytokine, Significantly Increases the Therapeutic Effects of Human Endometrial Stem Cells

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The major challenges of most adult stem cell-based therapies are their low therapeutic effects due to the loss of multilineage differentiation and homing capacities. Therefore, many researchers have recently devoted their efforts to identifying novel stimulating factors that can fundamentally increase the differentiation capacity and homing potential of various types of adult stem cells. Tryptophanyl-tRNA synthetase (WRS) is a highly conserved and ubiquitously expressed enzyme that catalyzes the first step of protein synthesis. In addition to this canonical function, we identified for the first time that WRS is actively released from the site of injury in response to various damage signals both *in vitro* and *in vivo* and then acts as a potent nonenzymatic cytokine that facilitates the self-renewal, migratory, and differentiation capacities of endometrial stem cells to repair damaged tissues. Furthermore, we also found that WRS, through its functional receptor cadherin-6 (CDH-6), activates major prosurvival signaling pathways, such as Akt and ERK1/2 signaling. Our current study will provide novel and unique insights into ways to significantly enhance the therapeutic effects of human endometrial stem cells in various clinical applications.

1217. Novel Autonomously Replicating S/MAR DNA Vectors for the Persistent Genetic Correction of Patient-Derived Stem Cells and Cell Therapy of Choroideremia

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Choroideremia is an X-linked recessive chorioretinal dystrophy caused by mutations in the gene that encodes the Rab Escort Protein 1 (REP1), leading to progressive blindness due to the degeneration of the retinal

pigment epithelium (RPE), photoreceptors, and choroid. The current state of the art genetic treatment for Choroideremia involves the subretinal injection of adeno-associated viral (AAV) vectors encoding REP1 to the RPE of patients. Despite the promising therapeutic outcomes emerging from current clinical trials, the successful clinical translation of AAV vectors for the gene therapy of Choroideremia is tempered not only by a transient transgene expression, but also by significant concerns arising from the adverse effects related to the administration of viral vectors in the patients' retina. Therefore, there is an emerging need for exploring and developing alternative, safer and more efficient gene therapy strategies for the treatment of Choroideremia. In recent years, our research has been focused on the development of non-viral, non-integrating, and autonomously replicating DNA vectors comprising scaffold/matrix attachment region (S/MAR) sequences, for the gene and cell therapy of human diseases, including Choroideremia. This new and promising DNA vector system is cost-effective and easy to produce, comprises entirely clinically approved sequences, replicates autonomously and extrachromasomally in dividing human cells, and does not cause any molecular or genetic damage to the targeted cells, thus ultimately resulting in the efficient, persistent and safe genetic modification of human cells. In this study, we describe the design, development and refinement of an S/MAR DNA vector platform encoding REP1, which we used to genetically correct human induced pluripotent stem cells (hiPSCs) derived from primary fibroblasts of Choroideremia patients. In *in-vitro* experiments, we have initially induced the reprogramming of the aforementioned human primary cells bearing a mutation in the CHM gene into hiPSCs, and successfully demonstrated the passive establishment of the REP1-S/MAR DNA vectors in these cells, without resorting to any drug selection. Remarkably, the genetically modified hiPSCs exhibited a persistent and high-level expression of both a reporter gene and transgenic REP1 for at least two months. The REP1-S/MAR DNA vectors were proven to be established episomally at 1-2 copies per cell in their nuclei, to autonomously replicate upon cell division, and to be rescue intact as plasmid DNA. Importantly, the corrected hiPSCs are isogenic and retain the pluripotent status, as evidenced by the expression of commonly assessed pluripotency markers, and are capable of directly differentiating *in-vitro* into cellular structures and express markers characteristic of the three germ layers. In addition, single-cell RNA sequencing and proteome analysis revealed that the DNA vectors have no significant impact on the molecular and genetic profiles of the cells. Finally, we intend to demonstrate that the REP1-S/MAR DNA vectors and transgene expression are maintained during the directed differentiation of the hiPSCs into RPE cells and retinal organoids, ultimately envisioned for retinal transplantation. We firmly believe that these findings represent a major breakthrough in the scientific fields of stem cell research and human gene therapy, thus paving the way for the further development of innovative cell therapy strategies for the treatment of Choroideremia and other genetic diseases.

1218. Improved Cellular Maintenance of hPSCs in a Xeno-Free, Serum-Free Culture System for Therapeutic Applications

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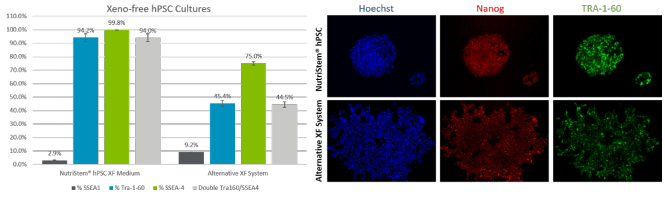
Introduction: As the desire to transition from bench to bedside becomes more prevalent, there is a growing need for higher-quality, clinically relevant culture methods. Currently, the recommendation for transitioning pluripotent stem cell research to clinic is to maintain cells in xeno-free (XF) culture conditions. However, determining the ideal XF culture condition for pluripotent cells remains challenging for a variety of technical and biological reasons. Of particular difficultly is establishing a supportive growth media that can enable scalable, long-term culture while maintaining cellular stability. Additionally, as culture systems become more defined, expected cell health and pluripotency maintenance may be compromised. **Methods:** Human iPSCs were cultured for five passages in multiple conditions utilizing either completely XF, clinically relevant culture media on a XF substrate, or conventional hPSC media on an animal-derived substrate. Cells from all culture conditions were then transitioned to the most supportive XF media while examining the cells' ability to adapt from traditional, poorly defined, animal-containing media to a cleaner, more clinically applicable condition. Throughout the duration of these cultures, analyses were performed to determine cellular pluripotency and stability, including marker expression and fluorescent imaging. **Results:** Cells cultured in the serum-free NutriStem® XF hPSC Medium seeded on vitronectin exhibited high levels of pluripotency markers TRA-1-60 and SSEA-4, as well as low levels of SSEA-1 as determined through FACs analysis. Conversely, cells cultured in the alternative XF culture system were found to exhibit lower levels of TRA-1-60 and SSEA-4 with higher expression of SSEA-1. While representative ICC staining for Hoechst, Nanog, and TRA-1-60 further confirmed the pluripotency of NutriStem hPSC cultures, cell colonies in the alternative XF system displayed atypical morphology and irregular, less intense staining (Figure 1). Finally, regardless of the original medium formulation, substrate, or cell stemness, each group tested could be successfully transitioned to NutriStem hPSC XF Medium and maintain all expected hPSC characteristics.



Conclusions: These studies determined that 1) serum-free, xeno-free culture methods exist that can effortlessly maintain pluripotency and cellular stability but not all systems are made equally, 2) transitions from standard culture conditions to clinically relevant ones can be seamlessly achieved with minor adjustments facilitating long-term success, and 3) more streamlined culture systems can easily support the maintenance of key hPSC qualities, for over 3 months in culture, essential for the downstream differentiation and future application of hPSCs.

1219. Gene Modified CD34+ Cells With Increased ALDH1 Expression Confers *In Vitro* Protection Against Cyclophosphamide

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Background: It has been demonstrated that increasing intracellular levels of ALDH1 confers resistance to cyclophosphamide (CY). This finding was originally used to increase doses of CY in oncology therapies. We hypothesize that low, non-myeloablative, doses of CY could be used for *in vitro* or *in vivo* selection of cells genetically modified to over-express ALDH1 plus additional genetic modifications that are known to promote a non-disease state, e.g. potentially to treat or cure HIV. We evaluated a lentiviral vector that incorporates a triple cassette to overexpress ALDH1, knockdown CCR5, a key HIV co-receptor (shR5) and an HIV fusions inhibitor C-peptide (C44), to produce cells that are potentially resistant to both HIV infection and CY. **Methods:** A CD34+ TF-1a cell line was transduced with a lentivirus vector with a ALDH1a cassette only (LV800), or transduced with a triple cassette lentivirus vector (ALDH1a/shR5/C44). Chemoprotection experiments were performed at 48, 72 and 96 hours, and with varied doses (0-50μM) of mafosfamide (MFA). MFA is an analogue of CY that is more stable and does not require liver enzymes, thus suitable for *in vitro* studies. The viability of treated cells was measured with Propidium Iodide (PI), or Annexin V with 7-AAD staining protocols. ALDH1a activity was evaluated with flow cytometry AldeFluor assay. Cell viability, total live cell count, and AldeFluor fluorescence level were analyzed using nucleocounter NC-200 and flow cytometry. **Results:** Comparable cell viability was found between LV800 and LV801 transduced cells following administration of mafosfamide *in vitro* (Fig 1A). However, LV800 transduced cells exhibited higher levels of AldeFluor activity (60%-70%) than LV801 transduced cells (~30%) (Fig 1B). Additionally, LV800 and LV801-transduced cells exhibited 2.5-3.5fold higher total viable cells compared to untransduced cells with a treatment concentration of 3.12μM MFA (Fig 2). The killing rate across groups equalized at 12.5μM MFA. Furthermore, we observed comparable viability data between both PI and Annexin-V with 7-AAD staining systems. The results from similar experiments with primary human CD34+ cells will be presented.

Fig 1A

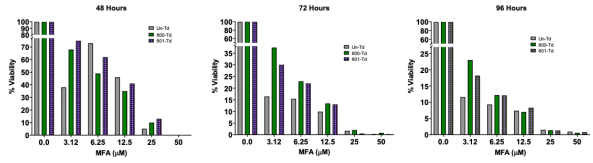


Fig 1B

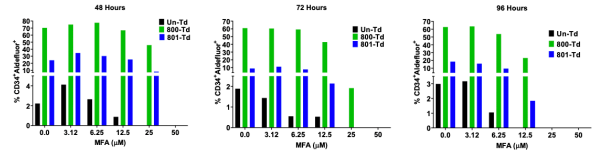
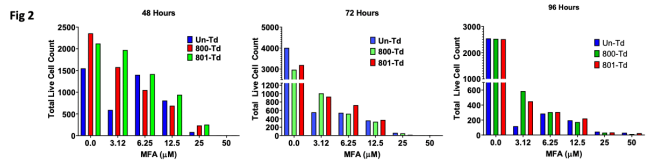


Fig 2



Conclusion: Although, as expected, ALDH1 expression was substantial in CD34+ cells transduced with both single and triple cassette vectors, the higher levels seen with the single cassette vector was likely due to the smaller size of the vector. However, the chemoprotection to CY was similar, suggesting that even relatively lower increases in ALDH1 was sufficient. The chemoprotective observed effects could be exploited in clinical interventions to increase *in vivo* engraftment of gene-modified cells potentially allowing for effective non-myeloablative treatment or cure of disease with known genetic mutations that confer a non-disease state, e.g. HIV infection.

1220. Induced Neurospheres as a Novel Drug Delivery Vehicle to Treat Breast Cancer Brain Metastasis with Intracerebroventricular Injection

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Background: Breast cancer is the deadliest cancer in women in the US as 10% - 15% of late-stage breast cancer patients develop brain metastasis. Common treatment strategies such as radiation therapy and surgical resection ultimately fail due to the invasive, migratory nature of the tumors. Studies have shown that neural stem cells possess innate tumor tropism. We recently discovered that transdifferentiation, a process that transforms somatic cells into other adult cell types, creates tumor-homing drug carriers capable of homing to tumors and delivering payloads that suppress tumor progression. In this study, we sought to advance this platform by generating induced neurospheres and investigated their tumor homing capability in the brain following intracerebroventricular administration. **Methods:** Induced neurospheres were generated by transducing human fibroblasts with lentiviral *Sox2,* followed by serially passaging the cells in stem cell media for 5 weeks. *In vitro* immunohistochemistry staining was performed to assess *Sox2* and *Nestin* expression levels in induced neurospheres. The relative expression level of known cell surface receptor genes related to neural stem cell tumor tropism was measured by qRT-PCR. An *in vitro* EVOS live cell migration assay was used to evaluate the migration of induced neurospheres towards MB231-Br tumor cells over 72 hours. Athymic nude mice were inoculated with fluorescent- and bioluminescent-labeled MB231-Br cells by intracranial injection. After 7 days, induced neurospheres labeled with fluorescent and bioluminescent markers were administered by intracerebroventricular injection. In order to investigate the tumor tropism of the induced neurospheres, mouse brains were harvested on days 3, 7, and 14 for sectioning. The persistence of induced neurospheres was investigated by injecting fluorescent- and bioluminescent-labeled induced neurospheres into the lateral ventricle of athymic nude

mice and measuring bioluminescence signal over time. **Results:** Immunohistochemistry analysis showed positive staining results for *Sox2* and *Nestin*, indicating that induced neurospheres possessed neural stem cell properties. qRT-PCR showed that induced neurospheres had significant increases in CXCR4, VEGFR1, IL6R, HGFR, and uPAR expression compared to human fibroblasts. Real-time single cell migration tracking analysis showed that induced neurospheres were able to directionally migrate towards MB231-Br cells *in vitro*. After intracerebroventricular injection, post-mortem histology showed the presence of induced neurospheres co-localized with the tumor as early as Day 3, and non-invasive kinetic imaging showed that induced neurospheres were able to persist in the mouse brain for as long as 50 days. **Conclusions:** These data suggest that induced neurospheres possess the migratory properties of endogenous neural stem cells. The tumor tropism and persistence of induced neurospheres following intracerebroventricular suggest that induced neurospheres can be a promising drug delivery vehicle for the treatment of brain metastases.

1221. Hope Biosciences Autologous, Adipose-Derived Mesenchymal Stem Cells for Adults with Active Rheumatoid Arthritis: Clinical Results

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The primary objective of this phase 1/2a clinical trial was to evaluate the safety of a single IV infusion of Hope Biosciences autologous, adipose-derived mesenchymal stem cells (HB-adMSCs) in 15 subjects with active Rheumatoid Arthritis (RA). Safety was assessed by the number and frequency of adverse events, and secondary organ injury. The secondary objective was to determine the efficacy of HB-adMSCs to alter RA-related inflammation, measured by swollen and painful joint counts, TNF-a, IL-6, CRP, and ESR. Subjects were evaluated 24hrs, 1, 4, 8, 12, 26, and 52 weeks post-infusion. All results included here are part of an interim analysis.There were no serious adverse events related to the study, and organ function was preserved, with all clinical laboratory results demonstrating static or improved values. Swollen/painful joints and markers of inflammation significantly decreased at 4, 12, and 26 weeks post-infusion. These results indicate a single IV infusion of autologous HB-adMSCs is safe and effective for adults with active RA.

1222. Repair of Retinal Degeneration Following Ex Vivo Minicircle DNA Gene Therapy and Transplantation of Corrected Photoreceptor Progenitors

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We describe retinal reconstruction and restoration of visual function in heritably blind mice missing the rhodopsin gene using a novel method of ex-vivo gene therapy and cell transplantation. Photoreceptor precursors with the same chromosomal genetic mutation, were treated ex vivo using minicircle DNA, a non-viral technique that does not present the packaging limitations of adeno-associated virus (AAV) vectors. Following transplantation, genetically-modified cells reconstructed a functional retina and supported vision in blind mice harboring the same founder gene mutation. Gene delivery by Minicircles, showed comparable long-term efficiency to AAV in delivering the missing gene, representing the first non-viral system for robust treatment of photoreceptors. This important proof-of-concept finding provides an innovative convergence of cell and gene therapies for the treatment of hereditary neurodegenerative disease and may be applied in future studies towards ex vivo correction of patient-specific cells to provide an autologous source of tissue to replace lost photoreceptors in inherited retinal blindness. This is the first report using minicircles in photoreceptor progenitors and the first to transplant corrected photoreceptor precursors to restore vision in blind animals.

1223. Rapid and Automated Isolation of White Blood Cells from Leukopaks Suitable for Cell Therapies, Using the Curate™ Cell Processing System

Alison Skelley, Yasna Behrmardi, Khushroo Gandhi, Roberto Campos-Gonzalez, Jason Walsh, Tony Ward

GPB Scientific, Vista, CA

CAR-T autologous cell therapies are delivering impressive results in the clinic. However, there are still significant challenges impeding rapid scale-up of therapeutic production to allow broad adoption. Two of the major challenges are the inconsistency in harvesting enough high-quality cells for transduction and the lengthy and laborious time to achieve dose. We previously presented a microfluidic solution using deterministic-lateral displacement (DLD) to effectively isolate and separate cells with the highest recoveries, no loss of a particular subtype, and no cell damage. We demonstrated significantly improved CD3/CD28 magnetic bead activation and expansion of DLD-prepared T-cells compared to Ficoll,

which when combined with higher initial WBC isolation efficiency led to >2-fold production of T-central memory cells from leukoreduction samples. Further, we achieved wash efficiencies of more than 3 log, as demonstrated by the removal of viral particles and soluble proteins. We have now further scaled that DLD technology into a highly-parallelized, disposable, closed fluid path, and automated platform, named the Curate™ Cell Processing System. Our initial studies demonstrate >96% WBC recovery, >99.9% removal of RBCs, and >99% removal of PLTs from undiluted leukopak samples. With a hands-on time of only 10min, the Curate system significantly reduces the time needed for processing leukopaks as compared with centrifugation and elutriation methods, being able to process a full leukopak (200-300 mL containing 0.5-12x10^9 total WBC) within 30-40min. The DLD microfluidic device can be used to achieve up to 30-fold increased concentration of the cells without requiring pelleting. The unique action of Curate's DLD enables processing the WBC fraction directly into more viscous media (*e.g.*, CS-10 cryopreservation media) during the debulking process, facilitating point of collection processing and freezing for subsequent transport to a cell processing facility. Further, following receipt, the thawed leukopak can be processed directly into cell culture or other media suitable for downstream manufacturing of CAR-T cells, again using the same instrumentation and disposable device solution. The closed-system, single-use cassette within the Curate system is a walk-away solution that also eliminates cleaning steps and avoids the potential contamination between samples. The compact nature and minimal hands-on time (<10 min) significantly reduce the intense performance and labor challenges in the manufacturing of medicines for cell therapy. Thus, GPB Scientific's Curate Cell Processing System enables a significant breakthrough in CAR-T processing.

1224. Human Vascular Networks Genetically Engineered to Overexpress and Release Full-Length Coagulation Factor VIII into the Bloodstream Restore Hemostasis in a Mouse Model of Hemophilia A

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Hemophilia A is an inherited bleeding disorder characterized by improperly folded coagulation factor VIII (FVIII) due to a defective F8 gene. Current treatments are expensive and involve repeated intravenous infusions of FVIII concentrates throughout the life of the patient causing tremendous discomfort and morbidity. In response to these limitations, we devised an approach to use patients' own cells to bioengineer a vascular network that could serve as a drug delivery system capable of sustained FVIII release into the bloodstream. To this end, we first isolated human endothelial colony-forming cells (ECFCs) from cord blood. These ECFCs were then transfected with a PiggyBac transposon system that inserted multiple copies human F8. The use of PiggyBac was critical particularly for its ability to insert the full-length sequence of F8, which remains too large (~7 kb) for conventional AAV and lentiviral mediated gene editing. We validated proper F8 gene insertion and protein expression in the modified ECFCs, which retained their inherent ability to form perfused vessels inside a hydrogel upon subcutaneous implantation in immunodeficient mice. Importantly, following implantation into immunodeficient f8-ko (hemophilic) mice, we demonstrated that our implants were able to significantly increase the levels of functional coagulation FVIII in blood plasma and to effectively restore blood coagulation in a tail-tip bleeding assay. Untreated mice or mice with implants containing unmodified ECFCs served as controls and were not able to restore hemostasis. We anticipate this PiggyBac mediated ex vivo gene therapy could become an alternative autologous cell treatment for sustained release of FVIII in hemophilia A.

1225. A Platform for the Generation of High-Quality Clonally-Derived Induced Pluripotent Stem Cell Lines for the Mass Production of Off-The-Shelf Cellular Therapeutics

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Cell therapy holds great promise for many diseases including cancer, diabetes, and neurological and cardiac diseases. However, cell therapies based on primary cells, especially if engineered, are hindered by limited proliferation capacity, donor to donor variability, poor and heterogenous engineering processes, costly and complex manufacturing and limited patient access. Induced pluripotent stem cells (iPSCs) have the unique potential to self-renew indefinitely and to differentiate into all cell types of the body and could serve as a renewable source of engineered products for cell therapies. Here we describe the development of a comprehensive platform for the generation, engineering and cryopreservation of high-quality clonal iPSC lines streamlined for clinical applications. For generation of footprint-free iPSCs, we evaluated multiple expression systems (Sendai viruses (SeVs), episomal plasmids and CRISPR activation system (CRISPRa)) for the delivery of reprogramming factors in diverse cell types (fibroblasts, adipocytes, keratinocytes and hematopoietic cells). The use of SeVs led to the highest reprogramming efficiency and fastest kinetics (e.g. 85.3% T cell-derived iPSCs on day 8), but the fraction of iPSCs decreased over time (28.3% iPSCs on day 26). Reprogramming with episomal systems or CRISPRa was markedly less efficient and slower but the reprogrammed cultures were more stable and the iPSC fraction continued to increase over time. There was significant donor-to-donor variability in the reprogramming efficiency of all cell types tested and methods used, with some donors showing resistance to plasmid-based reprogramming. Regardless of the variability observed using various reprogramming strategies, the process of single cell sorting and iPSC selection, resulted in the derivation of multiple clones shown to be bona fide iPSCs. To further mitigate donor variability and deficiencies of plasmid-based methods, optimized stage-specific media supplemented with small molecules were used to enhance the reprogramming efficiency and induce a naïve state of pluripotency. This led to enhanced reprogramming efficiency regardless of the expression method or the starting cell type and allowed for the use of transient plasmid-based methods and reduced set of reprogramming factors including elimination of oncogenes use in the reprogramming process. The use of the stage-specific media enabled

non-integrating and feeder cell-free generation of naïve iPSC lines with unique advantages. Global gene expression studies of generated naïve iPSC lines revealed enhanced expression of pluripotency and naïve gene networks and reduced spontaneous differentiation. The unbiased and naïve pluripotent phenotype allowed for highly efficient trilineage differentiation and specifically, large scale differentiation into homogenous NK and T cell populations. In contrast to the marked poor survival following single cell dissociation of iPSCs derived by conventional methods, we observed enhanced recovery and improved clonogenicity of iPSCs cultured in our media. Engineering (sequentially or concurrently) and cryopreservation of iPSCs were also improved. The single cell-derived and cultured iPSC clones maintained long-term genomic stability (tested by karyology and genome-wide microarray analysis) after multiple rounds of engineering and cryopreservation. Our data overall describe improvements in the generation, single cell maintenance, engineering, differentiation and cryopreservation of footprint-free iPSCs for use in regenerative medicine and off-the-shelf adoptive cell therapy.

1226. The Application of High Content Omics for Comparability Testing of an Allogeneic Cell Therapy

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Allogeneic cell therapies derived from primary cell sources often have a finite specification for expansion before the cells become exhausted or their phenotype changes due to climatization to the culture process. Consequently, these products may require the generation of new master cells banks to maintain clinical and/or commercial supply. The use of new master cell banks requires in-depth product knowledge and a comprehensive comparability study to ensure that any differences in quality attributes have no adverse impact on the safety or efficacy of the drug product. In this study we demonstrate an approach for product comparability testing of an allogenic therapy in Phase I/IIa clinical trials for the treatment of retinitis pigmentosa. The therapy is centred on the ability of foetal derived human retinal progenitor cells (hRPCs) to differentiate into mature photoreceptors while also establishing a neuroprotective microenvironment. Current strategies to maintain supply of the therapy for on-going clinical analysis include an increase in the passage level of the drug substance and the derivation of new master cell banks. Both approaches require comparability assessment as outlined in guidance documents such as ICH 5QE. Three new GMP master cell banks from primary tissue sources were established for the hRP cells. We then performed an extensive omic characterisation of the product (cell surface markers, proteomics, RNA-sequencing), and identified a panel of markers that may be associated with function, maintenance and regenerative characteristics of the cells. This panel was used to assess changes in gene expression at higher cell passages and across different donors. Analysis with multiple independent replicates demonstrated that the expression of key markers remained similar up to 6 additional passages, while only minor changes were observed in the new master cell banks. This similarity was also assessed using single-cell RNA-sequencing, a method which could potentially be used as a predictor of product potency. This omics-based approach provides a comprehensive characterisation of the current hRPC therapy product. It also allows an assessment of the impact of changes to both the manufacturing process and the cellular starting material. This work sets crucial groundwork for selecting critical quality attributes of the therapy and enables routine testing of new sources of hRPC material ensuring continued supply of the product through to commercialization.

1227. A NICE: Neoantigen-Cytokine-Chemokine Multifunctional Engager for NK Cell Immunotherapy of Solid Tumors

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The effectiveness of natural killer cell-based immunotherapy against solid tumors is limited by the lack of specific antigens and the immunosuppressive tumor microenvironment. To improve the clinical efficacy of NK cell therapy, we are designing, developing, and characterizing a new generation of multi-specific killer engagers, which consists of a neoantigen-targeting moiety, together with cytokine and chemokine-producing domains. Neoantigens are new antigens formed specifically in tumor cells due to genome mutations, making them highly specific tools to target tumor cells. We evaluated the responsiveness of NK cells to Wilms Tumor 1 (WT1) antigen in GBM by synthesizing an antibody that is able to block the WT1-HLA complex interaction. Incorporation of cytokine (namely IL-2, IL-15, and IL-21), which is essential for the maturation, persistence, and expansion in vivo, favors the proliferation and survival of NK cells in the tumor microenvironment, thereby leading to their enhanced clinical efficacy. Additionally, our previous data indicated that chemokines play an important role in the infiltration of immune cells into GBM, including CCL5 and CXCL10. Incorporation of a chemokine-producing element, which can secrete chemokines otherwise not present in GBM, but to which NK cells respond, into our construct further supports such NK cell behavior and may stimulate the recruitment of other immune cells. In summary, we are generating a novel multi-functional NK cell engager, combining neoantigen-cytokine-chemokine elements fused to an activating domain specific to NK cells, and investigating its ability to support and enhance NK cell-mediated cytotoxicity against solid tumors in vitro and in vivo. We hypothesize that taking advantage of our multi-functional engager, NK cells will exhibit superior ex vivo expansion, infiltration and antitumor activity in the treatment of GBM and other solid tumors.

1228. Non-Integrating Measles Virus Vector is a Promising Tool for Naive iPSCs Generation and T-cell Engineering

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Development of regenerative therapy using viral vector is expected to open new medicine. Currently both of retroviral or lentiviral vectors are considered to be very useful in this field. Particularly to establish induced pluripotent stem cells (iPSCs) both of the vectors have long been used. Both of the viral vector, however, have potential risk of insertional mutagenesis. To resolve these problems induced by using retroviral or lentiviral vectors, we have developed a novel vector based on non-integrating RNA viruses. Among many RNA viruses, we focused on measles virus because of its high infectivity to hematopoietic progenitor cells (HPC) and T cells even in the resting state, and rapid gene expression after infection without chromosome integration. Currently, Sendai virus vectors (SVs), which is also a *Paramyxoviridae* virus-based vector, are widely used for gene delivery into hematopoietic cells and establishing induced pluripotent stem cells (iPSCs), but the transduction to unstimulated T cells (UTs) is still challenging for SVs. We, therefore, established a new measles virus vector (MV) by modifying H gene and deleting F gene to eliminate cell membrane fusion-associated cytotoxicity. To determine if our MV is superior to SV in gene transduction of UTs, we examined gene transduction efficiency of these vectors. As expected, Our MVs allowed more efficient gene transduction to various hematopoietic cells including UTs and B cells than SVs. In addition, compared with SV-transduced cells, there was less apoptosis in MV-transduced cells probably due to rapid decrease of viral RNA after transduction into those cells.We also compared iPSCs generation efficiencies between MVs and SVs. We could establish iPSCs from UTs with MVs harboring reprogramming genes 50 times more efficiently than SVs harboring the same reprogramming genes. Gene expression profile of MV-induced iPSCs derived from $CD3^+$ T cells (MV-T-iPSCs) were similar to regular human pluripotent stem cells (hPSCs: embryonic stem cells and iPSCs), which are in primed state, in morphology and pluripotency. On the other hand, MV-induced iPSCs derived from $CD34^+$hematopoietic progenitor cells (MV-HPC-iPSCs) formed dome-shaped colonies and showed gene expression profiles close to naive iPSCs, which are more pluripotent than primed iPSCs (Figure). After culturing in primed induction condition, the MV-HPC-iPSCs showed primed-like features. Moreover, whole genome bisulfite sequencing analysis showed that MV-HPC-iPSCs had lower methylation than primed MV-HPC-iPSCs. These results strongly suggested that we could establish naïve iPSCs directly from HPCs by MVs without taking primed state. In conclusion, our MVs would be a promising tool for developing new regenerative medicine.

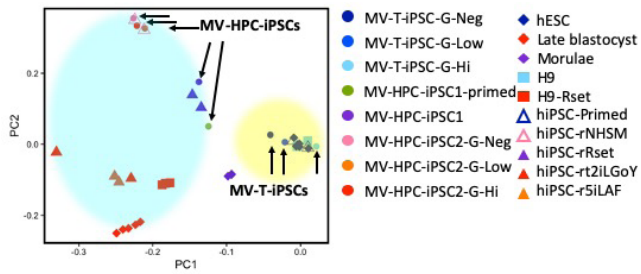

Figure. Principal component analysis of gene expression profiling: MV-HPC-iPSCs and MV-T-iPSCs shared characteristics of naive and primed pluripotent stem cells, respectively.

1229. Neural Induction Enhances the Engraftment of Mesenchymal Stem Cells in a Rat Stroke Model

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Stem cell therapy is a promising strategy for treating neurological diseases but its effectiveness is influenced by the route of administration and the characteristics of the stem cells. We determined whether neural induction of mesenchymal stem cells (MSCs) was beneficial when the cells were delivered intra-arterially through the carotid artery. MSCs were neurally induced using a retroviral vector expressing the neurogenic transcription factor neurogenin-1 (Ngn1). Ischemic stroke was induced by transluminal occlusion of the middle cerebral artery and 3 days later the MSCs were delivered intra-arterially through the internal carotid artery. Magnetic resonance imaging analysis indicated that compared to MSCs, MSCs expressing Ngn1 (MSCs/Ngn1) exhibited increased recruitment to the ischemic region and populated this area for a longer duration. Immunohistochemical analysis indicated that compared to MSCs, MSCs/Ngn1 more effectively alleviated neurological dysfunction by blocking secondary damage associated with neuronal cell death and brain inflammation. Microarray and real-time PCR analysis indicated that MSCs/Ngn1 exhibited increased expression of chemotactic cytokine receptors, adherence to endothelial cells, and migration ability. Neural induction with Ngn1 increases the homing ability of MSCs, enhancing their engraftment efficiency in the ischemic rat brain. Intra-arterial delivery of neurally induced MSCs/Ngn1 3 days after ischemic injury blocks neuronal cell death and inflammation, and improves functional recovery. Thus, intra-arterial administration of stem cells with neural properties may be a novel therapy for the treatment of ischemic stroke

1230. Development of an Off-The-Shelf Therapeutic Nk Cell Product Generated from Cord Blood Cd34+ Cells

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Background: Natural killer (NK) cells are a critical component of the innate immune system's toolbox in fighting cancer, killing

tumor cells without priming or prior activation through their complement of activating and inhibitory surface molecules. In comparison to T cell therapies, NK cells are emerging as a safer, more clinically accessible and cost effective allogeneic cellular therapy as they do not cause graft versus host disease or cytokine release syndrome. We are developing an allogeneic, cryopreserved, off-the-shelf NK cell product for the treatment of hematological malignancies. Our NK cell product is generated from cord blood derived CD34+ cells through a scalable and cost-effective 2-phase manufacturing process of (1) expansion and progenitor cell priming on a proprietary Notch ligand cell culture platform followed by (2) NK cell differentiation that is animal component- and feeder cell-free. Cells in the final product are characterized by immune phenotyping, cytokine expression, RNA sequencing, and functionality by in vitro cytotoxicity against a panel of different AML tumor targets, and anti-tumor activity is being evaluated in a mouse AML tumor model. **Results and Discussion:** A commercially and clinically viable number of NK cell doses per manufacturing run can be produced using this scalable platform with ~1500 NK cells generated per starting CD34+ cell. The expression profile of the NK cells is indicative of a highly active NK cell phenotype expressing a range of cell markers associated with functional cytotoxic NK cells, including CD16, NKp46, perforin, and granzyme B. RNA sequencing analysis has shown a similar cytotoxic gene signature as observed in adult NK cells. Proinflammatory cytokines are secreted in response to tumor target cells which may help to recruit host immune cells for further anti-tumor activity. Potent in vitro cytotoxicity has been observed across several AML tumor target cells, and AML xenograft studies are currently underway. These cells also retain their functional capacity with good cell recovery and viability post-cryopreservation, as demonstrated through in vitro cytotoxicity assays. The ability to scale up the manufacturing process to reduce costs of goods and improve lot-to-lot consistency, and to cryopreserve these cells and retain their functional capacity post-thaw, allows for generation of a truly off-the-shelf therapeutic NK cell product. Furthermore, this product has the capacity to be modified through genetic engineering at early stages of the manufacturing process to generate therapeutic cells targeted for specific indications through CAR expression or gene editing, for example. We are investigating opportunities to modify these cells, and are planning to initiate clinical trials using the unmodified cell product to treat patients with hematologic malignancies to assess the safety and potential efficacy of the product.

1231. Assessment of Factors that May Contribute to CRS Development During CAR-T Cell Therapy

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Chimeric antigen receptor (CAR) T cell therapies have great potential to treat many human diseases; however, inflammatory toxicities such as cytokine release syndrome (CRS) and neurotoxicity associated with CAR-T cell therapies have limited its potential. These toxicities can be highly variable between patients, which range from low-grade fever to severe inflammation resulting into death. To mitigate inflammatory toxicities, immunosuppressive drugs such as corticosteroids and Tocilizumab are commonly used; however, these drugs have heterogenous response in patients, can affect CAR-T cell function and may not be effective in all cases. More importantly, inflammatory cytokines such as IL-6 and IL-1 that are associated with CRS are not primarily produced by CAR-T cells but by myeloid cells. The mechanisms by which myeloid cells are activated during CAR-T cell therapy are poorly understood. Previous studies have found that GM-CSF secreted by activated CAR-T cells activate myeloid cells; however, GM-CSF neutralization does not completely prevent CAR-T cell associated inflammation in animal models suggesting presence of additional factors that contribute to CRS development. Consistent with this observation, we found that neutralization of GM-CSF released by activated primary human T cells does not prevent myeloid cell activation in all donors tested (N=7). Furthermore, we found that cell-free supernatant obtained from activated human T cell line (Jurkat) that did not contain detectable level of GM-CSF potently activated THP-1 myeloid cells. Using size exclusion columns, we found that a fraction within the size range 50-300 kDa activated THP-1 cells. Additionally, we found that cell-free supernatant obtained from another activated human cell line (HuT78) contained GM-CSF; however, GM-CSF neutralization by antibodies did not completely prevent THP-1 cell activation. Interestingly, exosomes obtained from activated Jurkat cells also activated THP-1 cells suggesting these factors may be also present in exosomes. Together, these data suggest that soluble factors other than GM-CSF released by CAR-T cells can activate myeloid cells and contribute to CRS development. Current studies are underway to identify these factors. Understanding mechanisms by which CAR-T cells activate bystander immune cells may aid to design novel CAR-T cells that are less inflammatory and/or develop better treatment protocols to manage CRS. Additionally, these studies may also aid to identify critical quality attributes (CQAs) to predict inflammatory toxicities during CAR-T cell therapy.

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1232. Abstract Withdrawn

1233. Human Stem Cell Derived Exosomes: Functional Studies Ex Vivo and In Vivo

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Extracellular vesicles (EVs), including exosomes and microvesicles, mediate intercellular communications and exert various biological activities via delivering unique cargos of functional molecules such as proteins to recipient cells. These membrane- enclosed but nuclei-free EVs are stable for weeks at 4oC and survive quite well after electroporation, unlike their parental mammalian cells. It is widely

considered that EVs produced from various cell types have different cellular contents that include membrane and cytosolic proteins, RNA, mRNA and metabolites. Therefore, it is of great interest to examine in details EVs derived from various types of human stem cells. Previous studies showed that EVs produced and secreted by human mesenchymal stem cells (MSCs) can substitute intact MSCs for tissue repair and regeneration. In this study, we examined properties and functions of EVs from human induced pluripotent stem cells (iPSCs) that can be cultured infinitely under a chemically defined medium free of any exogenous EVs. We collected and purified EVs secreted by human iPSCs and MSCs. Purified EVs produced by both stem cell types have similar sizes (~150 nm in diameter), but human iPSCs produced EVs 16-fold more efficiently than MSCs. When highly purified iPSC-EVs were applied in culture to senescent MSCs that have elevated reactive oxygen species (ROS), they reduced cellular ROS levels and alleviated aging phenotypes of senescent MSCs in 3 different aging models. Our discovery reveals that EVs from human stem cells can alleviate cellular aging in culture, at least in part by delivering intracellular peroxiredoxin enzymes that reduce intercellular ROS levels in recipient cells. We also tested whether human iPSC-derived EVs exert reparative or regenerative capacity in vivo after tissue injuries. The highly purified EVs were electroporated with SPIO magnetic particles. The SPIO-containing EVs were assessed by MRI in vivo after iv injection in 3 animal models with acute injuries, revealing that iPSC-EVs can selectively home to the injury sites and confer substantial improvement in treated mice. Our studies provide evidence that EVs from human iPSCs may represent a novel form of cellular therapy (without the use of intact cells) for treating aging/degenerative diseases and acute tissue injuries. We also describe a novel approach to track EVs by non-invasive MRI to assess in vivo whole-body distribution that helps optimize further development of EV-based cellular therapy.

Vector and Cell Engineering, Production or Manufacturing

1234. Development of Robust Quality Control Assays for Non-Clinical rAAV

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The University of Massachusetts Medical School (UMMS) and GE Healthcare Life Sciences have established a new large-scale viral vector manufacturing facility with the intention to provide high-quality recombinant adeno-associated virus (rAAV) vectors in quantities sufficient to support large-animal and high dose preclinical research. The facility, housed on the Worcester campus of UMMS, will alleviate a bottleneck that delays the start of some preclinical research. This facility was established by two of the originators of the Sf9 / baculovirus platform with extensive experience developing and optimizing the platform. The UMMS facility employs state-of-the-art upstream equipment including two Xcellerex 200 liter (XDR200) single-use bioteractors and downstream processing equipment (AKTA PILOT, AKTA FLEX). The instrumentation underwent IQ/OQ/PQ validation that will facilitate the technology transfer to clients for use in cGMP CDMO such as MassBiologics or other client specified manufacturers. We will also provide extensive QC and analytical services to ensure quality and reproducibility of the vector and process. The analytical services utilize droplet digital PCR, dynamic light scattering, and isopycnic density gradient. Several examples of the analytics are presented, such as physical characteristics of rAAV, full/empty ratio, capsid protein ratio, and genome size and heterogeneity based on assays developed using the QX200 ddPCR from BioRad and the Maurice CE-SDS from Protein Simple.

1235. Abstract Withdrawn

1236. Public Engagement in Novel Gene Therapies for Hemophilia and Blood Disorders: A Computational Analysis of Public Sentiments in Social Media

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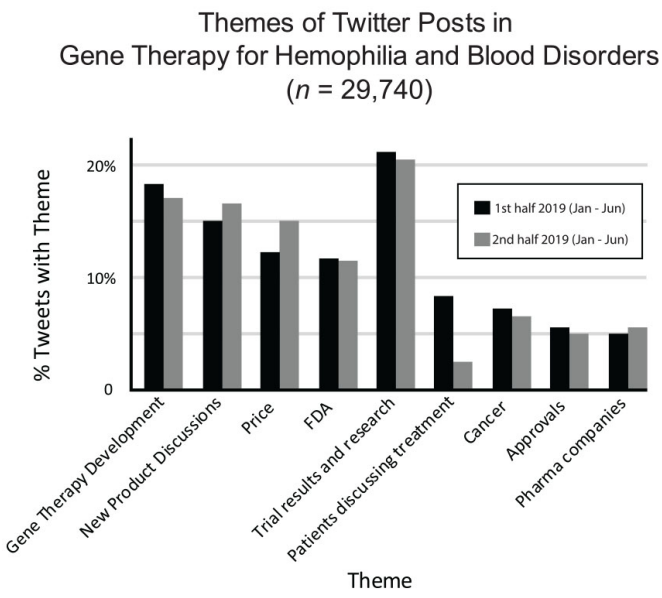
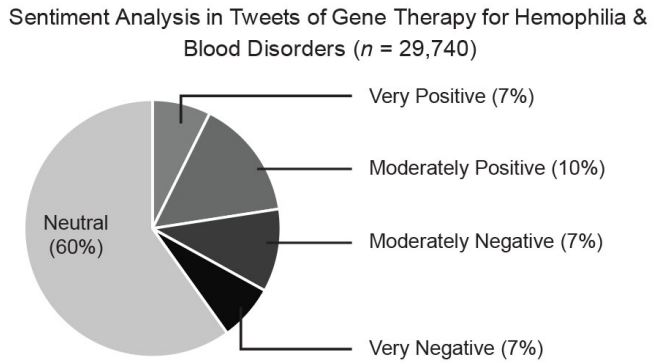
BACKGROUND: An increasing number of gene therapy products for hemophilia and blood disorder products are moving into the clinic, generating engagement from patients and the public. Public sentiment affects not only willingness to participate in gene therapy trials, but also patient advocacy, policy making, and adoption of gene therapy over conventional therapies. **OBJECTIVES:** Analysis of twitter text strings ("tweets") was conducted in order to empirically assess discourse topology and public sentiment surrounding gene therapies for hemophilia and blood disorders. **METHODS:** Social media text strings ("tweets") were extracted with a serial combination of manual NCapture (NVIVO 12, QSR International) and computational Perl script applying Boolean logic with the Twitter API. Tweets were extracted from from Jan 1 2019 to Jan 10 2020 that contained a combination of "hemophilia/haemophilia" OR "blood/bleeding disease/disorder" AND "gene therapy", excluding promotional strings primarily related to non-gene therapy products. Data were exported and analyzed in NVIVO 12 and SPSS using validated functions for sentiment analysis and grounded heuristics extraction for themes and content topology. **RESULTS:** A total of 29,740 tweets were analyzed, with monthly average volume of 2,478 per month. The primary themes in the data were development of milestones for gene therapies (18%), new product discussions (15%), pricing (12%), FDA (12%), trial results or updates (22%), first-hand discussions of patient treatment (8%), oncology (7%), drug market approvals (6%), and posts by pharma companies (5%). As expected, early results of trials and development milestones were commonly discussed in tweets by the companies developing the products; however, public engagement and third-party tweet volumes dramatically increased when the data was "re-tweeted" or originally tweeted by a news outlet or public figure. Interestingly, incidence of patient treatment discussions significantly more than doubled from 1st to 2nd half of 2019, and tweets related to pricing showed a steady increase throughout the year.



Sentiment analysis of tweets showed 60% were neutral, 7% and 15% were very or moderately positive, and 7% and 10% were very or moderately negative, respectively. ($p < 0.05$).



CONCLUSIONS: Patient and public engagement has dramatically increased for novel gene therapies for blood disorders in social media, largely responding to increased public news outlets and media coverage. Like other evolving high-tech markets in software and medical devices, this indicates a notable increase in public engagement going into 2020 that merits further study in context of policy, pricing, and adoption of gene therapies.

1237. Rapid Determination of rSIV.F/HN Lentiviral Vector Titre

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Our laboratory is developing non-viral and viral vectors for the treatment of a range of airway disorders. We have previously demonstrated that a recombinant simian immunodeficiency virus (rSIV) vector, pseudotyped with the fusion (F) and hemagglutinin-neuraminidase (HN) envelope proteins from Sendai virus (together termed rSIV.F/HN), has high tropism for multiple cell types found within both the conducting airway epithelium and the gas exchanging alveolar regions of the mouse, sheep and human lung. Historically, we have routinely used two titre assays to assess vector preparations and to normalise vector dose between studies: (i) a viral particle assay (reported as VP/mL) is derived from the concentration of viral RNA and is evaluated using qRT-PCR, (ii) a transducing assay (reported as TU/mL) is derived from the concentration of vector-derived DNA within cells transduced with a viral preparation and is evaluated using qPCR. Both approaches are widely used within the field, and accepted by multiple competent authorities. However, both approaches are relatively slow, taking serval hours (VP/mL) or several days (TU/mL) to report. We are routinely producing a large number (multiple lots per week; target >1e10 TU) of highly purified (anion-exchange/TFF), high-concentration (target >1e9 TU/mL) preparations of rSIV.F/HN. To refine this process and to produce vector lots with more consistent vector concentration, we are seeking a very rapid titre-dependent assay that could be incorporated into the downstream purification workflow that would allow an assessment of vector yield during the final stages of vector formulation. To this end, we have evaluated the utility of a simple hemagglutination assay in which sialic acid receptors on the surface of red blood cells (RBC) bind to the hemagglutinin-neuraminidase found on the surface of rSIV.F/HN. This creates a lattice structure of interconnected RBC and virus particles that prevents RBCs from settling under gravity. While a standard rSIV.F/HN-EGFP vector preparation demonstrated a consistent HA value (the highest virus dilution inhibiting RBC settling) of 800±0 (n=18), similar vector preparations pseudotyped with VSV-G or GP64 failed to show any evidence of hemagglutination 0±0 (n=8 and 12, respectively; $p<0.0001$ for both). Importantly, for multiple vector preparations, HA values remain essentially unchanged as RBC preparations are replaced ($p>0.9999$) or used repeatedly over 4 weeks ($p>0.9999$). As anticipated, HA values between virus preparations vary with VP/mL and TU/mL. In summary, the HA assay is rapid (<1 hour), inexpensive, requires no specialist equipment, training or interpretation, and allows vector concentration information to be incorporated into the choice of final vector formulation volume without extending the vector processing time. Using this approach will narrow variations in final vector concentrations and thus allow greater consistency in dosing volumes between vector lots - a particularly important property for rSIV.F/HN vectors where *in vivo* administration of a high concentration is anticipated.

1238. A Platform Approach for Analytical Methods to Support Adeno-Associated Virus (AAV) Gene Therapy Products

Yan Zhi

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Recombinant adeno-associated virus (rAAV) vectors have been widely used for in vivo gene therapy, with Luxturna™ and Zolgensma™ already approved by FDA as commercial products. Even though typical cGMP manufacturing of AAV gene therapy product consists of cell culture production (upstream process), chromatography purification (downstream process), and sterile filtration to fill / finish, unique

manufacturing challenges for each unit operation would warrant specific analytical methods to appropriately characterize and test AAV gene therapy products. In order to quickly characterize and test AAV gene therapy products, Charles River Laboratories (CRL) PA Biologics has established a platform approach for AAV vector genome titer quantification, residual human host cell DNA quantification and sizing evaluation, and replication competent AAV (rcAAV) detection, by adopting real-time PCR as well as ddPCR technologies targeting to sequences other than gene of interest (GOI) region, and using reference materials available from ATCC or biological materials created at CRL. Assay-specific data will be presented from in-house development and generic validation studies. Depending on the design and feature about any specific AAV gene therapy product (including vector serotype, production cell line, GOI, and clinical development phase, etc.), CRL can individualize its platform analytical methods by incorporating GOI-specific primer/probe set, preparing DNA standards from production cells, and identifying appropriate indicator cell lines and/or positive controls for each corresponding assay.

1239. Incorporation of Dual-Color Phenotyping Assay and Viability Assessment for Apheresis Using an Automated Cell Counter in cGMP CAR-T Cell Manufacturing

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The cGMP manufacturing of autologous CAR T-cell faces the challenge of highly heterogenous starting materials, typically patient apheresis products. As observed in more than 400 patient apheresis products that were processed in our laboratory, the content in T lymphocytes can be as low as 1% and as high as 70% . Moreover, high levels of monocyte contents in some patient apheresis products pose a further challenge for CAR T cell production. It is of interest to develop a manufacturing platform whereby T cells are selected from the highly variable apheresis products in order to start the CAR T cell manufacturing with purified lymphocytes. It is therefore desirable to develop a fast and reliable method to determine the percentage of CD3, CD4 or CD8 in the incoming apheresis product, which is required for the lymphocyte selection process. To this end, we tested the feasibility of using Nexcelom Spectrum with two optic modules, S1-534-470 (Ex: 475 nm and Em: 534 nm) and S1-605-527 (Ex: 525 nm and Em: 605 nm) to determine CD4+ and CD8+ cell contents in fresh apheresis products. The goals of this experiment were two folds: 1) to determine whether the readouts from Nexcelom Spectrum are comparable to those from the conventional FACS analysis; 2) to evaluate whether the turnover time of the assay was shorter than the conventional FACS analysis. We tested a dual fluorescence CD4/CD8 phenotype assay using CD4/CD8 antibodies conjugated with BB515 or PE fluorochromes. The quantification analysis was done using FCS Express coupled with Nexcelom software. Our initial results suggest that the protocol we tested using CD4-PE and CD8-BB515 antibodies generates CD4+ and CD8+ percentages readouts that are comparable to those obtained by flow cytometry analysis for fresh healthy donor apheresis products. We did find that this method is timesaving when compared to FACS analysis. We are in the process of further evaluationg the possibility of using the instrument for simultaneous determination of cell enumeration and CD4/CD8 composition. This Nexcelom Spectrum dual-color phenotype assay could potentially be developed into a routine assay to determine percentages of CD4 and CD8 cells in an incoming apheresis product.

1240. Strategic Formulation Development Approach for Early Stage rAAV Mediated Gene Therapy Programs for CNS Indications

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A strategic formulation development approach can be highly beneficial to support early stage rAAV mediated gene therapy programs for CNS indications. Until recently, a conventional "simple formulation" approach based on literature was widely accepted as a sufficient path forward. However, a refined approach to formulation developed has become a necessity as rAAV mediated therapies increase in demand and are implemented in more challenging routes of administration. We have developed a refined approach for early stage capsid selection and formulation development which takes into consideration rAAV specific issues such as limited material availability, challenges in analytics developments, and physiochemical properties of rAAV capsids, as well as unique considerations related to CNS administration. To this end, we characterized several available rAAV serotypes in wide ranges of pH and ionic strength. We leveraged this knowledge for our proprietary capsid and nominated a formulation that appropriately addresses considerations for CNS administrations and overcomes manufacturability challenges related to delivering a high concentration rAAV drug product.

1241. From Rocking Motion Bioreactors to Stirred Tank Bioreactors the Journey to Scalable AAV Gene Therapy

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Current manufacturing practices for rare and ultra-rare indications are likely adequate to supply the market for the near term. However, for indications were the patient population is larger than the rare market an appreciable scale-up will be required to meet demand. uniQure uses the baculovirus expression system to produce adeno associated virus vectors for gene therapy. This system has many advantages in scale-up of virus production. The baculovirus system does not require large volumes of plasmid for transfection of suspension mammalian cells. The fact that the Rep:Cap:Transgene is supplied by a virus which can infect insect cells removes the requirement for inefficient transfection of DNA. The scale up of baculovirus production is easy and can be accomplished in shaker flasks or in bioreactors allowing for the infection of 500L bioreactors and possibly larger reactors. uniQure has successfully made the leap from rocking motion bioreactors to 500L

stirred tank bioreactors using the baculovirus expression system to produce AAV. The AAV is produced to high titers and critical quality attributes are maintained in the scaled-up material.

1242. Development and Scale-Up of a Monolithic Chromatography Purification Method for Rhabdoviral Vectors

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Chromatography is the purification of choice for large-scale virus manufacturing, given its long history and demonstrated efficiency with the manufacturing of protein based pharmaceuticals. Furthermore, commercial and therapeutic applications of viral vectors are dependent on the establishment of an effective manufacturing protocol that complies with the contaminant limit specifications as stated by regulatory agencies. We have previously demonstrated the utility of a polymethacrylate CIMmultus monolithic column functionalized with hydroxy (OH) groups for the purification of Rhabdovirus vectors. Purification of 1 mL virus-infected cell supernatant via a linear gradient elution method with a 1 mL hydrophobic CIMmultus OH column consistently resulted in >90% virus recovery. We have tested this method with up to 250 mL of infected cell supernatant, and found that modifications to the infection medium and binding buffer were necessary to maintain high virus recovery. The dynamic binding capacity (DBC) of the 1 mL CIMmultus OH column for Rhabdovirus was found to be approximately 1e11 infectious units. This value was used as a starting point to determine the DBC of the 8 mL CIMmultus OH column. We will discuss the results obtained from these scale-up experiments and address issues that pose a challenge to Rhabdovirus chromatography purification.

1243. Production of rAAV2/HBoV1 Vector in Insect Cells

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We have developed a rAAV2/HBoV1 vector, which is highly tropic for human airway epithelium, by pseudopackaging an oversized rAAV2 genome (up to 5.8kb) in a human bocavirus 1 (HBoV1) capsid. In a recent study, we improved the rAAV2/HBoV1 production system in HEK293 cells and established an NS protein-free production system using three-plasmid co-transfection of HEK293 cells with two trans helper plasmids. One encodes VP1 and AAV2 Rep, and another one encodes VP2-3 and components from adenovirus. This improved system yielded >16-fold more vector than the original production system in HEK293 cells, without sacrificing the transduction efficiency. Baculorvirus expression vector system (BEVS), a versatile and robust protein manufacturing platform, has been applied for efficient rAAV vector production in Sf9 insect cells compatible with large scale preparation with serum-free suspension culture in bioreactor. In this study, we explored the possibility of utilizing the BEVS for rAAV2/HBoV1 production. Similar to the rAAV production in the BEV-Sf9 system, adenoviral helper is not required to support the replication of rAAV genome for pseudopacking. We expressed the AAV2 Rep gene and HBoV1 Cap gene (VP1, VP2, and VP3) in two baculovirus vectors. As NP1 is required to prevent premature termination of transcription of the Cap mRNA from the native viral genome in mammalian cells, we also compared the vector production efficiency with or without the expression of NP1 protein by an additional baculoviral vector. We found that, with the incorporation of the expression of HBoV1 NP1 protein, the rAAV2/HBoV1 vector production was enhanced to the similar level of rAAV2 vector production in Sf9 cells. However, at the same multiplicity of infection in polarized human airway epithelia, the transduction efficiency of the rAAV2/HBoV1 vector produced from Sf9 cells was lower than that packaged from HEK293 cells. We found a high percentage of empty capsids in the vector preparations from the BEV-Sf9 production system, which is thought to be responsible for its lower transduction potency. In the future, we will optimize the production for efficiently pseudopackaging the rAAV2 genome in HBoV1 capsid in Sf9 cells as well as the purification processing for high quality of vector preps. Taking advantage of the highly efficient BEVS, an improved AAV2/HBoV1 production in the easily scalable Sf6 cell culture will meet the need for large amount of vectors in order to test the efficacy of gene therapy for cystic fibrosis lung disease in large animal models.

1244. Engineered Primary T-Cells as Vectors for *In Situ* Protein Synthesis

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Systemic toxicity of drugs limits its application for diseases that evolve *in vivo* (e.g. cancer, viral infections, autoimmune disorders). We have developed an *artificial cell-signaling pathway* that capitalizes on T-cell's innate extravasation ability and transforms the cell into a living vector (antigen-specific T-cell Biofactory) for synthesizing engineered proteins *in situ* upon interacting with target cells. The T-cell Biofactory has three constant and two variable domains. The constant domains include - the *Receptor* that mobilizes the DNA-based *Actuator* to synthesize engineered proteins and the *Secretor* for transporting them into the extracellular space. The variable domains provide a broad applicability and include - a *Sensor*, part of the Receptor, to identify the disease-specific biomarker; and an *Effector* transgene with the potential to neutralize the pathology that triggered the T-cell Biofactory. The feasibility was validated by measuring the activity of a reporter enzyme, NanoLuc®, the DNA template for which can be replaced with a protein having desired therapeutic function. OVCAR3 and A2780cis cell lines, with or without endogenous expression of Folate Receptor alpha (FRa) and engineered for Luc2 expression were used as target and nontarget cells, respectively. Co-culture of FRa-specific primary T-cell Biofactory with target (FRa+) and non-target (FRaneg) cells demonstrated that the former exhibits the engineered functions *in situ* and specifically upon engaging with the target cells. This included statistically significant increase in (i) target cell lysis (~14-fold) compared to non-target cells, as measured by reduction in cell-viability marker Luc2 enzyme activity; and (ii) synthesis of a non-human protein (~4-fold), as measured by upregulation of NanoLuc® reporter enzyme activity. The signal-to-noise ratio (S/N), a parameter that defines the target-specificity, was calculated as the ratio of enzyme activities (NanoLuc for engineered protein synthesis) when the T-cell

Biofactory was stimulated by the target cells to when stimulated by the non-target cells. The S/N was found to be higher at lower T-cell Biofactory-to-target cell ratios. In conclusion, we have demonstrated the potential of an *in vivo* cell-based vector technology that can be used to synthesize proteins with desired properties *in vivo* and upon engaging with the tumor cells that upregulate an antigenic biomarker as a tumor-associated antigen.

1245. Generation of Lentiviral Inducible Producer Cell Lines with Accelerated Timelines and Production at Large Scale

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Lentiviral vectors (LVs) are a very potent tool for gene and cell therapy applications and to meet current and future demands for clinical use, efficient large-scale production platforms are necessary. Producer cell lines (PCLs) capable of generating high titre LVs at scale are therefore desirable to streamline the production process, while improving batch consistency and reducing cost. Oxford Biomedica (OXB) has previously generated adherent PCLs which have subsequently been adapted to a suspension growth mode. Production of LV from such a suspension PCL was further verified at large scale (50L bioreactor). However, this PCL generation approach is very time-consuming and resource intensive, and thus OXB has developed a new platform that enables the generation of inducible PCLs directly in suspension culture using solely chemically defined animal-free components. Requirements for the generation of adherent cell lines were thus bypassed and timelines were considerably reduced. Furthermore, in combination with OXB's high throughput Automated Cell Screening System (ACSS), the current workflow allows the screening of hundreds of clones for LV production directly from a pool of PCL suspension cells. This approach has been used to perform a large scale ACSS PCL campaign isolating and selecting inducible suspension PCLs for the production of a therapeutic LV. Here we describe the isolation and high-throughput evaluation of therapeutic suspension PCLs clones. Selection of the best clones resulted in the identification of suspension PCLs capable of producing therapeutic LVs of higher titre than those achieved using the standard transient transfection process at various scales. Furthermore, the best PCL demonstrated tight Tet Repressor (TetR) regulation of LV production in the off-state with titres increasing more than 2000-fold upon induction with doxycycline. This data demonstrates it is possible to generate PCLs directly in suspension that are capable of producing high titre therapeutic LVs. Further improvements in productivity are expected following process optimisation.

1246. Design and Testing of Vector Producing HEK 293T Cells Bearing a Genomic Deletion of the SV40 T Antigen Coding Region

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The use of the human embryonic kidney (HEK) 293T cell line to manufacture vectors for *in vivo* applications raises safety concerns due to the presence of SV40 T antigen-encoding sequences. We used CRISPR/Cas9 genome editing to remove the SV40 T antigen-encoding sequences from 293T cells, by transfecting them with a recombinant plasmid expressing Cas9 and two distinct sgRNAs corresponding to the beginning and end of the T antigen coding region. Cell clones lacking T antigen-encoding sequences were identified using PCR. Whole Genome and Targeted Locus Amplification Sequencing of the parental HEK 293T cell line revealed multiple SV40 T antigen-encoding sequences, replacing cellular sequences on chromosome 3. The putative T antigen null clones demonstrated a loss of sequence reads mapping to T antigen-encoding sequences. Western blot analysis of cell extracts prepared from the T antigen null clones confirmed that the SV40 large and small T antigen proteins were absent. Lentiviral vectors produced using the T antigen null clones exhibited titers up to 1.5 x 10^7 transducing units (TU)/ml, while the titers obtained from the parent HEK 293T cell line were up to 4 x 10^7 TU/ml. The capacity of the T antigen-negative cells to produce high titer AAV vectors was also evaluated. The results obtained revealed that the lack of T antigen sequences also did not impact AAV vector titers.

1247. Large-Scale Production of Recombinantadeno-Associated Viral Vectors

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Recombinant adeno-associated virus (rAAV) is relatively safe and demonstrate high gene transfer efficiency, low immunogenicity, stable long-term expression, and selective tissue tropism, and has been widely used in many clinical-stage gene therapy programs including 3 approved gene therapies. However, a main barrier for AAV-based gene therapy is the affordability of the drug product attributed to the high cost of manufacturing. Here we describe the use of HEK293T cells which have been adapted to grow in suspension without FBS in bioreactors to yield over 1×10^{14} vector genomes per liter (crude fermentation) . Furthermore, we also describe the use of recombinant baculovirus platforms which have been widely employed for large-scale production of rAAV in Sf9 cells. Sf9 suspension systems can be cultured at high cell densities and the cell capacity of rAAV is 1×10^4 to 1×10^6 vector genomes per cell. The manufacturing costs of large production of rAAV in our systems maybe are as less as 30% of current transient transfection of adherent HEK293 cells costs. The two cost-efficient manufacturing platforms are developed for large-scale production of rAAV.

1248. Development and Scalability of Transfection-Based Production and Purification of Novel Clade F Adeno-Associated Viruses Isolated from Human Hematopoietic Stem Cells (AAVHSCs)

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Homology Medicines is a clinical stage genetic medicines company developing gene therapy and gene editing adeno-associated viral (AAV) therapeutics for the treatment of rare diseases. Using a proprietary dual platform based on the production of novel Clade F capsids derived from hematopoietic stem cells (AAVHSCs), we have developed a GMP manufacturing process that delivers high-quality gene therapy and gene editing product candidates. The platform has been linearly scaled up to meet current and future clinical and commercial supply demands. The AAV production utilizes suspension HEK293 cells in a serum-free, transfection-based bioreactor process. This process has been successfully scaled up from 2L to 500L and has been executed over 400 times across more than 300 different constructs to produce consistent, high-quality product candidates. To enhance the platform process productivity, bioreactor optimization increased titer 1.5-3.5 times and our platform chromatography-based depletion of empty capsids was able to consistently deliver final product with >85% nucleic acid containing capsids. To support clinical expansion, we have built a 25,000 square foot internal manufacturing facility, are currently operating three 500L bioreactors , and have successfully produced GMP material at the 500L scale for multiple pipeline candidates. Additionally, a 2,000L bioreactor scale is also being executed. In summary, Homology has developed a scalable production and purification platform that has been utilized for external and internal manufacturing of AAVHSC vectors to support multiple product development and pipeline programs for both gene therapy and gene editing.

1249. Impact of Depth Filter Lot to Lot Variations on Yield of rAAV Clarification in Manufacturing Scale and the Approach to Overcome this Issue

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Takeda, Orth/Donau, Austria

Recombinant adeno associated virus (rAAV) emerged as one of the most relevant vectors for gene therapy in the past few years. rAAV is produced mainly in bioreactors with HEK293 cells in suspension with the need for an efficient clarification of the supernatant from the cells at the end of fermentation. The present study shows how observations of yield fluctuations in GMP production could be pinned down to lot to lot variability in depth filters used for primary clarification of the cell- and vector suspension. After the root cause could be confirmed, a strategy to overcome the fluctuating step yields regardless of the used filter lot was set up. In a Design of Experiment (DoE) approach different filters and cell suspension treatments were tested in small scale filtration experiments using a small-scale filter lot equivalent in its composition to disfavored GMP production lots. With optimal treatment of cell suspension, we could raise the step yield to similar numbers like we experience with non-problematic filter lots and thus compensate lot to lot variation. Our recent results and the approach to overcome such challenges will be presented in the poster.

1250. Purification of Recombinant Adeno-Associated Viral Vectors

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The recombinant adeno-associated viral (rAAV) has various serotypes which display differential cellular tropism, transduction efficiency and immunogenicity. Their purification strategy depend on not only the serotypes of the virus but also the adopted production approach. CsCl gradient centrifugation is somewhat laborious and time consuming. Purification by iodixanol gradient centrifugation offers a simpler method universally applicable for all serotypes of rAAV. Here, we describe single rounds of ultracentrifugation can yield vectors with sufficient titers and purity in research-grade and small-scale preparations. For large-scale production, multiple purification steps of scalable chromatographic methods including affinity or ion exchange chromatography, were carried out. Following POROS CaptureSelect AAVX,anion exchange chromatography was chosen to separate fully packaged particles from empty capsids of wide variety of serotypes. Thus, a scalable purification platform is established for GMP-grade vector production.

1251. Controlled Stirred Tank Bioreactors for Large-Scale Manufacture of Human iPSC Models for Cell Therapy

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Cell and gene therapies show promise in providing new therapeutic strategies for a wide range of indications. Advancements in induced pluripotent stem cell (hiPSC) technologies have substantially expanded access to many human cell types to accommodate the future demand for such therapies. However, the direct utilization of standard cell manufacturing equipment and methods in the differentiation and manufacture of iPSC-derived cells can face significant challenges in obtaining the necessary production scales, quality standards and high reproducibility between batches for cost-effective cell therapy research and clinical application. Currently, the development and production of iPSC-derived cell

types is often performed in a small-scale culture unsuitable for robust generation of a large number of cells. Using the example of cell-based therapy for heart failure, it is estimated that 10^9 cells are required per patient with a target population potentially requiring thousands of doses per year, as much as 10^{11} - 10^{14} cells. Stirred-tank bioreactors have emerged as promising culture systems for large-scale cell manufacturing from hiPSC sources. These systems allow full automation and conduction in closed systems that can be monitored and maintained at defined physiochemical levels, resulting in cultures with comparable characteristics from batch to batch. Closed-system, parallel processing with increased automation is also critical to minimize error and contamination from human interaction with cell products. We have established a controlled stirred-tank bioreactor platform that is shown to routinely yield high numbers of hiPSC-derived cardiomyocytes and additional cell models that are currently used in cell therapy, safety and efficacy applications. Using a Quality by Design approach, we demonstrate a robust and controlled process for large-scale manufacturing (>3x10^{10}) of iPSC-derived cardiomyocytes to a purity of >95% in a serum-free protocol. The bioreactor-derived cells are shown to be a relatively mature model recapitulating a human cardiomyocyte's contractile and electrophysiological profile. We demonstrate the implementation of a flexible process development workflow comprised of state-of-the-art bioreactor systems that allows for optimization of processes at 15 mL scale, validation of promising conditions at mid-scale (100 - 250 mL) and manufacture from a diverse set of hiPSC lines to yield the required scale in the tens of billions.

1252. Prescribing Gene Therapy: An Ethnographic Pilot Study of Decision-Making Among Non-Research Physicians

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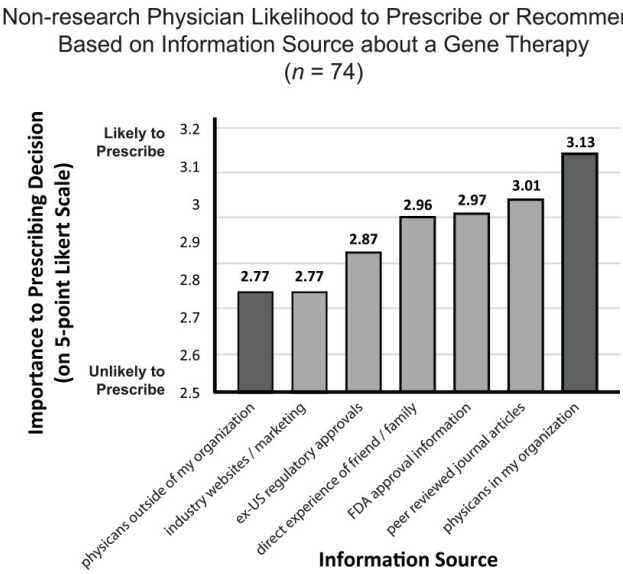
Background: By 2025, analysts predict that 10 to 20 new cell and gene therapy (CGT) products will be approved by FDA each year, resulting in unprecedented availability of CGT in clinical settings. However, these novel therapies may not be accessible to patients due to pricing, availability, and willingness of their regular physicians to recommend these therapies. While pricing has been widely discussed, more behavioral information is needed to examine recommending and prescribing decision-making behaviors among individual physicians as novel CGT products enter the clinic. **Methods:** An ethnographic study was conducted by triangulating results of an online survey of recommending/prescribing preferences ($N = 196$) and grounded observations from professional interviews ($N = 18$). Of surveys, the subgroup of 74/196 non-research physicians were included in this analysis (research professionals were excluded). Convergent heuristics were established using grounded theory methods in NIVO 12 (QSR International) and factorial analysis was performed in SPSS. **Results:** Of 74 non-research physicians surveyed, 84% (62/74) viewed gene therapy positively, while 55% (41/74) indicated they would be willing to recommend or prescribe a commercial gene therapy to qualifying patients in the future. Heuristic coding and triangulation revealed that negative responses were most commonly related to lack of first-degree professional connections with experience in GCT, small organization size, a preference for print over online information sources (all $p < 0.05$). Interestingly, physicians were significantly more influenced by information about gene therapy from other physicians within their own institution than by physicians outside their institution or industry marketing materials (both $p < 0.05$), as shown in Figure 1.



Conclusions: While recommending and prescribing gene therapy is a complex multifactorial behavior, these initial findings demonstrate the importance of institutional expertise in gene therapy adoption. To ensure equitable access of novel CGT therapies outside of research settings and large institutions, it will be necessary to increase institutional comfort with gene therapy through training, continuing education, and professional involvement of non-research physicians. Further study is needed to explore programs that provide information and increase expertise among non-research physicians and institutions as more GCT therapies enter the clinic.

1253. Lentiviral Gene Therapy of Hemophilia A: Expression and Functional Comparison of Universal and Tissue-Specific Promoters

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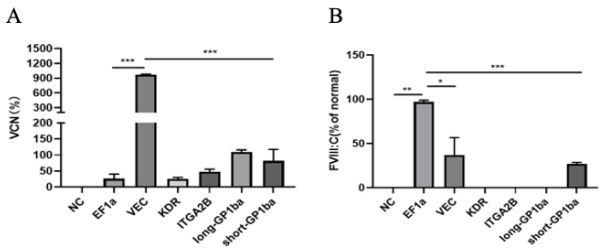
Hemophilia A (HA) is an X-linked coagulation disorder resulting from monogenic deficiency of the factor VIII (F8) gene in the intrinsic coagulation cascade. The current treatment of HA is based on protein replacement therapy (PRT) through plasma-derived coagulation factors or recombinant proteins produced in culture. The limitation of PRT includes short half-life, high cost of recombinant concentrates, and life-time requirement of the treatment. Thus, gene therapy has become the most promising treatment for HA. In this study, we have developed advanced lentiviral vectors carrying a universal promoter or tissue-specific promoters driving F8 transgene expression in different cell types. Selective codon optimization, full-length F8 and B-domain deleted F8 (F8BDD) genes were synthesized and sequence verified. The EGWI lentiviral vectors carried different tissue-specific

promoters including universal (EF1a), endothelial-specific (VEC), endothelial and epithelial-specific (KDR), megakaryocyte-specific (ITGA2B), and a long- and short-version of platelet-specific (Gp1ba) promoters, were characterized. *In vitro* assay based on K562 cells, a human erythroleukemic cell line, revealed that higher level of protein expression could be detected from EGWI-EF1a-F8BDD as compared with EGWI-EF1a-F8. To examine endothelial specific expression, EA-hy926, a human endothelial cell line was used. *In vitro* assay comparing different promoters demonstrated that EGWI-VEC-F8BDD displayed substantially higher transgene integration efficiency than the other lentiviral constructs; the lentiviral packaging efficiency of EGWI-VEC-F8BDD was 100-fold (>=10^9) greater than EGWI-EF1a-F8BDD and EGWI-KDR-F8BDD, and 10-fold greater than the other constructs. EGWI-VEC-F8BDD showed the highest vector integration copy number (VCN) when transduced at the same MOI (Figure A). Endothelial-specific transgene expression of EGWI-VEC-F8BDD was confirmed in EA-hy926 cells and illustrated by Western blot. Functional assay based on chromogenic assay using EA-hy926 cells demonstrated high F8 activity of EGWI-VEC-F8BDD (Figure B), which was in the therapeutic range (~36% of the normal level), as compared with EGWI-short Gp1ba-F8BDD (~26%) and EGWI-EF1a-F8BDD (~97%), while the other promoters did not show F8 activity (0%). Our study indicates that the EGWI-VEC-F8BDD gene therapy supports high transgene delivery and tissue-specific expression in endothelial cells, the cell type highly relevant to F8 function manifestation in human body. To support future clinical applications, *in vivo* studies are underway to illustrate translational potential of these tissue specific F8BDD genes in F8 knockout mice.



1254. Optimization of Clarification Process for Viral Vectors

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Viral-based vector systems such as lentivirus (LV) and adeno-associated virus (AAV) are more widely used and show great potential for delivery of genetic material to the target cells in gene therapy. Downstream processing of lentivirus and adeno-associated virus offers its own unique challenges to generate clinical products of high titer, high potency and high purity. The objective of this work is to identify an efficient clarification step for the separation of viral vectors from the range of impurities found in typical adherent and suspension based viral vector cell culture. These include: host cells, cell debris, aggregates and cell culture media components. The clarification step needs to combine high capacity for impurity removal, high product yield, and ease of scale-up to prepare for downstream operations. Our strategy includes evaluating various depth and membrane filters made up of different organic and inorganic materials for their virus clarification performances. Both AAV and LV processes can be adherent or suspension, each having their own challenges, and in this work, we show an optimized improvement in throughput and recovery for these viral vector feed streams. Thus, making the overall downstream process more efficient, scalable and economical.

1255. Improving Process Control of Transfection Unit Operation Increases Viral Titers in Suspension-Adapted Production Systems

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Lentiviral vectors (LVVs) play a key role in a number of gene therapy processes. Most commercialized LVV production processes utilize adherent cell cultures for production. While these processes have been able to meet commercial demand, they require scale out to increase batch size, limiting the maximum process scale. Adherent processes also require the addition of animal products like fetal bovine serum to maintain cell health. For these reasons, it may be advantageous to develop serum-free suspension processes to reduce the cost of LVV production by allowing single-vessel scale up, rather than scale out, and improve safety by eliminating the need for animal derived raw materials. A major challenge involved in developing an effective suspension system is maintaining culture productivity when scaling up. The transfection unit operation is a crucial step in developing a suspension process and has a high impact on culture productivity. Polyethylenimine (PEI) mediated transient transfection is a common method for introducing genes of interest to host cells in LVV production systems, and is an effective transfection technique for suspension production processes. While developing a serum-free suspension process for LVV production, we observed that PEI/DNA complex hold time was a highly time-sensitive process parameter that would present challenges during manufacturing scale-up. Using dynamic light scattering (DLS), we demonstrated that polyplex size increases with increased formation time, and that higher concentrations of DNA and PEI resulted in more rapid particle size increase. When co-transfecting the components of a 3rd generation lentiviral vector, we observed that maximum LVV titer is achieved by polyplexes of a specific size, and titer decreases as polyplex size increases beyond this optimal size. This presentation will focus on process optimization efforts aimed at improving process control and titer output in a serum-free suspension transient transfection of lentiviral vector components.

1256. Next-Generation Transfection Reagent for Large Scale AAV Manufacturing

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The number of ATMP therapeutic-based medicines for inherited genetic disorders is in constant growth, with a global 32% increase in new clinical trials in the last 4 years. ATMPs have demonstrated their success with already more than ten approved for commercialization. The success of AAV as the most promising viral vector for gene therapy is due to low immunogenicity, broad tropism and non-integrating properties. One major challenge for translation of promising research to clinical development is the manufacture of sufficient quantities of AAV. Transient transfection of suspension cells is the most commonly used production platform, as it offers significant flexibility for cell and gene therapy development. However, this method shows some limitations in large scale bioreactors: inadequate transfection protocol, reduced transfection efficiency and lower productivity. To address this concern, we present data on a novel transfection reagent showing: *i)* increased AAV titers, *ii)* improved transfection protocol for large scale bioreactors and *iii)* reproducibility of viral titers at different production scale. The aforementioned optimized parameters make this novel transfection reagent ideal for cell and gene therapy developers by combining the flexibility of transient transfection with scalability and speed to market.

1257. Controlling Electroporation Parameters for CAR-T Cell Manufacturing

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Although electroporation is a promising non-viral technology for manufacturing of therapeutics cells such as CAR-T cells, this seemingly straightforward process can become very inconsistent. Here we investigated several factors in electroporation and found out that they affect electroporation parameter selection. The first simple factor is cell density. Higher cell density requires higher voltage and we were able to explain by a physics analysis. The second factor is reagent type. We found that plasmid and mRNA use a similar optimal voltage but Cas9 ribonucleoprotein as well as siRNA requires a higher optimal voltage. The third factor is T cell preparation method. We found that there is some significant donor variation of optimal voltage for primary unstimulated T cells. Also frozen-thawed unstimulated T cells requires a higher voltage than fresh unstimulated T cells. For stimulated T cells, the overall voltage is much lower than primary T cells and the electroporation efficiency and viability decreases with prolonged culture. Our work suggested that the concept "one program for one cell type" is not suitable for real-life applications and we have proposed optimization strategies that could simplify the manufacturing of therapeutic cells.

1258. Testing Approaches for Cell Based Therapeutics: Rapid Testing and Regulatory Expectations

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Ensuring the safety of cell-based therapeutic products is achieved through a multi-tiered approach that examines several factors to establish product safety. The nature of these novel therapeutic strategies presents testing challenges when compared to traditional therapies that may include limited shelf life and limited testing sample size as well as a need for reduced assay turnaround times. As a consequence, existing testing approaches are often not suitable for these products. Rapid and sensitive new technologies are being developed that can be effective replacements for traditional assays. These new assays are often more sensitive and provide results in considerably less time. Regulators recognise the limitations that existing assays place on cell-based therapies and have revised guidelines to provide flexibility in the use state of the art technologies to replace current methods. This presentation provides an analysis of the rapid testing approaches used to ensure cell-based product safety. The implementation and validation of rapid assay methods and suitability of new testing platforms that allow for streamlining testing and reporting are also discussed.

1260. Pall Xpansion Bioreactor Supports Progenitor Cell Growth to 1 Million Cells/cm2 and Proper Cell Differentiation

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With the recent increase in early phase cell therapy clinical trials, there is a need for a manufacturing platform which can be implemented in research labs and easily scaled up to expedite process development studies, pre-clinical testing, and large-scale expansion of adherent human cells. Although various platforms such as traditional flatware and stirred tank bioreactors with microcarriers exist and have been well characterized, traditional flatware often does not allow for tight control of the cellular environment and is not scalable. Pall's Xpansion single-use bioreactor offers a tightly-controlled, scalable manufacturing platform for cell therapy applications, allowing for expedited process development, pre-clinical testing and large scale, high-density expansion of progenitor cells while maintaining their differentiation capacity. We have previously demonstrated efficient expansion of both epithelial cells (Vero, HEK293), human mesenchymal stem/stromal cells (hMSC), and progenitor cells in the Xpansion single-use bioreactor. Here we extend the progenitor cell findings by successfully expanding a proprietary progenitor cell to extremely high cell densities (> 1 million cells/cm^2) reproducibly for 8 batches. In summary, all batches resulted in 1) cell expansion to > 1 million cells/cm^2 and equivalent cell densities to the traditional flatware process control; 2) equivalent differentiation to a more mature cell fate as indicated by expression of key cell surface markers; and 3) self-assembly into characteristic three-dimensional structures.

1261. Production of Recombinant AAV Vectors in Chemically Defined Media

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Recombinant adeno-associated virus (rAAV) is a widely used viral vector for gene therapy applications. Manufacturing of rAAV vectors, using baculovirus expression system (BEVS) and insect (*Spodoptera frugiperda*) cells suspension culture, is commonly performed using a non-defined medium that contains extracts originated from microbial, plant or even animal sources. Although the product quality may not be impacted, the presence of the complex and non-defined components in the medium introduces the risk of inconsistent process performance, process control and regulatory challenges. In the present work, we demonstrate adaptation of *expres*SF+ cells into a chemically defined

medium (CDM) that is yeastolate-free, serum-free, protein-free and animal origin-free. The CDM adapted cells showed similar growth characteristics, e.g. population doubling time and maximum cell density, as original *expres*SF+ cells in non-defined media. The CDM adapted cells were used for propagation of baculovirus as well as for production of AAV in CDM and compared side-by-side with the performance of original *expres*SF+ cells in non-defined medium. The result shows that the baculovirus titers in both CDM and chemically non-defined media are comparable. The result also shows comparable production titers and quality attributes of AAV when produced in CDM and chemically non-defined media.

1262. Development of Novel Synthetic Promoters for CNS Gene Therapy

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Gene therapy for previously untreatable neurological diseases is now a clinical reality. However, neurological diseases are diverse with different brain regions and cell types affected and the development of custom design vectors to address the diversity of neurological disorders is required. Using Synpromics Ltd bioinformatics-based promoter design platform, novel synthetic CNS promoters were generated and evaluated *in vivo*.

A GFP reporter gene under the transcriptional control of 9 novel promoters were evaluated in rAAV9 vectors. Neonatal CD1 mice received titre matched vector by intracerebroventricular or intravenous delivery and euthanised at 5 weeks for GFP expression. CNS distribution was characterised in detail by free floating whole brain immunohistochemistry and systemic organ biodistribution evaluated by qPCR compared to human Synapsin 1 promoter. All novel constructs were active in the CNS with rostrocaudal gradient. Within these novel promoters, we identified a candidate with selective activity in midbrain dopaminergic neurons. To validate this novel promoter we generated a gene therapy construct for validation in the Dopamine transporter knockout mice as a model of Dopamine transporter deficiency syndrome: infantile parkinsonism dystonia. Heterozygous mice were time-mated to generate knockout pups that received intravenous gene therapy at P1. Untreated littermates were used as controls (n=4 per group). Here we present biodistribution of 9 novel CNS promoters and preliminary data on therapeutic validation of a novel midbrain dopaminergic promoter.

1263. LentiSoma-Based New Generation of Stable Packaging Cell Line for the Production of Lentiviral Vectors for Gene Therapy

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Lentiviral vectors (LVV) are currently present in more than 300 ongoing clinical trials of gene therapy and few have already entered in the commercial phase. The FDA foresees approving 10 to 20 cell gene therapy products each year by 2025. As the gene therapy field has surged forward, manufacturing has emerged as a persistent challenge: market's needs in the mid-term are challenging CMOs to evolve manufacturing platforms from the current methods that should be considered barely first-generation. Commercial-scale manufacturing represents one of the next significant barriers to the continued development of the field. The production of LVV has principally been based on the transient co-transfection of cells with three/four expression cassettes: *gag-pro-pol*, *rev*, *env* and the packageable construct. This approach suffers of low biosafety, high cost of raw materials and low reproducibility between production batches making manufacture unfeasible to reach commercial stages. **The hallmark of commercial manufacturing requires a much more consistent production with minimal batch to batch variability and a more cost-efficient process.** Different approaches have been followed and others are ongoing to solve these problems. The availability of suitable packaging cell lines (PCL) able to continuous production should be part of the solution. Despite this, since the 90's several strategies have been followed to generate PCLs, most still continue to present undesirable defects in biosafety, reproducibility, genetic and expression stability of the viral elements and importantly, viral titres achieved remains at most similar to the transient transfection strategy. VIVEbioTECH SL has secured exclusive exploitation license (patent WO2015/078999) of an innovative gene therapy vector, **LentiSoma** (LS). LS is derived from sinLV that behaves as an autonomous self-replicating episomal DNA. Upon transduction, LS-derived circular cDNA by means of eukaryotic genetic elements (an *ori* and a matrix attachment region (*S/MAR*) divides and segregate in unison with cellular chromosomes. LS represents the prototype of a new generation of viral vectors keeping almost all properties of LVV likewise pseudotyping, rooming capacity and stable expression but without integration. VIVEbiotech is developing a novel PCL (named **VIVESOMA**) based on pivotal benefits of LS for the production of lentiviral vectors through the episomal expression of helper genes. Positional-silencing, genetic stability and variability derived from integration are expected to be eliminated on the basis of the unique LentiSoma's properties thus achieving **reproducibility** and **robustness in production**. In addition, VIVESOMA is designed to express different helper genes by synthetic circuiting and guarantee higher biosafety concerns by eliminating all non-essential viral sequences and prokaryotic genetic elements as well. In conclusion, VIVESOMA is designed to display unprecedented properties of PCL to afford next-generation scaling-up of LVV manufacturing for commercial stages. The approach followed to afford such goals and the data available on LVV production will be presented.

1264. Development of a High-Yield Virus Production Platform Using a Nove Fixed-Bed Bioreactor

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The increasing importance of viral vaccine manufacturing has driven the need for high cell density process optimization that allows for higher production levels. Utilization of Vero cells is one of the more

popular adherent cell lines used for viral vaccine production; however, production is limited due to the logistical limitations surrounding adherent cell line processes. We have addressed this limitation with the establishment of a viral vaccine production system utilizing the novel single use scale-X™ carbo bioreactor from Univercells. The unit is compact and is scalable and allows for continuous in-line downstream purification and concentration. We present the results from a campaign featuring a proprietary Vero cell line for production of a live recombinant Vesicular Stomatitis virus vaccine platform. Metabolite analyses and viral yield comparison between traditional flasks, cell factories, and the scale-X carbo bioreactor were performed, and on average, the single use bioreactor produced up to 4 logs higher titers per surface area when compared to classical flatstock production. Overall, we describe a novel bioreactor platform that allows for a cost-efficient and scalable process for viral vaccine and/or gene therapy vector production.

1265. Abstract Withdrawn

1266. Process Development and Scale-up Comparisons for Transient Production of AAV

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Upstream cell culture production of adeno-associated virus (AAV) for the gene therapy field has grown from simple adherent flasks to complicated adherent systems to suspension-based stirred-tank bioreactor processes. Many production systems exist for generating AAV as a product for gene therapy including transfection of HEK 293 cells. However, most of the commonly used equipment for modern suspension mammalian biotechnology processes have not been specifically designed for large-scale transient production of AAV using HEK 293 cells. The challenge of producing an AAV in a suspension mammalian cell transfection process is expanded when comparing reactor scale and design. Thus, the need for the comparison of current bioreactor systems for the production of AAV. The impact of bioreactor configuration and mixing on transfection and AAV production will be compared for two separate processes and manufacturing cell lines. Bioreactors from 15 mL to 500 L were optimized for scalability and process performance including high-throughput automated reactors, bench-scale reactors, and production reactors. The effect of reactor settings and typical scale-up factors on cell growth rates, phenotypes and harvest titers were compared against several types of reactors at the varying scales. Key Words:scale-up, gene therapy, transfection, AAV

1267. An Automated CNS Histology Platform for Evaluation of Candidate Gene Therapy Vectors

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The central nervous system (CNS) is a complex tissue with multiple, distinct cell types arranged in larger functional assemblies. In this context, a successful gene replacement therapy (GTx) for monogenic CNS disease must both target the appropriate tissue and cell type, and also approximate the normal endogenous expression level of the affected gene. Underexpression in the affected cell types will lead to an ineffective therapy, whereas overexpression or off-target expression may lead to unwanted side effects. Means to measure GTx-candidate gene expression exist but as currently implemented these are typically low-throughput, semi-quantitative and non-comprehensive with regard to region and cell identity. Inadequate characterization of cell tropism and expression level of candidate gene therapies at the pre-clinical stage increases the risk of reduced efficacy, increased cost, adverse patient outcomes, and eventual clinical failure. The mission of Expressive Neuroscience is to provide FDA-compliant preclinical safety and efficacy data for CNS-directed gene therapies. This calls for efficient, rapid, quantitative, well-controlled and comprehensive measurements of GTx payload expression in the brain at the single cell level. To meet this mission, we have created the Expert System™: an industrialized experimental assay platform that tightly integrates highly-optimized immunohistology and imaging protocols with a custom relational database and automated analysis tools. Starting with a fixed brain, Expert extracts expression levels for proteins of interest in single cells from any neuroanatomical region. Expression levels are integrated with detailed experimental metadata in a relational database, facilitating rigorous quality control and powerful data visualization to guide preclinical triage decisions. Due to the level of automation and experimental efficiency implemented in the platform it is possible to achieve high precision, accuracy and throughput with high efficiency. Expressive offers this preclinical evaluation service to academic and industry partners seeking to accelerate the development of successful CNS-GTx.

1268. Development of a Scalable Serum Free Single-Use (SU) Suspension Platform for the Manufacturing of High Potency and Quality AAV Batches

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FREELINE is a clinical stage AAV gene therapy company with two programs currently in clinical development, and further programs moving towards clinical development as well. For commercial-scale manufacturing of AAV, FREELINE developed an iCELLis-based manufacturing platform. Combined with its proprietary plasmid system, this platform provides very high cell-specific productivity, overall yields and cost of goods (COG) comparable to that of a well-developed 200 l suspension bioreactor, but importantly also state-of-the-art vector quality and potency. 10 to 20 batches per year using one iCELLis500 device will be sufficient to meet peak market supply needs of our lead program. Nevertheless, as this platform relies on adherent cell culture requiring the addition of serum to the culture medium, FREELINE is also investing in the development of a suspension cell manufacturing platform completely free of animal-derived components (ADC). Our major development objectives are to increase cell-specific yields and to reduce the payload of undesired DNA packaging, aiming at a vector quality and potency comparable to that of iCELLis derived-AAV. We developed a fully single use (SU) serum free suspension platform based on a proprietary HEK293 cell line, capable of producing AAV of comparable quality and potency to the currently established

adherent platform. This SU platform showed scalability from 20 ml shake flasks to 2L SU bioreactors, with comparable metabolic, growth, titer, impurity and potency profiles. The advantages of such platform are: further decrease of cost of goods (COGs) compared to the adherent platform, decreased process complexity, higher flexibility for scaling up the process, and usage of chemically defined media. This further reduces ADC-contingent process variability and possible supply shortage issues. Here we present the current development status of this platform, in addition to, comparability data against our established adherent HEK293T platform.

1269. Site-Specific Integration Enables Rapid Cell Engineering for the Development of Precision Exosome Therapeutics

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Introduction: Incorporation of pharmacologically active molecules on the surface or the lumen of extracellular vesicles (EVs) is an important strategy for maximizing the therapeutic potential of EVs. Genetic engineering of producer cells by introducing DNA through random or site-specific integration are promising strategies for creating engineered EVs. Long-term stability with consistent transgene expression and therapeutic potency in the EV producer cells are crucial for biomanufacturing. We present a comprehensive study to investigate stability of transgene expression and potency of two potential therapeutic engineered EVs derived from stably selected pools transfected by either random integration (RI) or site-specific integration (SSI). **Methods:** Producer cells were engineered to make exosomes displaying interleukin 12 (IL-12) or interferon gamma (IFNg) by random integration (RI) or nuclease mediated site-specific insertion (SSI) into AAVS1 locus. Following puromycin (puro) selection, long-term cellular stability and transgene expression without selective pressure was investigated. EVs were generated from stable cell pools at 0, 1, and 2 months post-thaw and purified by density gradient ultracentrifugation. Purified EVs were biochemically characterized by NTA, BCA, Western blot, and cholesterol quantitation. Transgene expression and biological activity of EVs displaying IL-12 and IFNg were assessed by AlphaLISA and *in vitro* reporter assays. **Results:** Transfection by SSI resulted in faster recovery in puro selection compared to RI. All stable cell pools, regardless of integration method, resulted in comparable cell culture performance, EV yield, and lipid and protein content at all time points tested. The engineered EVs also demonstrated long-term stability of IL-12 and IFNg transgene expression and *in vitro* activity from both integration strategies. **Summary/Conclusion:** Both methods for generating stable cell lines were comparable in terms of cell stability, transgene expression, EV titer and potency, with SSI having the advantage of speed, allowing for more rapid iteration cycle times. Thus, both methods are suitable for the precision engineering of therapeutic EVs. This work demonstrates feasibility to manufacture therapeutic engineered EVs from stable cells from either integration strategy for clinical development.

1270. Quantitation of AAV Capsid Proteins by CE-SDS

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Adeno-associated virus (AAV) is a virus consisting of a protein shell which encapsulates a small genome roughly 4.7 kB in size and is dependent on co-infection with helper viruses to replicate. AAV particles are used extensively in R&D and clinical applications due to their nonaggressive nature in humans. Several human AAV serotypes with different tissue tropisms are under investigation in clinical trials as vehicles for gene therapy. The AAV genome contains Rep (replication), Cap (capsid), and assembly genes. The Cap gene produces three viral proteins (VP) known as VP1, VP2, and VP3 which form the outer shell of the capsid and perform host binding. SDS-PAGE followed by silver staining has typically been used to visualize the VP1, VP2, VP3 ratio. This approach is labor and time intensive but yields only qualitative data with poor reproducibility. Here, we describe the use of microfluidic CE-SDS for the characterization of capsid proteins from AAV serotype 8 as the rapid, quantitative, reproducible alternative to SDS-PAGE with silver stain.

1271. Evaluation of AAV Production in Insect Cells Using Chemically Defined Media

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Gene therapy is the fastest-growing market amongst all the regenerative medicines. Various viral vectors are used to facilitate the delivery of the gene of interest. The insect Sf9 cells are one of the many cell lines predominantly used for Baculovirus and Adeno Associated Virus (AAV) production. This study focuses on the evaluation of Lonza's new chemically defined, animal-origin free cell culture medium, which is intended to support the production of AAV as seen by rAAV2 and Anc80 (Lonza's proprietary ancestral AAV) data. This data is further broken down to show the amount of AAV that is intracellular versus extracellular domain. Lonza'sTheraPEAK™ SfAAV™ Medium ensured lot-to-lot consistency, stable doubling time, and increased AAV production compared to other media formulations.

1272. Development of a Scalable Manufacturing Platform for Viral Vectors: Case Study on AAV and Adenovirus Vectors

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The biotech industry is currently living through a period of exciting discoveries as more and more gene therapy treatments for previously unmet indications are entering the clinical trial pathway. However, the global availability and affordability of current and future gene therapy products is threatened by the challenges associated to their manufacture. Producing viral vectors using conventional technologies presents several limitations including scalability constraints and high manufacturing costs of goods (COGs) in the case of flask-based cell cultures, or low productivity and reproducibility when working with traditional fixed-bed bioreactors. In order to overcome these hurdles, manufacturing technologies must be re-designed with the aim of easing

scale-up and reducing COGs. Univercells has developed the NevoLine™ platform, a fully automated, low footprint flexible solution for high throughput cost-effective viral vector production. This platform capitalizes on the principles of process intensification, automation and chaining to miniaturize all the unit operations required for "end-to-end" drug substance manufacture and to integrated these into low-footprint modules. The process sequence inside of the modular units starts with cell culture and virus production within a high-cell-density structured fixed-bed scale-X™ bioreactor chained to an in-line concentration loop for continuous virus harvest and volume reduction. Virus production is followed by an in-line continuous clarification and purification. This presentation will narrate the successful application of the technology principles described above to viral vector production processes with the example of HEK293 cells for rAAV and Adenovirus manufacture. The case study will demonstrate how the use of a structured high-density fixed-bed bioreactor resulted in a significant increase in cell density, viral titers and homogeneity. Moreover, the case study was also extended to illustrate how the innovative design of our platform tackles both the operational and economic barriers to making gene therapy products available and affordable to those in need.

1273. Development of a Highly Productive and Reproducible Manufacturing Process for FX201, a Novel Helper-Dependent Adenovirus-Based Gene Therapy for Osteoarthritis

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INTRODUCTION: Gene therapy offers a new approach to providing long-term efficacy and disease modification in osteoarthritis (OA). FX201 (humantakinogene hadenovec) is an intra-articular (IA) gene therapy in development for knee OA. FX201 is a novel helper-dependent adenovirus (HDAd), an engineered human serotype 5 adenovirus with all viral genes replaced with non-coding stuffer sequences and an expression cassette. The expression cassette encoding for human interleukin-1 receptor antagonist (IL-1Ra) is controlled by an inflammation-inducible NF-κB promoter. Upon IA injection of FX201, joint cells are transduced and conditionally express the potent inflammatory inhibitor IL-1Ra, thereby reducing the inflammation associated with knee OA. Using a fit-for-purpose manufacturing process suitable for early development, we have produced 4 batches of drug substance enabling Good Laboratory Practice (GLP) toxicology, pharmacology, and Good Manufacturing Practice (GMP) clinical studies. One of the 4 batches replaced the expression cassette with the rat ortholog of FX201 (HDAd-ratIL-1Ra) to support in vivo rat pharmacology studies. Here we present the manufacturing and product quality data across the 4 batches, demonstrating a highly productive and reproducible early-stage manufacturing process. **METHODS:** A manufacturing process described in Suzuki et al. (*Hum Gene Ther*. 2010;21(1):120-126) was adapted for GMP. The 2 batches intended for non-clinical studies were produced at twice the scale of Engineering (ENG) and GMP batches due to material requirements needed to support animal studies and analytical development. These 2 batches (FX201 and HDAd-ratIL-1Ra) were used for toxicology and pharmacology studies in rats. The FX201 ENG batch was used to establish a lead-in stability program and create an analytical reference standard. The FX201 GMP batch was used to initiate a long-term stability program and manufacture 3 drug product batches for first-in-human studies. All batches were analyzed for productivity, purification yield, and product quality by measurements of physicochemical properties, infectious and genome titers, product- and process-related impurities, transgene expression, and safety. **RESULTS:** Across 3 batches of FX201 and 1 batch of HDAd-ratIL-1Ra, pre-infection total number of viable cells, pre-purification batch productivities, and post-purification batch yields were within 20%, 30%, and 10% of the respective averages of the 4 batches when normalizing across the process scales used. Process-related impurities, including host cell proteins, were mostly below the level of detection or quantification. An elevated level of residual DNA (rDNA) was observed in the 2 batches used in the GLP toxicology and pharmacology studies. The GLP toxicology study demonstrated that the rDNA-associated tumorigenicity and immunogenicity risks are low. Approximately 150-fold lower rDNA level was achieved in the subsequent ENG and GMP batches due to improvements made in rDNA removal steps. Human IL-1Ra expression was tested using a cell-based assay for 3 batches of FX201 and was within 20% of the average. **CONCLUSIONS:** Productivity, purification yields, and product quality were consistent across all batches produced. These data suggest that reproducibility was achieved across multiple batches of FX201 manufactured using this early-stage process. A single GMP batch of drug substance has yielded sufficient drug product for a Phase 1 study evaluating safety and tolerability of a 100-fold range of total genome copy number in up to 24 patients (NCT04119687). These results demonstrate a viable manufacturing process to support the FX201 gene therapy program through clinical development.

1274. Novel Nucleases Tailor Made for Use in Bioprocessing of Viral Vectors

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When producing viral vectors, biologics or vaccines, unwanted DNA in the final product is an issue that must be handled and monitored. For safety reasons, regulators have imposed limits on the amount and length of DNA-fragments in final dosages for human administration. The residual DNA can originate from several sources, the most common being chromatin from the host cell and plasmids from transfection. The industry standard is to remove residual DNA enzymatically using a bioprocessing grade nuclease during the manufacturing process. As residual nuclease in the final product must also be avoided, DNA digestion is commonly done early in the process, e.g. during the harvest/lysis step. For fragile viral vectors, it is often desired to perform digestion of DNA as close as possible to physiological conditions. When digesting DNA from productions of more robust vectors, high amounts of salt has proven to be beneficial to avoid aggregation and improve removal of DNA. The most used enzymes for DNA removal in bioprocessing originate from the *Serratia marcescens* endonuclease (e.g. Benzonase® and Denarase®). However, these nucleases do not perform optimally at the

physiological conditions found in cell media, nor do they tolerate the high amounts of salt beneficial in some manufacturing processes. Being the industry standard, manufacturers tend to adapt their workflow to conditions optimal for *S. marcescens* nucleases by adding e.g. desalting steps in the workflow to save cost of nuclease, thereby increasing processing time. Another option is to compensate for sub-optimal nuclease performance by using more of it, thereby increasing cost. Instead of adapting the process to an enzyme, using enzymes adapted to the process is favored. We present M-SAN® and SAN HQ®, two nonspecific endonucleases tailor made for viral vector bioprocessing. M-SAN HQ® is a novel nuclease with excellent performance at physiological conditions found in typical cell media. We show that this nuclease outperforms other commercially available nucleases for removal of typical DNA impurities at conditions often used for manufacture of fragile viral vectors such as Lentivirus. SAN HQ® is a nuclease with optimum performance at 0.5M salt. This nuclease allows efficient digestion of DNA directly in salt-supplemented media, resulting in improved DNA clearance and reduced processing time compared to other commercially available alternatives. We show that this nuclease is the superior choice for purification of more robust viral vectors such as Adeno associated viral vectors. Manufacturing viral vectors is a diverse art, with varying processes depending on the vectors manufactured. DNA removal can occur at many different steps in the process and the conditions where DNA removal is desired varies. Using nucleases selected and developed for optimal performance at the conditions relevant for viral vector manufacturing is a helpful tool to achieve purer product and higher titers, at a reduced cost.

1275. Isolator Design in Cell Therapy Facilities

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Cell and gene therapy manufacturing must be performed under aseptic conditions to ensure patient safety. The use of isolators can help address this critical need, but designing isolators for advanced therapy medicinal product (ATMP) processes can be challenging. With so many candidates reaching late-stage clinical development and nearing commercialization, demand for cell and gene therapy manufacturing capacity is rapidly increasing. Despite heavy investment by contract manufacturers to expand their capabilities, they cannot possibly meet the growing needs of the sector; many cell and gene therapy developers are, therefore, establishing in-house facilities. This presentation will address key decisions and challenges facilities are facing when moving to isolators in cell and gene therapy facilities. Below are a few key areas of focus: Quality control - Cell and gene therapies that are produced using viral vectors cannot be terminally sterilized. Therefore, very rigorous quality control is essential in their production, regardless of the batch size -- whether it is a patient-specific autologous therapy or a large-volume batch of an allogenic, off-the-shelf product. The entire production process must be performed aseptically, including extensive quality control testing, in a manner that ensures sterility and efficacy with a high degree of certainty. Open or close the process - The traditional approach has been to perform processes in biosafety cabinets within cleanrooms. However, these processes are not closed but are open to the environment. As such, the cleanroom must be Grade A or B, and with a Grade A biosafety cabinet in an environment that is very tightly controlled. The need for customization - Isolators for cell and gene therapy production are very complex. Considerations must be made for ergonomics to ensure that operators can easily perform necessary manipulations and material can be moved smoothly from one unit operation to another. Transfer in and out -- as with the removal of waste and the collection of samples -- are generally more complicated. Other issues that must be addressed in the isolator design include the heat load it will need to support and the electronic connections necessary to automate the equipment and for data transfer.

1276. Abstract Withdrawn

1277. 2-8ºC Storage Preserves and Extends the Quality of Fresh Whole Leukapheresis Products

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Fresh leukapheresis product (LP) is a critical raw material in providing primary cells for applications in research and manufacturing. While challenging, the preservation of the integrity of the fresh material is key to successful downstream workflows. This puts a constraint on short-term storage and shipping durations to avoid loss of cell viability and functionality. Multiple factors can influence the stability of the fresh product, including temperature. Many commercially available whole LP products are stored and transported at controlled room temperature (CRT), however, studies have shown that when transported under these conditions these products degrade rapidly within 72 hours. In this study, the stability of fresh LPs from unique donors was compared at 2-8ºC and CRT setpoints over 5 days to represent worst case international shipping durations. Samples were assessed daily for cell viability, functionality and activation potency. Results showed that LP Storage at 2-8ºC maintains LP quality across the assessment panel after Day 2 relative to CRT storage, making 2-8ºC a preferred condition for extending LP quality post collection. Transport and storage of these materials at 2-8ºC provides the added benefit of wider accessibility of fresh whole LP products for research use and clinical processing.

1278. Evaluating the Targeting Efficiency and Specificity In Vivo of Lentiviral Vectors Bearing Cell-Specific Ligannds

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Lentiviral vectors have been developed as effective agents for the introduction of DNA into cells ex vivo. However, due to their inherent ability to transduce both dividing and non-dividing cells and their ability to be retargeted through pseudotyping, there is also great interest in using lentiviral vectors to transduce cells in vivo. Recently, we have been working to improve the in vivo use of lentiviral vectors by pseudotyping them with non-human tropic Tupaia paramyxovirus

(TPMV) glycoproteins fused to the IL-13 ligand, in order to target interleukin 13 receptor α2 (IL-13Rα2) expressing tumor cells *in vivo*. Two roadblocks to the *in vivo* use of pseudotyped lentiviral vectors are the inactivation of the vector by pre-existing neutralizing antibodies and off-target transduction events due to non-specific interaction of the ligand with receptor homologs. To address these issues, we have: a) determined the cell-specific transduction efficiency *in vivo* and b) evaluated the on and off-target transduction of the vector system in vivo in a pilot study utilizing a combination of digital droplet PCR (ddPCR) and IHC confocal microscopic assays. This study was conducted using a mouse model of lung cancer with cells expressing IL-13Rα2 and a lentiviral vector pseudotyped with the TPMV H glycoprotein containing an IL-13 ligand. After systemic administration of the lentiviral vector to the tumor-bearing mice, transduction efficiencies and on and off-target vector distribution were assessed. Going forward, we plan on further exploring the combined utility of these, as well as other, methods and adding additional transgenes to simultaneously evaluate the potential therapeutic application of such novel pseudotyped lentiviral vectors.

1279. *SerLESS*, an Adherent HEK293-Derived Producer Cell Line for Manufacturing Lentiviral Vectors in Serum-Free Formulated Media

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R&D, VIVEbiotech, San Sebastian, Spain

Growing interest in the use of lentiviral vectors (LVV) for gene therapy applications has resulted in demand for production processes that are amenable to large scale. However, up-scaling LV manufacturing poses a number of challenges for process developers in order to cost effectively generate a gene therapy product in large quantities to commercial phases. VIVEbiotech is CDMO specialized in LVV manufacturing to clients based in UE, US and Australia ongoing phase I/II clinical trials. However next steps in the development of these treatments will require facing commercial phases. VIVEbiotech production is based on adherent HEK293Tgrowth in fixed bed bioreactors, a system designed to almost linear scaling-up to satisfy phase III and commercial needs. Commercialization requirements limit the use of products derived from animals during manufacturing. The use of serum from animal origin is the main product that compromises standardized lots introducing high variability and to develop a cost-effective production platform as well. VIVEbiotech has developed a new process using formulated media allowing for cell line derived from HEK293T (SerLESS) to grow in adherence without serum supplement. The cell line has been characterized in its growing profile, transfectability and LVV productivity in serum free conditions in both culture flasks and 3D cultures in bioreactors. PEI transfection and production process have been tuned to obtain in the serum free process identical level to the serum-supplemented manufacture process. The talk is aimed at (1) presenting how formulated-media strategy allow to accomplish LVV-production with cell line able to grow in absence of serum, maintaining their essential growing characteristics and state-of-the-art production yields and (2)summarizing the challenges faced and ongoing projects related to infectivity of LVV and DSP-related issue.

1280. Phase-Appropriate Viral Clearance Strategy for Sf9/Baculovirus Based Manufacturing of Gene Therapy Products

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Due to the inherent risk of adventitious and endogenous virus contamination in the manufacturing process of a bio-therapeutic modality, clearance of both classes of virus is a vital objective in the development of a robust purification process. In this study, we evaluate the clearance of model endogenous and adventitious viruses with different physico-chemical characteristics in recombinant Adeno-associated virus (rAAV) material produced in the Sf9/Baculovirus production platform using both early and late stage purification. The ability to successfully demonstrate the clearance of potential adventitious viruses ensures the safe and continuous supply of clinical and commercial products. Results from this study show the reduction of both model adventitious and endogenous viruses using four orthogonal purification steps. The results of this study also provide guidance in choosing viral clearance parameters for rAAV processes and related implications for commercial manufacturing of rAAVs for gene therapy

Pharmacology/Toxicology Studies or Assay Development

1281. Validating QPCR Assays for Gene Therapy Applications - Interpreting and Applying the BMV Validation Guidelines

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There are only a handful of bioanalytical methods that are used to support a wide variety of Cell and Gene Therapy molecules (e.g. modified viral gene therapies, stem cell therapy, CAR-T, oligonucleotides, gene editing...etc), with methods like Flow, Mass Spectrometery and ELISA being used. However, the most common and powerful tool still used in both the preclinical and clinical studies is the Quantitative Polymerase Chain Reaction (QPCR) - a sensitive and robust method for amplifying and detecting concentrations of DNA. Currently there are no specific regulations for the development and validation of QPCR assays, with what limited information there is available dating back more than 10 years. The current QPCR assays are being developed and validated in isolation which has resulted in differing approaches and variability in what parameters are assessed (and to what extent these parameters are being assessed and what acceptance criteria is applied). The number of Cell and Gene Therapy molecules being developed is expected to increase significantly over the next 10 years, and as these molecules will all need to be reviewed and approved by various regulatory authorities around the world there is a requirement to ensure a consist approach to how the QPCR assays are being developed and validated. In light of this there is a current movement that is gaining momentum to apply the existing Bioanalytical Method Validation guidance to the QPCR assays. This

poster will the review the applicability of the Bioanalytical Method Validation and how this guidance can be applied to the QPCR assays. The poster will assess the Bioanalytical Method Validation guidance in detail and present guidance on how the QPCR assays should be developed and validation for future Cell and Gene Therapy molecules.

1282. Toxicology Study of IL-8 Receptor-Modified CD70 CAR T Cells for Clinical Trials

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BACKGROUND: Cancer immunotherapy using chimeric antigen receptor (CAR) modified T cells is a promising new treatment strategy for advanced malignancies, including glioma. Because of tumor heterogeneity, immunosuppressive microenvironments, and inadequate migration/persistence, CAR T-cell therapy targeting solid tumors has stagnated. To tackle these obstacles, we developed the IL-8 receptor (CXCR1 or CXCR2) linked with CD70CAR (8R-70CARs) to extort T-cell intratumoral trafficking and persistence from overexpressed or post-radiation induced IL-8-secreting tumors. Preclinical studies using multiple xenograft mouse models (glioblastoma, ovarian and pancreatic cancer) to test the 8R-70CAR T cells demonstrated enhanced intratumoral trafficking and antitumor response of the 8R-70CAR T cells. However, animal study with an immunocompetent model is required to evaluate potential toxicities, including cytokine release syndrome, neurologic toxicity, and on-target/off-tumor effect, before clinical trial(s). **METHODS:**An all-murine version of the IL-8 receptor (CXCR2) modified CD70CAR (m8R-70CAR) retroviral construct was generated and used for mouse CAR T cell transduction. The transduction efficiency and anti-tumor response of the m8R-70CAR T cells were confirmed *in vitro* by flow cytometry analysis, and IFN-γ production using murine glioma cell line, KR158 overexpressing CD70. C57BL/6J female mice (6-8 week-old) were stratified into 4 groups (6 mice/group): 1) Healthy mice without any treatment. 2) Mice with total body irradiation, TBI (5Gy). 3) Mice with a single dose of the m8R-70CAR T cells ($2x10^6$). 4) Mice with TBI and m8R-70CAR T cells. The CAR T cells were administered via tail vein injection on day 0. The body weight and clinical signs of toxicity were monitored weekly, and blood hematology, serum chemistry, serum cytokines, gross pathology, and histopathology of 12 vital organs were analyzed 3, 30 and 100 days after the m8R-70CAR T cell injection. **RESULTS:**The transduction efficiency of the m8R-70CAR was 72.1% based on the CXCR2 expression on CD3+ T cells, and the CAR T cells highly recognize murine CD70 expressing glioma cells. Overall, TBI treatment affects nearly all parameters tested, including the body weight (12.9% decrease beginning in the first week and returning to normal 2 months after the TBI), the white blood cell (WBC) counts (~90% decrease on day 3 and recovered beginning on day 30 post TBI), and cytokines in serum. No significant difference was observed in blood chemistry among the 4 groups at all time-points. Histopathologically, marked-to-severe lymphoid and/or hematopoietic depletion of spleen, cervical lymph nodes, thymus, and bone marrow were revealed in the TBI treated groups starting on day 3, and partial recovery was observed on day 30. Signatures consistent with typical long-term irradiation effects were also observed, including minimal-to-mild severe cortical lymphoid depletion in the thymus and decreased hematopoietic cellularity in the bone marrow, as well as increased incidence of minimal hyaline casts within renal tubules on day 100. However, we did not find that m8R-70CAR T cells affected any of the tests, i.e., no CAR T cell related toxicity was observed. **CONCLUSION:** No CAR T related toxicity was observed. The m8R-70CAR T cells are safe in a syngeneic animal setting. The first phase I trial using 8R-70CAR T cells in patients with newly diagnosed, MGMT-unmethylated glioblastoma, will be initiated soon at the University of Florida.

1283. Purity and Identity Characterization of Adeno-Associated Virus Capsid Particles by Intact and Bottom-Up Based Liquid Chromatography-Mass Spectrometry Methods

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Introduction: Adeno-associated viruses (AAVs) are the main viral vectors for gene therapy and have been successful in treating inherited retinal diseases and spinal muscular atrophy (SMA). AAV is composed of an icosahedral protein shell with a single-stranded genome of approximately 4.7kb. The intact AAVs act as a vehicle to protect and deliver oligonucleotide therapeutics. As AAVs continue to be explored as therapeutic delivery platforms, it is vital to ensure that all the critical quality attributes (CQA) of the therapeutic product are maintained. Here we developed liquid chromatography mass spectrometry (LC-MS) methods for AAV characterization for reduced subunit and peptide mapping analysis. Our results confirm both molecular weight with accurate mass information, and post-translational modifications (PTMs) of all three viral capsid proteins. **Methods:** For intact analysis, both empty and full AAVs were incubated with dithiothreitol at 60°C for 1 hour. Capsid proteins were then chromatographically separated on a 2.1 x 150 mm Zorbax RRHD Diphenyl column and analyzed on a 6545XT Q-TOF mass spectrometer. For maximum sensitivity, a large molecule SWARM autotune was performed. For peptide mapping, proteins were reduced, alkylated, and incubated with trypsin overnight (37°C). Peptides were separated on a 2.1 x 150 mm AdvanceBio Peptide Mapping column and iterative MS/MS analysis on a 6545XT Q-TOF mass spectrometer was used for data acquisition. MassHunter BioConfirm 10.0 software was used for confirmation and relative quantitation of intact protein mass measurements, peptide sequences, and site-specific PTMs. **Preliminary Data:** Characterizing viral capsid proteins yields several challenges. The protein shell is composed of three capsid proteins, VP1, VP2 and VP3, that assemble into a 3.9 megadalton structure in a ratio of 1:1:10 with 60 capsids per virion. In addition to the low molar ratios of VP1 and VP2, all three proteins have overlapping sequences at the C-terminus; VP1 and VP2 are very difficult to separate with some serotypes. Another challenge and question in AAV characterization is quantifying the actual ratio of VP1, VP2, and VP3. An LC/MS method was developed to chromatographically separate each capsid protein and their major

variants. High resolution mass spectrometry data yields excellent mass accuracy, under 15 ppm for all proteins and variants and under 10 ppm in most cases. Relative quantitation of each protein with its variants was determined to elucidate the ratio of VP1, VP2, and VP3. A peptide mapping method was developed to confirm sequence coverage and relative quantitation of site-specific PTMs, such as phosphorylation, deamidation, and oxidation. Excellent sequence coverage was obtained for each protein, over 95% with MS/MS confirmation.

1284. Development and Optimization of A qPCR Method to Assess Biodistribution of an AAV5 Vector in Nhp and Mouse Studies

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Biodistribution and shedding studies are a prerequisite for the development of AAV-based gene therapy. There are several regulatory guidelines supporting gene therapy development, however there is limited guidance related to the associated bioanalytical requirements. During validation of these assays there are several challenges to be addressed, such as appropriate nucleic acid extraction methods for the different matrixes, appropriate normalization references, qPCR assays with high sensitivity (50 copies per microgram of genomic DNA), assessment of PCR inhibitory effects and others. In this study, we describe the development and optimization of a multiplex qPCR assay by determining analytical parameters such as extraction efficiency, matrix effect, linearity and dynamic range, inhibition control, amplification efficiency, sensitivity, specificity, precision and accuracy. Firstly, we optimized the DNA extraction from different types of matrixes by using an automated nucleic acid extraction platform. Secondly, upon selection of appropriate primers and probes, we tested different Taq polymerase mixes, using 2x and 4x concentrated Taqman and different primer-probe concentrations. The most optimal primer-probe concentration was chosen and tested for duplex qPCR set up and optimization. This duplex qPCR amplification combined the use of FAM-labeled probe for the target region and a VIC-labeled exogeneous internal positive control (exo IPC) in a single reaction, in order to control for PCR inhibition. The better-performing Taqman enzyme, with a significantly higher PCR efficiency, was finally compared in duplex qPCR in the absence and presence of NHP or mouse brain DNA matrix. Combining the automated nucleic acid extraction and a qPCR with a significantly higher PCR efficiency we developed an high-through put and highly sensitive assay.

1285. Utilization of the N-PLEX™ Platform for the Detection of Antisense Oligonucleotides (ASOs) in Plasma

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Introduction: The number of FDA-approved drugs that are revolutionizing treatment strategies for cancer and other diseases is on the rise. One of the categories of drugs that is gaining prominence is antisense oligonucleotides (ASOs), which are modified, single-stranded DNA or RNA molecules (generally less than 30 nucleotides). These ASOs are complementary to specific mRNA sequences and alter production of the various corresponding proteins. Due to their short length, however, it is difficult to measure ASOs in circulation for PK studies. HPLC-MS/MS is frequently used to detect ASOs in biological samples, which provides good specificity but low sensitivity. Hybridization ELISA techniques have increased sensitivity and have also been described, but greater sensitivity is still needed in many cases to enable thorough PK studies. **Methods:** Using MSD's highly sensitive electrochemiluminescence technology, we paired oligonucleotide ligation assays (OLAs), T4 ligase-mediated probe ligation assays, and RNase protection assays with detection on the N-PLEX platform. This achieved sub-pM detection of a model, 20-mer DNA ASO in plasma samples. For OLA- and T4 ligase-mediated detection, two probes were used that, when bridged by the ASO, were ligated together by either *Taq* DNA ligase or T4 DNA ligase, respectively. One probe contained a sequence complementary to a capture sequence that was bound to N-PLEX plates, whereas the other probe contained a 5' phosphate group for ligation and a 3' biotin for detection via streptavidin bound to the MSD® proprietary SULFO-TAG™ reagent. OLAs use multiple ligation cycles, whereas T4 ligase-mediated detection is limited to one ligation event. The RNase protection assay uses a chimeric probe that contains a 5' DNA sequence complementary to the plate-bound capture oligo, followed by an RNA portion that is complementary to the ASO of interest and a 3' biotin for detection via a streptavidin SULFO-TAG label. Once the probe has been hybridized to the ASO and the plate, RNase is added to the plate to degrade the RNA portion of the probe to release biotin if the probe is not bound by the ASO. **Results:** In plasma samples, the OLA-mediated, the T4 ligase-mediated, and the RNase protection assays reproducibly provided detection of ASOs of approximately 100 fM or lower when performed on the N-PLEX platform. All three of these assays can easily be completed in one working day, with T4-mediated detection affording the shortest time to answer (~4.5 hours). Factors to consider when designing assays for the detection of ASOs on the N-PLEX platform include the length and modifications of the specific ASO, type of biological matrix, assay time, and requirements for sensitivity and specificity. **Conclusions:** These data indicate that, paired with various techniques, the N-PLEX platform is a viable option for highly sensitive detection of ASOs in complex biological matrices.

1286. Analytical Testing Strategy for AAV Vectors Produced by the Baculovirus/Sf9 System

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The baculovirus/Sf9 system is a scalable expression platform that can produce large quantities of high quality rAAV product suitable for gene therapy delivery. Voyager Therapeutics has been developing a strategy to efficiently and comprehensively test AAV vectors produced in Sf9 cells using baculovirus. This approach uses both platform assays that only need to be optimized for different vectors and assays developed for specific targets. This strategy also needs to include assays specific for the idiosyncrasies of the baculovirus/Sf9 platform, as well as assays for the characterization of the baculovirus-infected insect cell stocks used as starting reagents. The analytical methods used can be functionally

grouped into the following categories: Safety, Identity, Purity, and Potency, and include vector titer, infectivity, residual protein and DNA analysis, and empty/full capsid quantitation. The introduction of more robust, higher-throughput, next generation assays is also presented. This approach has been utilized on multiple AAV vectors produced at Voyager for pre-clinical Toxicology Studies and Clinical Trials.

1287. Determining the Metabolic Consequences of Pharmacological Modulation of T Cell Activation

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T cells are one of the most actively investigated immune cell types for cell-based therapies, particularly in the immuno-oncology area. *In vitro* T cell activation and subsequent proliferation/differentiation are widely used as a standard platform to design and engineer specific T cells activity for therapies, as well as to scrutinize and predict the function and regulation of T cells *in vivo*. Recent findings have demonstrated that dynamic metabolic reprogramming is highly associated with T cell activation and appears to act as a key regulatory node determining the proliferation and differentiation of T cells through its life cycle. This deeper understanding is now being exploited pharmacologically to further expand. In this study, as many T cell-targeting pharmacological agents are considered to be employed in immune cell therapy strategies, the effects of several of these agents on the metabolic response of T cells during and post-activation was examined. The Agilent Seahorse XF Analyzer enables rapid quantitative measurement of the immediate-early glycolytic response to CD3/CD28-mediated T cell activation, as well as the assessment of real-time ATP production rates across the T cell life cycle. By using this combinatorial approach, the effects of immuno-directed agents can be comprehensively investigated from a metabolic perspective. For example, the modulation of T cell receptor signaling by tyrosine kinase inhibitors reveal a dose-dependent suppression of early activation-associated glycolytic reprogramming. In contrast, immunosuppressive agents, such as cyclosporin, which target downstream pathways, only modulate metabolic events associated with later stages of the T cell life cycle, impacting both mitochondrial and glycolytic ATP production rates. These data highlight the utility of accessible, functional metabolic analysis in underpinning a comprehensive understanding of T cell metabolism that will be key to informing strategies to deliver optimum therapeutic efficacy and efficient cell production.

1288. Adenovirus Titer Determination by Infectivity and Plaque Assay in Plasma and Tissue

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Viruses are a major etiological cause of disease, but are also increasingly used in multiple therapy approaches, such as gene and oncolytic therapy. To accurately track infection or characterize virotherapeutic agents, it is important to accurately determine viral levels, either it be in formulation suspension, blood, excretions, or tissues. This determination can take multiple forms, such as genome quantification by qPCR, detection of structural proteins by ELISA, or histological staining. Accurate determination of infectious titer involves laborious laboratory work that is difficult to scale up for high throughput assays and that usually requires manual evaluation of results. However this determination is part of the FDA guidance for industry documentation on Cell and Gene Therapy (Section IV) for the measurement of total particles vs infectious particles and the detection of replication competent adenoviruses (RCA) in gene therapy. This characterisation also evaluates the shedding of infectious virus. In this poster we are presenting an implementation of infectivity evaluation using TCID50 and plaque forming unit determination that uses quantitative methods that are automated and not user subjective. In addition, we also demonstrate limits of matrix concentration and the ability to detect infectious viral particles across multiple sample types typical of adenovirus infection. For TCID50 determination, we are using a colorimetric assay to detect cell survival upon infection in a 96 well format. This increases the read speed of infectivity results and standardizes detection of cytopathic effect. Plaque forming assay was performed using standard methodology in regards to viral growth and staining. However, plates were read using the automated BioTek Cytation 5 Cell Imaging Multi-mode reader. This system, along with a custom designed automated analysis protocol allows the consistent and objective counting of plaques along with producing high definition images of each well. This prevents user bias in counting and allows all data to be saved electronically. The developed methods allow for objective and standardized detection of viral titer.

1289. Strategies to Improve Data Quality and Maximize Throughput of Sedimentation Velocity Analytical Ultracentrifugation Experiments to Characterize AAV Gene Therapy Products

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Sedimentation velocity analytical ultracentrifugation (SV-AUC) is the gold-standard method to quantify major species present in AAV gene therapy products, including empty, partially filled, full, and overfilled particles, lower-molecular weight species, and aggregates. Chromatographic methods have largely replaced SV-AUC for protein therapeutics due to their relative ease in performance and greater throughput, but the development of robust chromatographic methods for gene therapy products has proven to be challenging. Subsequently, SV-AUC is required to measure critical quality attributes, despite the inherent challenges of the method, largely centered around the expertise required to successfully perform. Here, we will present strategic optimizations that can be implemented by any lab to improve data quality, maximize throughput, and minimize sample data turnaround times.

1290. Molecular Combing, an Innovative Quality Control Assay for Gene Therapy Development and Production

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Gene and cell therapies have surfaced as promising treatments for several diseases, including viral infections, hereditary disorders and cancer. Recent advances in the development of gene editing technologies, based on the CRISPR-Cas9 system, have enabled rapid and accessible genome engineering in eukaryotic cells and living organisms. New methods continue to be developed with the aim of providing consistent and safer gene therapies. Albeit these efforts, these methods can still result in a wide range of unintended modifications that hamper their full translation into the clinic. Here we present a new quality control assay (QCA) for gene and cell therapy based on molecular combing, a proprietary technology to directly visualize single DNA molecules. Associated with a unique detection strategy the Genomic Morse Code, the technology allows, in a single experiment, to analysis mid-size to large structural variants (>2kb to several Mb) at the target locus. Our QCA is highly sensitive, requires no DNA amplification and is compatible with a variety of sample types (heterogeneous cell lines, organs (liver), blood…). We will present and discuss different examples that illustrate how our molecular combing based QCA allows for (i) the digital quantification of rare and complex on-target rearrangements induced by CRISPR-Cas; (ii) the analysis of transgene copy number and pattern of integration (single vs multiple copies, tail to tail vs head to tail, intact vs rearranged copies…). As such, our QCA presents a powerful analytical tool to generate high-quality cell lines and monitor their stability throughout the manufacturing process, a critical step in the development and production of biological drugs. The advantage of using molecular combing as a quality control assay for the fine genetic characterization of cell line stability and transgene integration patterns over conventional methods as well as its use to accurately quantify the efficiency, precision and safety of novel gene editing based therapies will be discussed.

1291. Challenges and Strategies for Regulated Enzyme Activity Assay Bioanalysis

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Introduction: Cell and gene therapy (CGT) is a rapidly growing therapeutic area. CGTs are often treatments for rare genetic mutations that affect endogenous enzyme production or function. Enzymatic activity assays are a critical part of the pre-clinical and clinical bioanalytical strategy to assess the pharmacokinetics and efficacy of gene therapies. There is limited regulatory guidance that pertains specifically to bioanalytical enzymatic assays and the general industrial consensus is to use the FDA BMV and Biomarker guidance documents as a framework for enzymatic assay development and validation. However, enzyme activity assays present a unique set of challenges with limited regulatory guidance for the bioanalytical scientist to ensure that the assay can reliably measure enzymatic activity in a regulated environment. Enzyme activity assays present distinct challenges including sensitivity to laboratory conditions, e.g., temperature, reaction time and pH, lack of a true reference material and enzyme stability. Normal laboratory and analytical conditions may lead to assay drift and require increased monitoring during the life cycle of an enzymatic assay. These challenges require solutions that are tailored to each assay and may be different to the process and system controls used for traditional chromatographic and ligand binding (LBA) assays used in the same context. Here, we present an overview of our experiences with regulated enzyme activity assay bioanalysis, including case studies, recommendations for enzyme assay characterization and life cycle management.
Bioanalytical Challenges: Discussion and Case Studies:
Robust Analytical Process Control
Enzyme assays tend to be more sensitive to laboratory and reagent variability than LBA or chromatographic assays. Analytical process parameters including, laboratory temperature and buffer pH, need to be closely monitored to ensure that activity assays are precise and accurate.
Enzyme Activity Calibrator Approach
Enzyme activity assays generally use a non-detectable molecule that is specifically cleaved by the enzyme of interest into a detectable subunit of the parent molecule. The cleaved product is then detected using colorimetric or fluorescent spectrophotometry. Enzyme activity can be determined using either a calibration curve prepared from the enzyme cleavage product (static curve) or a calibration curve prepared from an enzyme stock of known activity (dynamic curve).
QC Sample Preparation and Monitoring
Quality control samples in enzyme assays must be able to monitor both the assay system (system suitability controls) and represent patient samples (endogenous enzyme controls). Setting acceptance criteria for enzyme activity assays has been particularly challenging due to the dynamic nature of the assay system. The statistical approach used to set acceptance ranges should be statistically appropriate to the assay.
Conclusion: Enzymatic activity assays are a critical component to the bioanalytical package of CGT. Due to the dynamic nature of enzyme activity assays, close control and monitoring of assay performance is required. The lack of true reference material and complex assay system also require novel approaches to assay calibration material to ensure reliable long-term assay performance. Assay performance should be monitored using quality control samples that represent both the assay system and patient samples. Further, rational statistical approaches need to be used to set acceptance criteria and monitor assay performance. As the regulatory landscape of biomarker and enzymatic bioanalysis evolves, it will be important to incorporate the expertise of bioanalytical laboratories into new guidelines. This will allow for more reliable and reproducible bioanalytical results to support emerging cell and gene therapies.

1292. A qPCR Assay for the Detection of VSV-G Sequences in Total Peripheral Blood Leukocytes and Whole Blood for Replication Competent Lentivirus Testing in Patient Follow-Up Visits

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Current FDA guidance specifies that clinical trial patients receiving gene therapy products involving the use of retroviral vectors receive replication competent retrovirus (RCR) testing for at least one-year post treatment.

Detection of RCR-specific sequences in peripheral blood mononuclear cells (PBMCs) by qPCR is the recommended methodology to meet the guidance. In the case of lentiviral vectors, the Vesicular stomatitis virus glycoprotein (VSV-G) sequence is the most common target. Isolation of PBMCs, by design, removes neutrophils from the cell population. Although direct transduction of neutrophils by VSV-G pseudotyped lentivirus is controversial, transduction and integration of upstream stem cells is possible. Therefore, as neutrophils compose 40-60% of all leukocytes, they may serve as a large reservoir of RCR sequences. As part of characterizing a new qPCR assay for VSV-G sequences, we sought to qualify detection in the entire peripheral leukocyte population by testing DNA isolated direct from whole blood (collected in EDTA) or leukocytes isolated by red blood cell lysis. The detection range of VSV-G sequences is based on a five-point standard curve of 1E1 to 1E6 copies of linearized VSV-G plasmid spiked into genomic DNA. The purpose of the genomic DNA is to allow for a multiplexed qPCR assay of a reference gene (Apolipoprotein B) for confirmation of the presence of 200 ng of test article DNA per well. Our preliminary data based on multiple, independent runs shows that the VSV-G qPCR assay averaged slopes of -3.39, R-squared values of 0.99, and 97% reaction efficiencies (the Apolipoprotein B assay has similar values). The inter-assay %CV was < 15 for each standard. Back calculated VSV-G copies from each standard replicate revealed %CVs between 1-16 at each standard concentration suggesting the assay has excellent precision. Sensitivity was demonstrated by spiking DNA from whole blood or isolated leukocytes with 20 copies of linearized VSV-G plasmid and recovering 60-140% of the expected value. Specificity was shown by a lack of amplification in unspiked genomic DNA from whole blood or isolated leukocytes. These data suggest an apparent limit of detection of at least 10 VSV-G copies and a limit of quantitation of 20 VSV-G copies in 200 ng of DNA. Taken together, our assay provides an acceptable platform for VSV-G sequence detection in whole blood and peripheral blood leukocytes for patient follow-up where an EDTA blood sample could be sent to a central laboratory. It is anticipated the assay could also be used to test manufactured cell therapy products for lot release.

1293. Distribution and Frequency of Anti-AAV Neutralizing Antibodies in Different Species of Non-Human Primates

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Introduction: Adeno-associated virus (AAV) vectors have been widely used for gene therapy-based medicines and pre-existing immunity in the form of neutralizing antobodies (NABs) against the viral capsids of AAV vectors is a major hurdle for successful AAV-based gene transfer in non-human primates. For this reason we wanted to investigate the incidence, frequency and distribution of AAVs in the two major species of non-human primates used in preclinical research. Methods: More than a thousand naive Cynomolgus monkeys (Macaca fascicularis) and more than two hundred naive Rhesus monkeys (Macaca mulatta) were screened for NABs against the major AAVs using cell-based neutralizing titer assays. Results: The distribution and frequency of anti-AAV NABs is described in the Table below:

Distribution of Anti-AAV Nab							
Species	AAV1	AAV2	AAV3b	AAV4	AAV8	AAV9	AAVrh10
Cynomolgus		93%	96%		79%	60%	48%
Rhesus	68%			22%	78%	59%	

Conclusion: Our data indicates that the incidence of NABs for the major clinically-relevant AAV strains is moderate to high in the two Macaca species investigated. Our data clearly demonstrates that individual anti-AAV NAB pre-screening is necessary before any AAV-driven gene therapy study. These data also support the importance of having access to large populations of naive non-human primates in order to be able to identify enough sero-negative animals for conducting preclinical studies.

1294. Abstract Withdrawn

1295. Express AAV Titration ELISAs - Finish it Faster

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A growing number of academic and industrial labs are using AAV vectors for the development of gene therapies leading to an increase in the demand for effective and reliable analytical AAV tools for R&D and manufacturing. To enable safe and effective AAV gene therapies, a dependable and reproducible quantification of accurate rAAV titers is needed to ensure secure and reliable gene transfer. PROGEN's AAV Titration ELISAs are widely used for the quantification of intact AAV capsids, thus to determine the total capsid titer including full & empty capsids. In combination with additional quantification methods, characterizing different AAV titers (e.g. genomic titer), PROGEN´s AAV Titration ELISAs are a robust and reliable tool for the comprehensive characterization of AAV preparations. In order to reduce the assay time from 4-5 hours (standard AAV ELISA) to less than 2 hours, we have adjusted the kit components and the protocols for the titration ELISAs for AAV2, AAV8 and AAV9. The corresponding adjustments resulted in the shortening of the incubation steps from 60 to 20 minutes each, thus leading to a reduction of the assay time of more than 50%. This was achieved by adapting reagent concentrations without changing the composition of the kit's core characteristics, allowing a seamless and risk-free transition of users from the standard AAV ELISAs to the faster version. Here we demonstrate that these faster titration ELISAs show the same accuracy and reproducibility as the corresponding standard ELISA kits but save a significant amount of time for the users. With these faster AAV ELISAs, users can rely on the well-established quality of PROGEN's reagents for accurate titer determination of AAV samples.

1296. Abstract Withdrawn

Clinical Trials Spotlight Symposium

1298. Resolution of Sickle Cell Disease (SCD) Manifestations in Patients Treated with Lentiglobin Gene Therapy: Updated Results from the Phase 1/2 HGB-206 Group C Study

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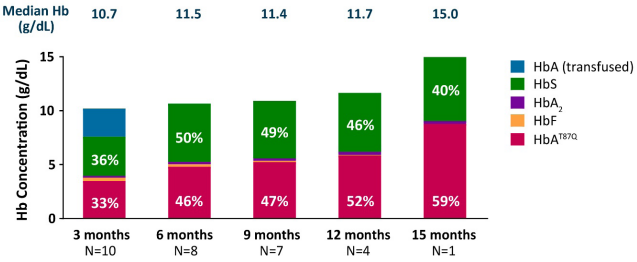
LentiGlobin for SCD gene therapy contains autologous CD34+ hematopoietic stem cells (HSCs) encoding β-globin with the anti-sickling T87Q substitution ($\beta^{A\text{-}T87Q}$). It is being evaluated in patients with SCD in the ongoing phase 1/2 HGB-206 Study (NCT02140554). The initial 7 patients (Group A) were treated with LentiGlobin made from bone marrow harvested HSCs. To increase gene therapy-derived hemoglobin (HbA^{T87Q}) production, changes were made to the protocol and manufacturing process (Group B, n = 2). Further, in Group C, which is discussed here, HSC collection by plerixafor mobilization and apheresis was instituted. CD34+ HSCs were collected by apheresis following plerixafor mobilization in adults with SCD who had complications such as recurrent severe vaso-occlusive crises (VOC) and acute chest syndrome (ACS). CD34+ HSCs were transduced with the BB305 lentiviral vector to make LentiGlobin drug product (DP), which was infused into patients after they received myeloablative busulfan conditioning. Data are shown as median (min-max). As of 7 March 2019, 13 Group C patients were treated with a follow-up of 9.0 (1.0-15.2) months. They received DP with vector copy number of 3.8 (2.8-5.6) copies/diploid genome, 80% (71%-88%) of transduced cells, and 4.5 (3.0-8.0) x 10^6 CD34+ cells/kg CD34+ cell dose. All but 1 patient had neutrophil and platelet engraftment at the data cut date. In patients with ≥ 6 months follow-up (n = 8), median HbS was ≤ 50% of total Hb (Fig 1). Without transfusions, total Hb at last visit in these 8 patients was 11.5 (10.2-15.0) g/dL, with HbA^{T87Q} of 5.3 (4.5-8.8) g/dL. Six of the 8 patients had a history of VOCs or ACS and their annualized VOC+ACS rate decreased from 5.3 (3-14) pre-treatment to 0 (0-2) post-treatment. Hemolysis markers also were decreased post-treatment. The most common non-hematologic Grade ≥ 3 AEs post-infusion were febrile neutropenia (n = 10) and stomatitis (n = 7). Six patients reported serious AEs post-DP, most commonly nausea and vomiting. No cases of DP-related AEs, graft failure, vector-mediated replication-competent lentivirus, or clonal dominance were observed. Patients in HGB-206 Group C show sustained expression of gene-therapy derived Hb, with median total Hb > 10 g/dL and median HbS ≤ 50% of total Hb in those with ≥ 6 months follow-up. The decrease in SCD-related complications and hemolysis in this cohort demonstrate a strong therapeutic benefit of LentiGlobin in patients with SCD.

Figure 1: Median total Hb and Hb fractions at various follow-up time points in HGB-206 Group C



1299. First-In-Human Gene Therapy for Tay-Sachs Disease: Report of Two Infants Treated on an Expanded Access Clinical Trial of rAAVrh8-HexA/HexB (AXO-AAV-GM2)

Terence R. Flotte[1], Oguz Cataltepe[2], Ajit Puri[3], Rita Batista[1], Richard Moser[2], Diane Mckenna-Yasek[4], Catherine Douthwright[4], Gwladys Gernoux[1], Meghan Blackwood[1], Christian Mueller[1], Scot Bateman[5], Spiro Spanakis[6], Julia Parzych[6], Allison M. Keeler[1], Aly Abayazeed[3], Laura Gibson[7], Robert Finberg[8], Matthew Gounis[3], Robert H. Brown[4], Heather Gray-Edwards[1], Miguel Sena-Esteves[1]

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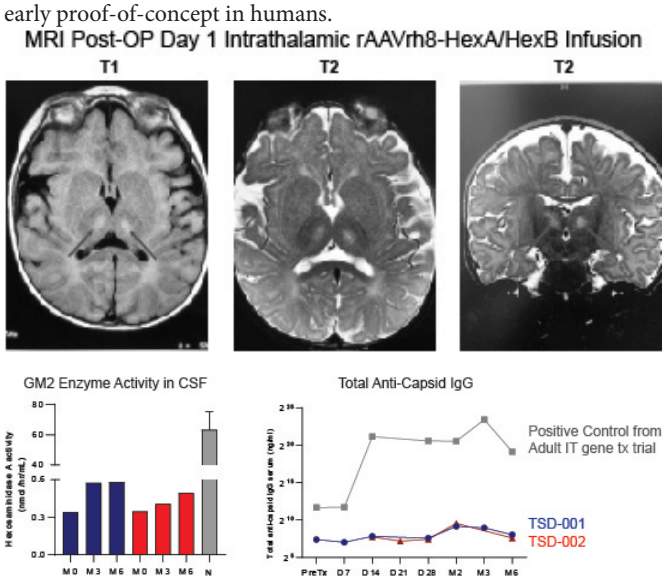
Tay-Sachs Disease (TSD) is a monogenic disorder due to deficiency of Hexosaminidase A (HexA). Previous work demonstrated safety and efficacy of rAAVrh8-HexA/HexB CNS gene therapy in preclinical models. An expanded access trial was undertaken in two patients with infantile TSD. The first patient (TSD001) demonstrated neurodevelopmental regression by 8 months of age and was treated at 30 months of age. The patient underwent immunosuppression with sirolimus, corticosteroids, and infusion of anti-CD20 antibody. An equimolar mix of 1x10^{14} vg rAAVrh8-HexA/rAAVrh8-HexB (AXO-AAV-GM2) was administered intrathecally (IT), with 75% of the dose delivered to the cisterna magna (CM) and 25% at the thoraco-lumbar junction. The second patient (TSD002) was treated at 7 months of age after similar immunosuppression with a total dose of 4.6x10^{13}vg. TSD002 underwent stereotactic, robotic-assisted intrathalamic (Thal) injection of 0.18 ml of vector into each thalamus, while the remainder of the vector was given IT as in TSD001. These procedures were well tolerated and have shown no vector-related adverse events to date. Serum and CSF HexA enzyme activity increased from baseline, and the post-treatment CSF HexA enzyme activity was greater than the

serum Hex activity in both patients, and remained stable. The maximal post-treatment CSF enzyme activity, 3 to 6 months post-treatment, was greater than 0.5 nmol/hr/ml in each of the two patients, and was approximately double the pre-treatment baseline. Both patients have been seizure-free since vector injection. In TSD002, MRI, DTI and neurodevelopmental assessments showed stabilization of disease at 3 months post, with slow decline at 6 months. In summary, administration of AXO-AAV-GM2 by IT and ITh routes was safe, increased HexA activity and enabled ongoing myelination, providing early proof-of-concept in humans.



1300. Lentiviral Gene Therapy with Autologous Hematopoietic Stem and Progenitor Cells (HSPCs) for the Treatment of Severe Combined Immune Deficiency Due to Adenosine Deaminase Deficiency (ADA-SCID): Two Year Follow-Up Results

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Background: ADA-SCID is a rare disorder caused by *ADA* gene mutations leading to compromised immune function. OTL-101 is an experimental gene therapy (GT) consisting of autologous $CD34^+$ HSPCs transduced *ex vivo* using a self-inactivating lentiviral vector (EFS-ADA LV) encoding the human *ADA* cDNA sequence under the control of a shortened human elongation factor 1α gene promoter. Here we report the safety and efficacy of OTL-101 in 30 ADA-SCID subjects (sbj) with 2 yr follow-up (FU), compared to a historical cohort of 26 ADA-SCID patients (pts) treated with hematopoietic stem cell transplant (HSCT) without or with a matched related donor (MRD). **Methods:** GT Group: Fresh or cryopreserved OTL-101 was infused into 30 sbj [13 male, 17 female; aged 4 - 51 months (mo)] in two clinical trials (NCT01852071, NCT02999984). Prior to infusion, busulfan was given as a single dose (4 mg/kg) or in 2 doses, the first at 3 mg/kg and the second adjusted to target a total area under the curve of 4,900 μM*min (20 mg/L*hr). Historical Control Group: 26 pts (aged 0 - 118 mo) were treated with allogeneic HSCT (without MRD, n=14; with MRD, n=12) at GOSH, UK (n=16) or Duke University Children's Hospital, USA (n=10) from 2000-2016. **Results:** Sustained engraftment and metabolic detoxification were observed in 29/30 GT sbj by 6 - 8 mo; 1 sbj was withdrawn due to lack of engraftment. At 2 yr FU, the GT group had higher rates of overall survival (OS), event-free survival (EvFS, survival in the absence of enzyme replacement therapy

or rescue HSCT), and cessation of immunoglobulin replacement therapy (IgRT) compared to the HSCT group without or with an MRD (**Table**). 12 GT sbj experienced one or more serious adverse events, 1 of which was considered treatment-related (bacteremia due to product contamination). In the GT group, there were no autoimmunity events or graft vs host disease (GvHD); in contrast, 8 HSCT pts experienced GvHD, 1 of whom died. **Conclusions:** Treatment of ADA-SCID with OTL-101 has a favorable benefit-risk profile with sustained gene correction and restoration of immune function in all engrafted sbj through 2 yr FU. In the GT group, higher rates of OS, EvFS and IgRT cessation were observed compared with the HSCT group.

2 Year Follow-up Results				
	OTL-101 (n=30)	**HSCT Overall (n=26)**	**HSCT without MRD (n=14)**	**HSCT with MRD (n=12)**
Overall Survival, %	100	88	86	91
Event-free Survival, %	97	56	50	64
IgRT cessation, % (n/N)	86 (25/29)	55 (12/22)	42 (5/12)	70 (7/10)

1301. Natural Killer T Cells Expressing a GD2-CAR and IL-15 for Children with Neuroblastoma - A First-In-Human Phase 1 Trial

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Background: Despite remarkable progress in B cell malignancies, T cells expressing chimeric antigen receptors (CARs) remain largely ineffective in solid tumors in part because T cells do not traffic effectively to or survive in tumor tissues. Due to their natural anti-tumor properties and ability to localize to the neuroblastoma (NB) tumor site, Vα24-invariant natural killer T cells (iNKTs) are promising candidate immune effectors for CAR-based immunotherapies targeting NB and other solid tumors. In pre-clinical studies, iNKTs expressing an optimized GD2-CAR with CD28 costimulatory endodomain and IL-15 (CAR.GD2.IL-15) demonstrated superior antitumor properties in a xenogeneic NB model. These results provided rational for clinical testing of iNKTs expressing CAR.GD2.IL-15 in NB patients. **Methods**: Following validation of current good manufacturing practice (cGMP) protocols for generation and clinical-scale expansion of CAR-iNKTs, we initiated a phase 1 clinical trial to assess the safety, persistence, and efficacy of autologous CAR-iNKTs in patients with relapsed/refractory NB (NCT03294954) using a 3+3 dose-escalation design. Five patients enrolled to date have received a single injection of therapeutic cells; three patients were treated on dose level 1 ($3x10^6$ CAR+iNKTs/m^2) and two patients on dose level 2 ($10x10^6$ CAR+iNKTs/m^2) following lymphodepletion. **Results**: Patient-derived iNKTs were successfully expanded to produce ≥10^9 CAR-iNKTs within 9-17 days, resulting in 95.6% median iNKT purity (90.4-97.17% range) and 46.9% median CAR expression (20.18-81.68% range). Lymphodepletion-induced toxicities were detected as expected. Patients did not experience Grade 2 or higher toxicities related to CAR-iNKTs. All five treated patients showed evidence of CAR-iNKT expansion peaking at three weeks post-infusion; expansion continued through week 4 in one patient (**Fig 1 A,B**). Peripheral blood IL-15 levels initially increased and returned to close baseline by week 2, IL-7 remained close to baseline throughout. We detected an increase in Fms-like tyrosine kinase 3 ligand (FLT3L) serum concentration for 3-4 weeks in two of three evaluated patients. CAR-iNKTs in these patients expanded and persisted better than those from the other evaluated patient, suggesting that FLT3L could contribute to this phenotype. Analysis of tumor biopsies showed CAR-iNKT trafficking to metastatic lesions in three patients. Clinically, two patients progressed and two patients had stable disease at 4-6 weeks post-infusion. One patient with two established metastatic bone lesions prior to therapy experienced complete regression of a right femur lesion and near complete regression of a large sternum lesion as determined by single-photon emission computed tomography imaging at 4 and 8 weeks post-infusion (**Fig 2**).

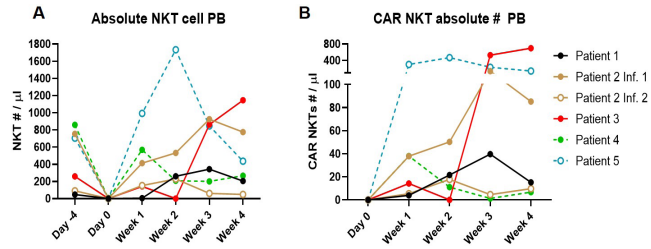

Figure 1. CAR-NKTs expand and persist in patients with relapsed/refractory NB. **A**) Absolute NKT numbers in peripheral blood as determined staining for CD3+iNKT+Vβ11+ cells by flow cytometry. **B**) Absolute number of circulating CAR-NKTs as determined by staining for CD3+iNKT+Vβ11+CAR+. Patient 2 received one additional infusion after partial response. Inf: infusion.

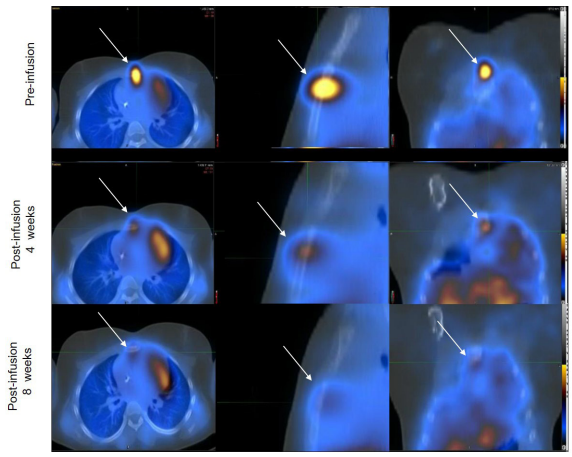

Figure 2. CAR-NKTs mediate tumor regression. Merged single-photon emission computed tomography (SPECT) and metaiodobenzylguanidine (MIBG) uptake scans showing metastatic sternum lesion at indicated time points.

1302. A Phase 2 Trial of MGTA-456 Cell Therapy Demonstrates Rapid and Durable Long-Term Improvement in Disease-Specific Outcomes in Inherited Metabolic Disease (IMD) Patients

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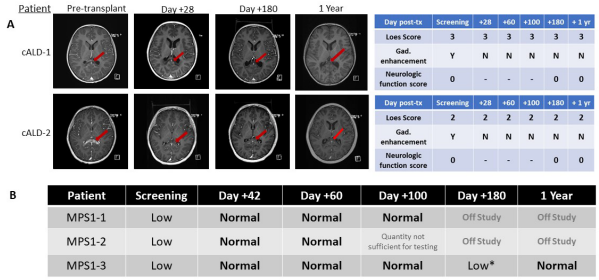
Background: Fatal neurodegeneration in IMDs can be treatable through hematopoietic stem cell transplant (HSCT), which allows cross-correction of enzyme deficiency via engraftment of donor CD34$^+$-derived microglia in the brain. Although umbilical cord blood (UCB) is the favored unrelated donor source, delayed engraftment and graft failure can result due to low CD34+ cell doses. Furthermore, higher cell doses are correlated with improved outcomes and cross-correction of disease. MGTA-456 is a novel cell therapy which provides a high dose of CD34+ cells and is being investigated to assess the safety and long-term outcomes in IMD patients in an ongoing Phase 2 clinical trial. ***Methods***: IMD-001 (NCT03406962) is enrolling ~12 pediatric patients with a diagnosis of cerebral onset adrenoleukodystrophy (cALD), mucopolysaccharidosis type IH (MPS I), metachromatic leukodystrophy (MLD) or globoid cell leukodystrophy (GLD). Patients receive a myeloablative conditioning regimen followed by either fresh (Cohort 1) or cryopreserved (Cohort 2) MGTA-456. Patients are followed for 1 year after transplant and eligible patients may enroll in a long-term follow-up study, IMD-002 (NCT04008849). ***Results***: In Cohort 1, 2 patients with cALD and 3 with MPS I, have been treated per protocol and with follow-up to 1 year. The cALD patients showed resolution of MRI gadolinium enhancement within one month post-transplant which was sustained throughout the follow-up period (Fig 1A). Loes scores, a measure of brain disease severity, and Neurologic Function Scores remained stable over this time period consistent with an arrest in disease progression. Neurocognitive function is being evaluated using Wechsler IQ Scale and Vineland Adaptive Behavior Scales. MPS I patients showed normalization of blood a-L-iduronidase and decreased excretion of MPS I-specific urinary glycosoaminoglycans from baseline over the period of observation (Fig 1B). Neurologic development and function scores as measured by Bayley Infant Development and Vineland Scales are being collected. Patients received a median CD34$^+$ dose of 110 x 10^6 cells/kg and TNC dose of 26 x 10^7 cells/kg with a median duration of neutropenia of 1 day (range 0-9) compared to a median of 8 days for historical controls. Myeloid chimerism was ≥98% donor in evaluable patients by day +14 and immune reconstitution of CD4 and CD8 T cell subsets were comparable or better than historical controls. Two steroid-responsive episodes of skin-only aGVHD were observed and no cGVHD developed. Data on additional patients receiving cryopreserved MGTA-456 will be reported. ***Conclusions:*** Treatment with MGTA-456 in patients with IMDs shows early, robust engraftment and immune reconstitution, features that are highly associated with improved clinical outcomes. Disease-specific outcomes up to one-year post-transplant in MPS I and cALD patients show crucial, durable stabilization in biochemical, MRI and clinical/neurodevelopmental assessments. In summary, the high CD34+ cell doses delivered by MGTA-456 shows compelling potential to rapidly and durably improve outcomes in IMD patients.



Day post-tx	Screening	+28	+60	+100	+180	+ 1 yr
Loes Score	3	3	3	3	3	3
Gad. enhancement	Y	N	N	N	N	N
Neurologic function score	0	-	-	-	0	0

Day post-tx	Screening	+28	+60	+100	+180	+ 1 yr
Loes Score	2	2	2	2	2	2
Gad. enhancement	Y	N	N	N	N	N
Neurologic function score	0	-	-	-	0	0

Patient	Screening	Day +42	Day +60	Day +100	Day +180	1 Year
MPS1-1	Low	Normal	Normal	Normal	Off Study	Off Study
MPS1-2	Low	Normal	Normal	Quantity not sufficient for testing	Off Study	Off Study
MPS1-3	Low	Normal	Normal	Normal	Low*	Normal

Figure 1. Disease-specific assessments at screening and post-MGTA-456. A) cALD: Brain MRI neuroinflammation-disease severity and neurologic assessment. B) MPS1: Blood leukocyte IDUA level.
*Poor sample quality for blood leukocyte IDUA. Day +180 plasma IDUA in normal range.

1303. CoupledCAR™Technology for Treating Thyroid Cancer

Xingchen Liu[1], Keshu Zhou[1], Yu Liu[2], Yong Huang[2], Chengfei Pu[3], Zhiyuan Cao[3], Cheng Lu[3], Hang Yang[3], Xi Huang[3], Xiaogang Shen[3], Yongping Song[1], Renbin Liu[2], Zhao Wu[3], Lei Xiao[3]

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Chimeric antigen receptor modified T cells (CAR-T) have demonstrated remarkable clinical efficacy in the treatment of B cell malignancies and multiple myeloma. Significant challenges restrict their application across solid tumors due to multiple obstacles, including the lack of robust *in vivo* CAR-T cell expansion and persistence, the immunosuppressive tumor microenvironment, and tumor escape due to heterogeneous tumor cell composition with a potential loss of the targeted tumor antigen. To address these difficulties, we generated CAR-T cells using a novel CoupledCAR™ technology. Specifically, we engineered CoupledCAR T cells with lentiviral vectors encoding an anti-thyroid stimulating hormone receptor (TSHR) CAR molecule. Immunohistochemistry (IHC) results showed that TSHR was highly expressed in thyroid cancer cells making it an ideal tumor-specific target antigen. *In vitro* co-culture experiments showed that TSHR CAR-T cells specifically recognized and consequently killed TSHR-positive tumor cells. Animal experiments showed that TSHR CAR-T cells inhibited the proliferation of TSHR-positive tumor cells. To evaluate the clinical safety and efficacy of anti-TSHR CoupledCAR T cells on refractory or relapsed thyroid cancer, we treated refractory/relapsed post-thyroidectomy thyroid cancer patients according to an IRB approved protocol. We treated two patients using anti-TSHR CoupledCAR T cells and observed the rapid expansion of CAR-T cells and enhanced the killing of tumor cells. Both patients achieved PR (Partial Response). Patient Profile: Patient 1 Male, 64Y, Papillary Thyroid Carcinoma. In May 2017, Thyroid cancer was diagnosed, bilateral total thyroidectomy, and right cervical lymph node functional dissection were performed in June, followed by iodine 131 isotope therapy. In December 2018, bilateral multiple cervical lymph nodes were enlarged, especially on the right side. In February 2019, right neck lymphadenectomy was performed. Patient 2 Female, 60Y, Thyroid

Carcinoma. In Aug 2013, a "double lobectomy of the thyroid gland" was performed. From Oct 2013 to Jan 2014, she received iodine 131 isotope therapy. In Sep 2014, she was diagnosed with iodine - resistant thyroid cancer. From Sep to Jan 2016, 5 cycles of chemotherapy were performed. In Jun 2016, she enrolled in the Anlotinib experimental group. In Mar 2019, multiple metastases in both lungs and multiple enlarged lymph nodes in the mediastinum were observed. Observations and Results: Patient 1: One month after infusion (M1), the patient was evaluated as PR: lymph node metastasis became undetectable and the size of the thoracic paratracheal tumor nodules decreased significantly. Three months after infusion (M3), the patient was evaluated as having a durable response, and the tumor tissue was substantially smaller than M1. Patient 2: M1, the patient was evaluated as PR (Partial Response): the tumor volume in the right lower lobe of the lung was reduced by approximately 67.51% (decreased from 65*55mm to 42*39mm). Three months after infusion (M3), compared with that before, the tumor volume was reduced by approximately 73.54% and SUV max value decreased from 14.9 to 2.8, therefore, the patient was evaluated as nCR (near complete remission). We show that TSHR is a good target for treating thyroid cancer, and our anti-TSHR CoupledCAR T cells are safe and effective for treating thyroid cancer. Recruitment is ongoing to evaluate the safety and efficacy of our CoupledCAR T cells. Further, since our CoupledCAR™ technology is a platform technology, we are developing it to treat other solid tumors using different target markers.

1304. Novel CoupledCAR™Technology for Treating Colorectal Cancer

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Chimeric antigen receptor (CAR) T cell therapy has made significant progress in the treatment of blood cancers such as leukemia, lymphoma, and myeloma. However, the therapy faces many challenges in treating solid tumors. These challenges include physical barriers, tumor microenvironment immunosuppression, tumor heterogeneity, target specificity, and limited reactive cell expansion *in vivo*. Conventional CAR-T cell therapy has thus far shown weak cell expansion in solid tumor patients and achieved little or no therapeutic responses. Here, we developed CAR T cells based on a novel CoupledCAR™ technology to treat solid tumors. In contrast to conventional CAR-T cells, CoupledCAR T cells significantly improved the expansion of the CAR-T cells *in vivo* and enhanced the CAR-T cells' migration ability and resistance to immunosuppression by the tumor microenvironment. The enhanced migration ability and resistance allow the CAR-T cells to infiltrate to tumor tissue sites and increase anti-tumor activities. Specifically, we engineered CoupledCAR-T cells with lentiviral vectors encoding an anti-GUCY2C (guanylate cyclase 2C) CAR molecule. Further anti-GUCY2C CAR-T cells showed anti-tumor activities *in vitro* and *in vivo* experiments. To verify the safety and efficacy of CoupledCAR T cells for treating solid tumors, we conducted several clinical trials for different solid tumors, including two patients with colorectal cancer. After the infusion of anti-GUCY2C CoupledCAR T cells, these two patients showed rapid expansion of CoupledCAR T cells and the killing of tumor cells. Spherically, we observed that CoupledCAR T cells expanded significantly in the patients and infiltrated tumor tissue sites, demonstrating enhanced anti-tumor activities. Both patients achieved PR (Partial Response). Patient Profile: Patient 1: Male, 55Y, Colon Adenocarcinoma. In May 2016, 8 cycles of XELOX chemotherapy and 1 dose of radiotherapy were performed. In Step 2016, "radical rectal resection and terminal ileum double ileostomy" was performed. After surgery, gemcitabine chemotherapy was performed for 2 cycles. In January 2018, relapse and metastasis of prostate and left lung were observed. Starting from March 2018, seven cycles of "Iritican + Retitrazepam + Epitope Chemotherapy," one cycle of "Iritican + Retitrazepam," implantation of radioactive particles, and three cycles of "Oxaliplatin + Capecitabine" were performed. In April 2019, relapse and metastasis were observed. Patient 2: Female, 57Y, Colon Adenocarcinoma. In December 2014, DT46Gy/2Gy/23 radiotherapy was performed. In December 2014 and January 2015, the single drug chemotherapy of Xeloda was taken orally. In February 2015, laparoscopic radical resection of rectal cancer was performed. In April, May, June, and July 2015, mFOLFOX6 chemotherapy was performed. In June 2019, CT showed tumor metastasis. In June, July, August 2019, irinotecan + fluorouracil regimen chemotherapy was performed. Observations and Results: Patient 1: One month after infusion (M1), the patient was evaluated as PR; most of the target lesions were significantly reduced by more than 50%, and the primary tumor volume was reduced by ~45%. Patient 2: M1, the patient was also evaluated as PR; the tumor in the left upper lobe tip posterior segment was reduced by approximately 75%. The clinical data demonstrated that CoupledCAR-T cells effectively expanded, infiltrated tumor tissue sites, and kill tumor cells in patients with colorectal cancer. We are recruiting more colorectal cancer patients to further test the safety and efficacy of anti-GUCY2C CoupledCAR T cells. Further, since our CoupledCAR™ technology is a platform technology, we are developing it to treat other solid tumors using different target markers.

AAV Vectors - Clinical Studies

1305. RGX-314 Ocular Gene Therapy: Overview of Phase I/IIa Ongoing Trial for Neovascular Age-Related Macular Degeneration (nAMD) and Future Directions

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Ocular anti-VEGF injections are approved to treat nAMD and prevent vision loss. Real world evidence shows patients lose visual acuity over time due to noncompliance with a high treatment burden. Gene therapy offers the potential to minimize the treatment burden of nAMD. Preclinical studies of subretinal delivery of AAV8 vector gene therapy has demonstrated efficient and strong transduction of retinal pigment epithelium (RPE). RGX-314 gene therapy utilizes an AAV8 vector to deliver a transgene for potential continuous production of a soluble

anti-VEGF fab to treat retinal disease. The Phase I/IIa clinical trial is ongoing and designed to evaluate the safety and signals of efficacy of RGX-314 in previously treated nAMD subjects (n=42). This trial is a multi-center, open label, 5-cohort dose-escalation study. RGX-314 is delivered in the subretinal space following a standard vitrectomy in the operating room. The ongoing study results show that as of the data cutoff date of December 6, 2019, the drug is well tolerated. A dose-dependent increase in protein production was observed at one month across all cohorts. Baseline serum neutralizing antibodies to AAV8 did not impact transduction or protein production of RGX-314. The highest dose (cohort 5 at 2.5e11 GC/eye (n=12)) has shown the highest clinical effect with stable visual acuity (+3 letters), improved central retinal thickness (-83 μm), and 73% of subjects being injection free at 6 months. Subretinal administration of RGX-314 has been well-tolerated and initial results show potential for a one-time administration of RGX-314 to provide sustained clinical outcomes in the treatment of nAMD. The suprachoroidal space is a potential space between the choroid and sclera that traverses the circumference of the eye's posterior area and can potentially allow a targeted delivery to the posterior eye (retina) to be administered in an in-office procedure. We have recently shown that AAV8 vectors delivered suprachoroidally in animals demonstrated a similar and widespread transduction of RPE and photoreceptors as observed in subretinal delivery. With positive outcomes of subretinal delivery of RGX-314, suprachoroidal delivery as a less invasive in-office procedure may demonstrate similar outcomes. RGX-314 injected suprachoroidally is currently being evaluated for safety and efficacy in preclinical studies with plans to proceed to clinical trials moving forward.

1306. AAV8-Mediated Liver-Directed Gene Therapy as a Potential Therapeutic Option in Adults with Glycogen Storage Disease Type Ia (GSDIa): Results from a Phase 1/2 Clinical Trial

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Introduction: Glycogen storage disease type Ia (GSDIa) results from deficiency of the enzyme glucose 6-phosphatase (G6Pase), essential for glycogenolysis and gluconeogenesis. Decreased endogenous glucose production causes severe hypoglycemia during periods of fasting. DTX401 is an AAV8 vector that expresses the human *G6PC* gene under transcriptional control of the normal G6Pase promoter. **Methods:** The GSDIa phase 1/2 gene therapy study (NCT03517085) is a global, open-label dose escalation trial evaluating the safety, tolerability, and efficacy of a single DTX401 IV infusion in adults with GSDIa. Cohorts of subjects were treated with DTX401 at doses of 2.0 x 10^{12} Genome Copies (GC)/kg and 6.0 x 10^{12} GC/kg. **Results:** Three subjects each in Cohort 1 and Cohort 2 received DTX401 at 2.0 x 10^{12} GC/kg and 6.0 x 10^{12} GC/kg, respectively. An additional 3 subjects in Cohort 3 (confirmatory cohort) received DTX401 at 6.0 x 10^{12} GC/kg under an amended protocol to optimize the reactive steroid approach to vector-induced immune response. DTX401 was generally well-tolerated. No infusion-related or treatment-related serious AEs were reported. All AEs were mild or moderate in severity. Most subjects had transient mild elevations in ALT, similar to previous observations with AAV-based gene therapy. In all subjects, time to hypoglycemia (<3.3 mmol/L) during a controlled fasting challenge increased from baseline, while total daily cornstarch use and MRI hepatic fat fraction (a surrogate measure of hepatic glycogen storage) decreased from baseline. Most subjects also experienced weight loss and all experienced decreased fasting lactate concentrations. **Conclusions:** DTX401 had an acceptable safety profile and sustained improvement in biological G6Pase activity to Week 52 in Cohorts 1 and 2, with increased time to hypoglycemia during a controlled fasting challenge, reduced daily cornstarch requirements, and reduced MRI hepatic fat fraction compared with baseline. A minimum of 12 weeks of efficacy and safety data from all subjects in Cohort 3, as well as additional follow-up for subjects in Cohorts 1 and 2, will be available at the time of the meeting.

1307. First China International Gene Therapy Study in Leber's Hereditary Optic Neuropathy

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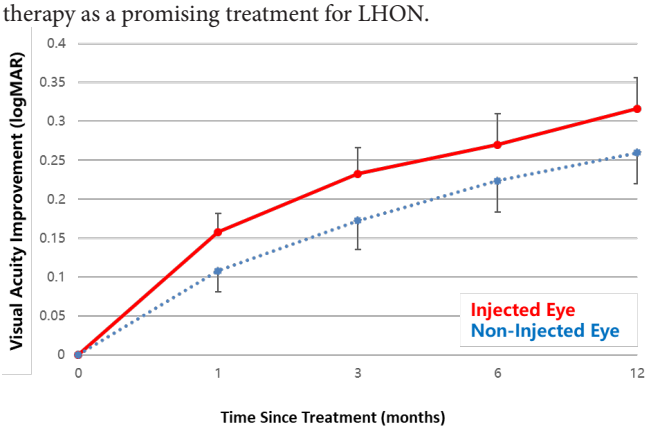
Purpose: Currently, there is no effective treatment for Leber's hereditary optic neuropathy (LHON), a mitochondrially inherited disease manifests with acute or subacute painless central vision loss. We previously reported the safety, efficacy and durability up to 7.5 years of a single unilateral intravitreal (IVT) injection of NR082-1, a recombinant adeno-associated virus (rAAV) expressing the NADH ubiquinone oxidoreductase subunit 4 (*ND4)* gene (ClinicalTrials.gov number, NCT01267422), in 9 patients with visual loss and mutated 11778G>A of the ND4-associated LHON. Here, we conduct LHON gene therapy study investigating a second generation of the rAAV2-ND4 (NR082-2) with improvement in manufacturing. ***Methods:*** Individuals aged between 6 and 60 years old with LHON carrying the ND4-G11778A mutation were enrolled in a prospective, international, open-label gene therapy study (NCT03153293) evaluating the safety, tolerability, and efficacy of a single 1.0x10^{10} vector genome unilateral IVT injection of NR082-2. The primary efficacy endpoint was best-corrected visual acuity (BCVA). Safety endpoints included adverse event reporting, ophthalmic examination (intraocular pressure, Humphrey visual field, visual evoked potential, optical coherence tomography, anterior segment and fundus photography), physical examination and laboratory testing, including chest radiograph and electrocardiogram. ***Results:*** As of the data cutoff date (02-December-2019), 159 subjects [149 subjects from China; 10 subjects from Argentina] ranging from 6.87 to 49.42 years old were intravitreally injected with NR082-2 into one eye; 127 treated patients completed 6-month follow-up and 133 treated patients completed 12-month follow-up. Of the 127 treated patients (completed 6-month follow-up) and 133 treated subjects

(completed 12-month follow-up), 63.8% (81 out of 127 subjects) and 61.8% (82 out of 133 subjects) had clinically significant BCVA improvement defined as ≥0.3 using Log of the Minimal Angle of Resolution (LogMAR), respectively (Figure 1). No adverse events such as uveitis, vitreous inflammation, keratitis, crystal damage and immune response were observed. ***Conclusions:*** Similar to previous reporting of NR082-1, we found that a single IVT injection of NR082-2 vector consistently leads to BCVA improvement, without acute or long-lasting toxicity. This international ND4 study validates the potential of gene therapy as a promising treatment for LHON.



1308. A Systematic Review of Clinical Safety and Efficacy of AAV Gene Therapies

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Gene therapy is an emerging therapeutic modality that demonstrated significant progress in treating otherwise unaddressed genetic disorders. In the past few years, the adeno-associated virus (AAV) emerged rapidly as a vector of choice for *in vivo* gene therapy, with widespread clinical presence and an extremely deep pipeline of drug candidates. However, the literature lacked a disciplined analysis of its safety, clinical study design implications, tolerability and efficacy. This work provides a comprehensive, systematic review of all registered clinical trials involving AAV as a vector for *in vivo* gene therapy, as of January 1st, 2020 (n=283). This study includes the first complete database of such clinical trials and clinical candidates; a review of safety events, their design and development implications; and an analysis of the efficacy of such therapies. We report significant novel systematic evidence showing that AAV is generally safe and well-tolerated, demonstrating success across 100% of reported safety endpoints in all clinical trials to date. This work provides a detailed analysis of side effect patterns for different routes of AAV administration, as well as different steroid regimens. We also report the exhaustive analysis of efficacy, demonstrating an overall efficacy rate of over 65% across all reported endpoints, and upwards of 80% on fully reported trials (n=72). We review the efficacy and clinical trial success rates across different therapeutic areas and routes of administration. We hope that the results of this work will be of use to the clinical trial design community, as well as the wider healthcare industry and clinicians.

1309. Clearance of Vector DNA from Bodily Fluids in Patients with Severe or Moderate-Severe Hemophilia B Following Systemic Administration of AAV5-hFIX and AAV5-hFIX Padua

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Introduction: Current adeno-associated viral (AAV) vector-based gene therapy strategies for hemophilia rely on systemic administration of the vector. Durable expression of the transgene over the span of years has been reported from several trials, yet information on the clearance of vector material from bodily fluids is still limited and monitoring of "shedding" is required during trials despite lack of evidence of environmental or transmission risk. Here, we examined the magnitude and duration of the presence of vector DNA in bodily fluids from participants from a Phase I/II study of an AAV5-hFIX wildtype construct (AMT-060; NCT02396342) and from a Phase IIb study utilizing the enhanced version, AAV5-hFIX Padua (AMT-061; NCT03489291). **Methods:** Adult male participants with severe or moderately severe hemophilia B received a single intravenous infusion of either AMT-060 at $5x10^{12}$ genome copies(gc)/kg (low dose) or 2×10^{13} gc/kg (high dose), or AMT-061 at $2x10^{13}$gc/kg in one of two ongoing trials. Assessments in both trials included efficacy and safety outcomes as well as vector shedding in whole blood and semen. Vector shedding was also measured in nasal secretions, feces, urine, and saliva in participants receiving AMT-060. Vector shedding was analyzed using a validated quantitative real time polymerase chain reaction (qPCR) based assay measuring presence of vector DNA in the bodily fluids. Vector clearance was reached when vector DNA was either zero or below the limit of detection (LOD) for three consecutive measurements. The range in time to the first and the third consecutive negative measurement for each dose group are provided. **Results:** Treatment with AMT-060 resulted in sustained improvement in FIX activity for up to 4 years [Mean FIX activity: 5.1% (low dose group at 4 years); 7.5% (high dose group at 3.5 years)] and treatment with AMT-061 resulted in mean FIX activity of 41% at 52 weeks. AMT-060 and AMT-061 reduced the mean number of annualized bleeds by between 77%-100% respectively. AMT-060 and AMT-061 reduced the requirement for exogenous FIX administration by 90%-100% respectively. Both AAV5-hFIX and AAV5-hFIX Padua were safe and well tolerated and no unexpected TRAEs have been observed with longer-term follow up. AMT-060 at the higher dose was cleared from semen, feces, urine, nasal secretions and saliva in all participants by week 78 (range 7-78 weeks) and in blood by 3.7 years (range 1.8-3.7 years). AMT-061 vector DNA was cleared from blood in 1 participant at 40 weeks and was low but detectable (<lloq) in the other 2 participants. amt-061 vector dna was cleared semen of 1 participant by week 52 and <lod on consecutive tests a second participant. **Conclusions:** Post-

AMT-060 treatment, vector DNA was undetectable in all participants in the high dose group by 10 months and considered cleared by 18 months in all bodily fluids except blood. AMT-060 was cleared from the blood in all participants by 3.7 years. With AMT-061, vector DNA was cleared from blood in 1 participant by week 40 and from semen in 1 participant by week 52. The presence of vector DNA in bodily fluids assessed was not associated with any adverse safety or efficacy findings.

1310. Improved Motor Function in Children with AADC Deficiency Treated with Eladocagene Exuparvovec (PTC-AADC): Interim Findings from a Phase 2 Trial

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Background: Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare autosomal recessive disorder caused by mutations in the gene encoding the enzyme AADC. Resulting neurotransmitter deficiencies hinder normal motor development early in life, leading to missed motor milestones and subsequent movement disorders. Gene therapy has been proposed to accomplish what current standard-of-care treatments do not: restoring AADC-mediated neurotransmitter production and improve motor function. Eladocagene exuparvovec (PTC-AADC), a recombinant adeno-associated virus vector containing human cDNA encoding the AADC enzyme, has been evaluated for treatment of AADC deficiency in a phase 1 compassionate use study and phase 1/2 trial. We report interim findings from a phase 2 study intended to expand on the phase 1/2 trial, including assessment of a higher dosage in a subset of younger patients. **Methods:** This is an interim analysis of data from 8 out of 10 planned participants in a prospective, open-label trial. Children with AADC deficiency underwent bilateral intraputaminal injection of PTC-AADC at the following total doses: $1.8x10^{11}$ vg for children ≥3 years and 2.4×10^{11} vg for children <3 years. The primary efficacy endpoint was the proportion of patients achieving key milestones at 1 year as measured by the Peabody Developmental Motor Scale, Second Edition (PDMS-2). Secondary efficacy endpoints included changes in PDMS-2, Alberta Infant Motor Scale (AIMS), and Bayley Scales of Infant Development, Third Edition (Bayley-III) scores; change in body weight; and neurologic examination findings related to AADC deficiency symptoms. Safety endpoints included treatment-emergent adverse events (TEAEs) and viral shedding. **Results:** Patients received doses of $1.8x10^{11}$ vg (n=3; mean age, 55.0 months) or 2.4×10^{11} vg (n=5; mean age, 24.8 months). Mean follow-up was 11.5 months; data for 1 patient in the high-dose group were available only through month 6. Baseline PDMS-2 and AIMS total scores were low. At 1 year measurement, some patients had achieved motor milestones (table).

Table. Patients Achieving Motor Milestones at 12 Months

Milestone	Low-dose group (n=3)	High-dose group (n=4)	Total (N=7)
Full head control, n	1	3	4
Sitting unassisted, n	0	1	1

Increases from baseline in PDMS-2, AIMS, and Bayley-III total scores at 1 year were statistically significant ($P<0.0001$, $P\leq0.0016$, and $P\leq0.0004$ respectively, both doses). Mean body weight increased from baseline to year 1 in both groups, with a transient decrease between months 3 and 6 in the low-dose group. The number of patients with hypotonia, oculogyric crises, and limb dystonia decreased during the first year. No viral shedding was detected. No apparent differences were observed between the safety profiles for the 2 doses. All patients experienced ≥1 TEAE, most of mild intensity and none were considered definitely related to treatment. Mild dyskinesia episodes (high dose: n=2; low dose: n=3) were considered possibly related to therapy. Patients experienced a total of 21 serious AEs, all of which resolved and were considered unlikely to be related to study treatment. **Conclusions:** Children with AADC deficiency achieved meaningful gains in motor function 1 year after PTC-AADC administration. No new safety signals were identified at either dose. The findings of this interim analysis add to the growing body of data supporting the efficacy and safety of PTC-AADC in AADC deficiency and suggest a benefit of early treatment.

1311. Improved Delivery in a New Clinical Trial of AAV2-BDNF Gene Therapy for Alzheimer's Disease

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Postmortem histological study of clinical trial tissues can improve upcoming trials of human gene therapy. A recent multicenter, sham surgery controlled Phase 2 clinical trial of nerve growth factor (NGF) gene therapy for Alzheimer's disease (AD) found that AAV2-NGF delivery to basal forebrain was safe but did not improve cognition. Patients were treated by manual stereotactic injection of AAV2-NGF targeting the nucleus basalis of Meynert: $2x10^{10}$ vector genomes in 20 µL were injected at 3 sites per brain half. We examined the targeting and spread of AAV2-NGF at 15 injection sites in 5 brain halves by immunolabeling of NGF and the NGF receptor p75. AAV2-NGF was expressed for up to 7 years but spread only a mean distance of 0.96 ± 0.34 mm. Due to limited spread and inaccurate targeting, NGF did not directly engage the target cholinergic neurons at any of the 15 injection sites. Our upcoming clinical trial of brain derived neurotrophic factor (BDNF) gene therapy for AD will use convection-enhanced infusion and real-time MR guidance to improve the spread and targeting of AAV2-BDNF delivery to the entorhinal cortex. In rhesus macaque monkeys, spread of the MR contrast agent gadoteridol accurately predicted the distribution of AAV2-BDNF expression. Infusions successfully targeted the entorhinal cortex and AAV2-BDNF expression persisted throughout the experimental period of 30 months. Peripheral tissues were not transduced (by qPCR) and BDNF protein was not elevated in CSF and serum (by ELISA) in five aged rhesus monkeys surviving 12 to 30 months. These methods for highly consistent gene

delivery to entorhinal cortex are adaptable to humans and will improve the spread and targeting of AAV2-BDNF gene therapy in upcoming clinical trials for AD.

AAV Vectors Preclinical and Proof-of-Concept Studies in CNS Disorders

1312. PR006, an AAV Gene Therapy Vector Expressing Progranulin, Improved FTD-GRN Phenotypes *In Vitro* and *In Vivo*

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Prevail Therapeutics, New York, NY

Mutations in *GRN* account for 5-10% of all cases of frontotemporal dementia (FTD-GRN). These loss-of-function mutations in *GRN,* which encodes the glycoprotein progranulin, result in lysosomal dysfunction and neuropathological changes. By administering PR006, our AAV gene therapy vector transducing a copy of the human *GRN* gene, we aim to increase progranulin levels in FTD-GRN patients. We first evaluated the efficacy of PR006 *in vitro* using iPSC-derived neurons from individuals with *GRN* mutations; these express reduced levels of progranulin and exhibit lysosomal dysfunction. PR006 transduction of these neurons led to a dose-dependent increase in secreted progranulin expression and promoted the physiological processing of the lysosomal enzyme, cathepsin D. Additionally, we assessed the therapeutic effects of PR006 in *Grn* knockout (KO) mice, which lack progranulin protein expression and exhibit progressive lysosome-related neuropathology and neuroinflammation. Intracerebroventricular (ICV) delivery of PR006 resulted in broad vector genome presence and increased progranulin expression in the brain. In addition, PR006 ameliorated the FTD-GRN-related phenotypes observed in *Grn* KO mice, including lipofuscinosis, ubiquitin accumulation, and neuroinflammation. Nonhuman primates were used to assess safety and tolerability. PR006 treatment in nonhuman primates was well-tolerated and resulted in broad vector biodistribution and progranulin expression in the central nervous system and the periphery. This, together with our observations that PR006 administration resulted in expression of progranulin that ameliorated FTD-GRN-related phenotypes in both *in vitro* and *in vivo* FTD-GRN preclinical models, support further development of PR006 to slow or stop disease progression in patients with FTD-GRN.

1313. Gene Therapy for the Progressive Visual Pathology in Late Infantile Neuronal Ceroid Lipofuscinosis Patients Receiving Enzyme Replacement Therapy

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Late infantile Neuronal Ceroid Lipofuscinosis (LINCL) is a childhood neurodegenerative disease that appears between 2-4 years of age and progresses with visual, motor, and mental decline, with a life expectancy of less than two decades. Most cases of LINCL are caused by deficiency in the soluble lysosomal enzyme TPP1 as a result of mutations in the *CLN2* gene. TPP1 is a mannose-6-phosphate decorated enzyme that can be used for cross-correction of deficient cells by enzyme replacement or gene therapy. Recently, Cerliponase alfa (Brineura®), an enzyme replacement therapy for TPP1 deficiency was approved. Cerliponase alfa is given bi-weekly and has shown remarkable benefit in treated children with slowing of neurological progression. However, the recombinant protein does not reach the retina, which over time will result in progressive visual loss despite the positive impact on clinical disease. As such, we have developed a gene therapy approach to treat the ensuing visual loss. To test the translatability of our approach, we injected non-human primates with AAV2.TPP1, and assessed TPP1 levels in the aqueous humor and in retinal tissues. In a pilot study, we found the ratio of TPP1 in aqueous humor to that in the retina was 1:15, providing us the important opportunity to assay for retinal expression levels in living animals using sampled aqueous humor as a proxy. In subsequent dosing studies, eyes injected 1-month earlier with 5.0 E9 to 8.3E9 vg of AAV.hTPP1, we achieved TPP1 expression between 60- and 140- fold of wild type levels in aqueous humor with no side effects. These data suggest a combination therapy for a more complete treatment of progressive LINCL disease that will greatly improve the quality of life of affected children. Finally, we evaluated morphological (optical coherence tomography, or OCT) and functional (pupilometry) measures to be used as endpoints for evaluation of progression of retinal disease in children with TPP1-disease. OCT data demonstrated a very consistent and rapid reduction in central subfield thickness (CST) and inner segment/outer segment (IS/OS) junction defects, indicating photoreceptor dysfunction or loss. The slope of decline of the IS/OS junction defect is greater than CST. Functional measures with a hand-help pupilometer demonstrated a gradual loss of pupillary constriction that correlated significantly with central retinal thickness. These morphological and functional measures provide us with potential endpoints for therapeutic efficacy in children with retinal disease caused by TPP1 deficiency.

1314. A Transient Burst of Transgene Expression Promotes Regeneration of Mature Vestibular Hair Cells

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Loss of vestibular hair cells can result in crippling balance disorders. A variety of insults ranging from ototoxic drugs to natural ageing can kill hair cells. In contrast, the supporting cells that interdigitate with hair cells in the sensory patches of the inner ear often remain intact after hair cells are lesioned. Thus, supporting cells are a natural source of cells that could potentially be stimulated to regenerate replacement hair cells and restore function. We have developed an AAV-based gene therapy, DB-201, that robustly regenerates vestibular hair cells in a mouse model in vivo. By combining single-cell RNA-Seq and ATAC-Seq profiling, we identified a novel promoter whose activity is highly restricted to vestibular supporting cells and limits off-target expression of the transgene. RNAScope labeling of vector genomes in treated tissue demonstrated broad viral tropism and confirmed the specificity of the promoter. We also show that the level of transgene expression in supporting cells is critical for maximizing the regenerative effect by comparing three promoters with different strengths. Molecular characterization of the regenerative process with single-cell profiling demonstrated that the supporting-cell-specific promoter is naturally silenced as the cells adopt a hair cell fate. This silencing effect promotes maturation of the new hair cells, whereas continuous expression of the transgene with a ubiquitous promoter appears to suppress later stages of the differentiation process. In vivo, the regenerated hair cells grow new stereocilia bundles, become reinnervated, and establish new synapses. Thus, DB-201 demonstrates the benefits of precision gene therapy for regenerative medicine, and it is a novel candidate for restoring sensory function to patients that suffer from vestibular deficits.

1315. In Vivo Postnatal Base Editing Restores Sensory Transduction and Transiently Rescues Hearing in a Mouse Model of Recessive Deafness

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Most genetic diseases arise from recessive point mutations that require correction, rather than disruption, of the pathogenic allele to benefit patients. Base editing has the potential to directly repair point mutations and provide therapeutic restoration of gene function. We developed a base editing strategy to treat *Baringo* mice, which carry a recessive, loss-of-function point mutation (*c.A545G*, resulting in the substitution p.Y182C) in transmembrane channel-like 1 (*Tmc1*) that causes deafness. *Tmc1* encodes a protein that forms mechanosensitive ion channels in sensory hair cells of the inner ear and is required for normal auditory function. We found that sensory hair cells of *Baringo* mice have a complete loss of auditory sensory transduction that causes profound deafness. To repair the *Baringo* mutation, we tested several optimized cytosine base editors (CBEmax variants) and guide RNAs in *Baringo* mouse embryonic fibroblasts. We packaged the most promising CBE, derived from an activation-induced cytidine deaminase (AID), into dual AAV vectors using a split-intein delivery system. The dual AID-CBEmax AAVs were injected into the inner ears of *Baringo* mice at postnatal day 1. Injected mice showed up to 51% reversion of the *Tmc1 c.A545G* point mutation to wild type sequence (*c.A545A*) in *Tmc1* transcripts. Repair of *Tmc1 in vivo* restored inner hair-cell sensory transduction, hair-cell morphology, and partial low-frequency hearing four weeks post-injection. These findings provide a foundation for a potential one-time treatment for recessive hearing loss and support further development of base editing to correct pathogenic point mutations.

1316. Investigational AAV Gene Therapy LYS-GM101 for GM1-Gangliosidosis

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GM1-gangliosidosis is an autosomal recessive disease caused by mutations in the GLB1 gene, which encodes the lysosomal enzyme acid beta-galactosidase (β-gal). The resulting enzyme deficiency leads to accumulation of GM1 ganglioside, causing cellular damage

in different organs, in particular neurons of the brain and spinal cord. Children affected by GM1-gangliosidosis suffer from progressive neurodegeneration, with severe and eventually lethal motor and developmental deficits. Delivery of functional β-gal to the central nervous system (CNS) offers the potential to halt disease progression and correct disease manifestations in GM1-gangliosidosis. To this end, Lysogene developed LYS-GM101, an AAVrh.10 vector carrying the human GLB1 cDNA. Preclinical studies were conducted to assess CNS delivery of LYS-GM101 in mice and nonhuman primates and pharmacological efficacy in a mouse model of GM1-gangliosidosis. Following administration into the cerebrospinal fluid (CSF) of GM1-gangliosidosis mice, LYS-GM101 led to a dose-dependent increase of β-gal enzymatic activity in the brain and spinal cord, which was accompanied by a reduction in GM1 ganglioside storage. Infusion of LYS-GM101 into the CSF of nonhuman primates led to significant increases of β-gal enzymatic activity in the brain and spinal cord, with broad enzyme distribution throughout the primate brain. Taken together, these results validate CSF delivery of LYS-GM101 as a promising method to achieve widespread enzyme distribution and correction of disease pathology in GM1-gangliosidosis and support the initiation of an international first in human open-label adaptive design clinical trial to assess the safety and efficacy of LYS-GM101 in children affected by GM1-gangliosidosis.

1317. Preclinical Results in Rodents Strongly Support Clinical Evaluation of scAAV9/MFSD8 as a Potential Gene Therapy for CLN7 Patients

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The variant late infantile neuronal ceroid lipofuscinosis type 7 (vLINCL7 or CLN7 in short) disease is a lysosomal storage disease caused by a mutation in the gene named *Major Facilitator Superfamily Domain Containing 8* (*MFSD8*). It is characterized by the accumulation of lysosomal storage material including autofluorescent ceroid lipopigments and subunit c of mitochondrial ATP synthase (SCMAS). The clinical presentation of CLN7 disease varies from a mild, late-onset version with non-syndromic visual deficits to a severe, early-onset version that manifests as neurological signs with progressive deterioration in intellectual and motor capabilities, seizures, muscle spasms, visual deficits, and even premature death. There is no approved treatment for CLN7 disease and patient care is limited to symptomatic intervention. To test our hypothesis that a self-complementary adeno-associated virus 9 (scAAV9)/*MFSD8* gene replacement therapy might provide a meaningful and long-term therapeutic benefit, preclinical efficacy and safety evaluations were conducted. An *in vitro* study demonstrated that scAAV2/*MFSD8* dose-dependently rescued lysosomal function in cultured CLN7 patient fibroblasts. In an ongoing *in vivo* efficacy study, *MFSD8* knockout (KO) mice were administered intrathecally (IT) with high (5×10^{11} vg/mouse) or low (1.25×10^{11} vg/mouse) doses of scAAV9/*MFSD8* at postnatal day (PND) 7-10 (pre-symptomatic cohorts) or at PND 120 (early-symptomatic cohorts). scAAV9/*MFSD8* administration to *MFSD8* KO mice led to clear age and dose effects with early intervention and high dose achieving the best therapeutic benefits. For instance, high dose of scAAV9/*MFSD8* injected IT at PND 7-10 resulted in: 1) significantly higher levels of *MFSD8* mRNA in all tissues and brain regions assessed compared to negative control animals (*MFSD8* Heterozygotes) and *MFSD8* KO vehicle-treated animals at 4.5 months post injection; 2) dramatic amelioration (~50%) of SCMAS immunoreactivity in all affected brain regions and spinal cord at 4.5 months post injection; 3) complete rescue of impaired open field and rotarod performance at 6 months post injection; 4) complete normalization of a survival (94%) in vector-treated *MFSD8* KO mice at 11 months post injection, compared to 95% and 0% in *MFSD8* Heterozygotes and *MFSD8* KO vehicle-treated animals, respectively; and 5) no neurological abnormalities or body weight changes up to 11 months post injection, indicating no apparent long term adverse effects. In parallel, a non-GLP single IT dose one-year toxicity study in C57BL/6J wild type (WT) mice and a GLP single IT dose three-month toxicity study in Sprague Dawley WT rats were conducted to further evaluate the safety of AAV9/*MFSD8* administration. Both studies concluded that administration of scAAV9/*MFSD8* was well-tolerated. Taken together, these results demonstrate that scAAV9/*MFSD8* vector is both effective and safe in preclinical rodent models, strongly supporting the initiation of a Phase I/II clinical trial of scAAV9/*MFSD8* as a potential gene therapy for CLN7 patients.

1318. Gene Therapy for Tuberous Sclerosis Complex Type 2 in a Mouse Model by Delivery of AAV9 Encoding a Condensed Form of Tuberin

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Tuberous sclerosis complex (TSC) is an autosomal-dominant syndrome caused by loss of a tumor suppressor gene - *TSC1* or *TSC2,* encoding hamartin and tuberin, respectively. These proteins act as a complex which inhibits mTORC1-mediated cell growth and proliferation. Loss of either protein leads to overgrowth of cells in many organs, most commonly affecting the brain, kidney, skin, heart and lung. Gene therapy using adeno-associated virus (AAV) vectors is a promising strategy to correct this disease by delivering the missing protein. Since the cDNA for human tuberin exceeds the packaging capacity of AAV vectors, we designed a "condensed" form of tuberin (cTuberin) such that its cDNA fits into an AAV vector. Functionality of cTuberin was verified in culture. Gene therapy was tested in a stochastic mouse model of TSC2 in which complete loss of tuberin is achieved in multiple cell types in the brain by AAV-Cre recombinase disruption of *Tsc2*-floxed alleles at birth, leading to shortened life span (mean 60 days) and brain pathologic findings consistent with TSC. Remarkably, when these mice were injected intravenously on day 21 with an AAV9 vector encoding cTuberin, most survived over 450 days in apparently healthy condition with reduction of brain pathology. This demonstrates the potential of treating life-threatening TSC2 lesions with intravenous injection of an AAV vector encoding cTuberin. This gene therapy approach has the advantage that a single treatment may be beneficial, in comparison to long term treatment with drugs which have side effects.

Gene Regulation and Delivery Technologies

1319. In Situ Gene Editing of HSPCs by TAGE, a New Biologics Class of Targeted Cas Ribonucleoproteins

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Despite remarkable advances in gene editing, the full therapeutic potential of this promising modality is hampered by delivery. To address the multiple limitations of viral- and nanoparticle-mediated delivery for gene editing, we are developing a new class of biologics called Targeted Active Gene Editors (TAGE). TAGE are modular, programmable ribonucleoproteins (RNPs) comprising a cell-targeting domain linked to a Cas protein loaded with sgRNA. These dual function TAGE are uniquely designed for genome editing of selected cell types in situ. Here, we demonstrate preclinical proof of concept by editing hematopoietic stem and progenitor cells (HSPCs). Gene editing of human HSPCs was achieved directly using naked RNP, bypassing the need for an exogenous delivery vehicle. Moreover, direct intraosseous injection of TAGE in the Ai9 reporter mouse resulted in editing of bone marrow cells, including long-term progenitor HSCs (LSK $CD150^+$ $CD34^-$).

1320. Crispr Cas9 Mediated Gene Knockout of KLKB1 to Treat Hereditary Angioedema

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Hereditary angioedema (HAE) is a rare genetic disorder characterized by severe swelling attacks in various parts of the body, which can be debilitating or fatal. This disorder is caused by inactivating mutations in the C1 esterase inhibitor (C1-INH) protein, a key regulator of kallikrein that itself mediates the conversion of high-molecular-weight kininogen into bradykinin. Uncontrolled regulation of plasma kallikrein in the absence of C1-INH results in high bradykinin levels. Bradykinin is a potent vasodilator, and high levels of this peptide result in increased vascular permeability and swelling. Gene knockout of kallikrein (gene: *KLKB1)* is expected to diminish plasma kallikrein activity, reducing production of bradykinin and, therefore, has great potential as a treatment for HAE. Here, we show *in vitro* data supporting development of CRISPR/Cas9 sgRNAs targeting the human *KLKB1* gene. Using our modular LNP-based CRISPR/Cas9 delivery system to knock out the *KLKB1* gene *in vivo*, we demonstrate the ability of these sgRNAs to edit the human *KLKB1* gene in a huKLKB1 mouse model, resulting in >90% reduction in total circulating kallikrein. Lastly, we demonstrate a similar >90% decrease in plasma kallikrein activity in cynomolgus monkey, achieving therapeutically relevant reduction in both nonclinical models. The data supports knockout of the *KLKB1* gene as a potential one-time treatment option for patients with HAE.

1321. Engineering the DNA-Binding Specificity of CTCF to Restore Enhancer-Mediated Gene Expression in Human Cells

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CCCTC-binding factor (CTCF) is a ubiquitous DNA-binding protein involved in higher-order topological organization of the genome, DNA repair, and gene regulation. CTCF binding sites (CBS)s are frequently mutated in cancer and developmental diseases leading to loss of CTCF binding and gene mis-regulation. Here we demonstrate a general method to restore gene regulation at mutated CBSs by creating engineered CTCF variants with altered DNA-binding specificities that can bind specifically to mutated CBSs. The central DNA binding domain of CTCF is composed of an eleven-finger zinc finger array which recognizes a twelve-nucleotide motif known as a CTCF binding site (CBS). Using a bacterial-two-hybrid reporter system, we first identified the nucleotides in a consensus CBS that are essential for CTCF binding and generated five different variant (v) CBSs (vCBSs) bearing multiple mutations that are no longer bound by wild-type CTCF. Using protein engineering performed with the bacterial two-hybrid system, we then identified CTCF variants that could bind specifically to each of the five different vCBSs. To show that our CTCF variants could function in human cells, we used the endogenous proto-oncogene *MYC* locus as a reporter. *MYC* is normally regulated in human K562 cells by CTCF-directed looping between a CBS located ~2 kb upstream of the *MYC* transcriptional start site (TSS) and distal enhancers positioned ~2 Mb downstream of the TSS. Previous studies have shown that disruption of CTCF binding at this CBS results in loss of *MYC* expression. To test our variant CTCFs, we first replaced this critical CBS upstream of the *MYC* TSS with each of our five different vCBSs in K562 cells and found that introduction of these mutated sites led to the loss of endogenous CTCF binding at this site and the expected reduction in *MYC* expression. Importantly, expression of a cognate CTCF variant capable of binding to its associated vCBS both restored the looping of the mutated CBS to downstream enhancer regions and rescued *MYC* expression. These results show that CTCF variants can be used to restore normal gene expression programs in cases where mutated CBSs result in loss of CTCF function (e.g., as has been reported for some gastrointestinal cancers and melanomas).

In sum, our results provide an important proof-of-principle for how engineered CTCF variants can be expressed *in trans* to rescue gene expression for cells bearing CTCF CBS mutations. In addition, our findings delineate a novel system for performing structure-function studies of CTCF without disrupting normal activities of endogenous wild-type CTCF and suggest a novel strategy for performing targeted epigenetic editing by altering three-dimensional genome topology in human cells.

1322. In Vivo HSC Gene Therapy with Base Editors Allows for Efficient Reactivation of Fetal Gamma-Globin in Beta-YAC Mice

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A major concern with current genome-editing technologies using CRISPR/Cas9 is that they introduce double-stranded DNA breaks (DSBs), which may be detrimental to host cells by causing unwanted large fragment deletion and p53-dependent DNA damage responses. Base editors are capable of installing precise nucleotide mutations at targeted genomic loci and present the advantage of avoiding DSBs. Here, we tested the efficacy of base editors by targeting critical motifs regulating γ-globin reactivation. We established a novel delivery platform for base editors using HDAd5/35++ vectors, the helper-dependent adenoviral vectors with chimeric fiber for HSC tropism. Through alternative codon usage to minimize repetitive sequences and the optimized design of promoters, we successfully rescued a panel of cytidine and adenine base editors targeting the GATAA motif in BCL11A enhancer or recreating naturally existed HPFH mutations in the HBG1/2 promoter region. In HUDEP-2 cells, all five tested vectors efficiently installed target base conversion and led to substantial γ-globin reactivation. In single-cell derived clones, monoallelic or biallelic target base conversion conferred 100% of HbF-positive cells. We observed ~60% -113 A to G HPFH mutation in HBG1/2 promoter of mixed HUDEP-2 cells using an ABE vector HDAd-ABE-HBG#2. This vector was therefore chosen for downstream animal studies. We used mice that carry 248kb of the human β-globin locus (β-YAC mice) and thus accurately reflect globin switching. An EF1α-mgmt^{P140K} expression cassette flanked by *FRT* and transposon sites was included in the vector for allowing in vivo selection of transduced cells. After in vivo transduction with HDAd-ABE-HBG#2 + HDAd-SB and selection with low doses of O6BG/BCNU, an average of 35% of HbF-positive cells was measured in peripheral red blood cells. In one out of eight mice, a near complete -113 A to G conversion and 90% of HbF-positive cells were achieved. No alterations in blood cell counts were found. The cellular composition of bone marrow samples was comparable to that of untransduced mice, demonstrating a good safety profile of our approach. Bone marrow lineage minus cells were isolated from primary mice at week 14 after transduction and infused into lethally irradiated C57BL/6J mice. The percentage of HbF-positive cells was maintained in secondary recipients over 16 weeks indicating genome editing occurred in long-term repopulating mouse HSCs. Our observations demonstrate that base editors delivered by HDAd5/35++ vectors in vivo represent a promising strategy for precise genome engineering for the treatment of hemoglobinopathies.

1323. Nanomembrane-Derived Extracellular Vesicles for the Delivery of Crispr-Cas9/Grna for Therapeutic Exon Skipping in Duchenne Muscular Dystrophy

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CRISPR-Cas9 has tremendous potential as a therapeutic tool for treating human diseases. However, prolonged expression of the nuclease and gRNA from viral vectors in an *in vivo* context may cause off-target activity and immunogenicity. While extracellular vesicles have been recently demonstrated to be a promising option to transiently deliver the CRISPR-system, sufficient packaging of both Cas9 protein and gRNA is critical to achieve efficient genome editing in hard-to-transfect cells and tissues, such as skeletal muscle. Here, we developed a novel ribonucleoprotein delivery system utilizing two distinct homing mechanisms. The first is by chemical induced dimerization to recruit Cas9 protein into extracellular nanovesicles. The second utilizes a viral RNA packaging signal and two self-cleaving riboswitches to tether and release sgRNA into nanovesicles. We term our fully engineered delivery system NanoMEDIC (nanomembrane-derived extracellular vesicles for the delivery of macromolecular cargo) and demonstrate efficient genome editing in various hard-to-transfect cell types, including human induced pluripotent stem (iPS) cells and myoblasts. Furthermore, NanoMEDIC production is scalable for industrial production as a xeno-free suspension culture system. As a disease model, therapeutic exon skipping in the dystrophin gene locus was targeted and resulted in over 90% exon skipping efficiencies in skeletal muscle cells derived from Duchenne muscular dystrophy patient iPS cells. Finally, we generated novel luciferase-based reporter mice to demonstrate that NanoMEDIC could induce exon skipping and sustain skipping activity for over 160 days, even though NanoMEDIC itself was rapidly degraded within 3 days, indicating its utility for transient *in vivo* genome editing therapy of DMD and beyond.

1324. AAV-CRISPR Gene Editing is Negated by Pre-Existing Immunity to Cas9

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Significance/Background: Adeno-Associated Viral (AAV) vectors are leading candidates for the delivery of CRISPR/Cas9 for therapeutic genome editing. However, AAV-based delivery involves persistent

expression of the Cas9 nuclease, a bacterial protein. Recent studies indicate a high prevalence of neutralizing antibodies and T-cells specific to the commonly used Cas9 orthologs from *Streptococcus pyogenes* (SpCas9) and *Staphylococcus aureus* (SaCas9) in humans. Pre-existing exposure to Cas9 could be a serious obstacle to therapeutic gene editing in humans. Despite progress in somatic genome editing, major questions remain concerning the feasibility and safety of this approach in solid organs, such as the liver. We tested in a mouse model whether pre-existing immunity to SaCas9 would pose a barrier to liver genome editing with AAV packaging CRISPR/Cas9. **Methods:** C57BL/6 mice were immunized against either SaCas9 or ovalbumin. One week later, AAV8 vectors containing either a gRNA targeting the Ldlr gene and SaCas9 or GFP were co-injected intraperitoneally. Mice were euthanized 2, 3, 6, and 12 weeks after AAV injection. T-cell infiltration was measured via flow cytometry and qPCR. Liver damage was measured via a Serum Alanine Transaminase (ALT) assay and cell proliferation in the liver was measured by Ki-67 mRNA expression. Total AAV genome copies in the liver were measured by qPCR, and SaCas9 protein was observed via western blot. *Ldlr* indels in the liver were measured via deep sequencing. **Results:** Our study indicates that although efficient genome editing occurred in mice with pre-existing SaCas9 immunity, this was accompanied by infiltration of the liver by $CD8^+$ T-cells. This cytotoxic T-cell response was characterized by hepatocyte apoptosis, loss of recombinant AAV genomes, and near complete elimination of genome-edited cells followed by compensatory liver regeneration. Our results raise important efficacy and safety concerns for CRISPR/Cas9 based *in vivo* genome editing in the liver.

1325. Abstract Withdrawn

Physical, Chemical and Exosomal Gene Delivery

1326. Assessment of Clinical Feasibility of Image-Guided Liver Specific Hydrodynamic Gene Transfer in Non-Human Primates

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Image-guided, liver-specific hydrodynamic gene delivery was evaluated for its efficiency and safety in non-human primates. Reporter plasmids (pCMV-Luc) were hydrodynamically injected into the liver through a 9-Fr balloon catheter inserted from the jugular vein and placed at the proximal end of the hepatic vein in the targeted liver lobe under fluoroscopy image guidance. The hydrodynamic injection of the plasmid was driven by the CO_2-pressurized injector under the control of *HydroJector600* software. Animals were sacrificed 24 h after the plasmid injection, and samples from these animals were collected for the luciferase assay. When 2.5× lobe volume of plasmid DNA solution (100 μg/ml) and an injection pressure of 150 psi were used, the average level of luciferase gene expression was 10^6-10^7 RLU/mg of extracted protein in the injected lobe compared with 10^3 RLU in the non-injected lobe. Long-term effects of the hydrodynamic gene delivery with the use of pBS-HCRHP-FIXIA plasmid (provided by Dr. Carol Miao) were evaluated in four baboons. In total, 3 different injection volumes, at 2×, 2.5×, or 3× lobe volume, with the same injection pressure of 150 psi were used to deliver the plasmids (100 μg/ml). The effects of the gene delivery were assessed by the plasma concentration of human FIX (hFIX), as determined by an ELISA and the coagulation assay using factor IX deficient human plasma. At 10 days after the gene delivery, the highest level of the hFIX gene expression with hFIX concentration of 31.4 μg/ml and coagulation activity of 53% were achieved in the animals receiving 4 sequential injections with 2.5× lobe volume into the right lateral, left lateral, right medial, and left medial lobes. The therapeutic level with 30% coagulation activity was observed in the animal 200 days after the gene transfer. In contrast, the animals injected with 2× lobe volume into 4 lobes, 3× lobe volume into 4 lobes, and 2.5× lobe volume into 3 lobes only achieved 2.6, 3.2, and 6.6 μg/ml of the hFIX concentration, and 35%, 36%, and 40% of the coagulation activity, respectively, at 10 days after gene transfer. Subsequently, these animals received sequential injections into all 4 lobes with 2.5× lobe volume after 250 days. Compared with the results in the first injections, three baboons showed higher levels of the plasma hFIX concentration of 19.3, 12.0, and 30.5 μg/ml, respectively, and the coagulation activity of 51%, 49%, and 47%, respectively, at 10 days after the second injection. Safety evaluation showed no significant changes in heart rate, blood oxygen saturation, body temperature, carbon dioxide concentration in expiratory air, and electrocardiogram during and immediately after the procedure. A transient increase of 10-20-fold elevation of serum level of aspartate aminotransferase and alanine aminotransferase, and 2-3-fold increase in serum concentration of lactate dehydrogenase were seen 24 h after the injections, which returned to the normal range within a week. In addition, serum concentrations of interferon-γ, interleukin-2, and interleukin-8 remained the same at the baseline level; however, a transient increase of vascular muscle stretching-related cytokine, such as tumor-necrotizing factor-α from in average of 8.0 pg/ml to 459.8 pg/ml, monocyte chemoattractant protein-1 from 81.6 pg/ml to 476.1 pg/ml, and interleukin-6 from 40.3 pg/ml to 108.4 pg/ml, were observed. Results from this first non-human primate study demonstrated that the procedure of hydrodynamic gene transfer using a computer-controlled, image-guided system is safe and effective in gene delivery to liver and clinically feasible.

1327. A Novel Serum-Insensitive Fusogenic Polyvalent Cationic Lipid for *In Vivo* mRNA Delivery

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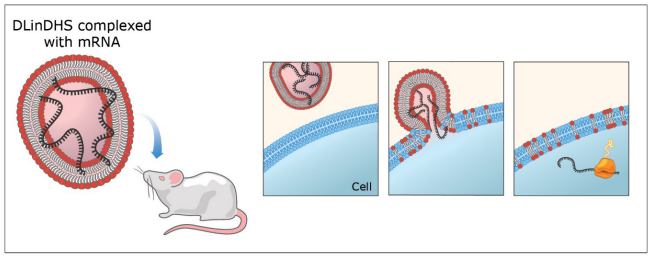
Cationic nanoparticles are powerful tools for delivering nucleic acids to cells *in vitro*. However, current nanoparticle formulations suffer from inhibition by biological macromolecules, such as serum proteins, and from endosomal retention, limiting their use *in vivo*.

We synthesized a series of novel polyvalent ionizable cationic lipids and tested their ability to deliver mRNA to cells *in vitro* and *in vivo*. Using mRNA encoding GFP, we found that delivery was influenced by both headgroup size and degree of unsaturation in the fatty-acid tail. We identified a compound, N^1,N^4-dilinoleyl-N^1,N^4-di-(2-hydroxy-3-aminopropyl)-diaminobutane (DLinDHS or "DLin"), capable of delivering nucleic acids (including mRNA, siRNA, and plasmid DNA) to a variety of cell types and in serum concentrations as high as 100%. DLin/mRNA lipoplexes exhibit ionic strength-dependent particle size and pH-dependent zeta potential. High-resolution, time-resolved fluorescence microscopy using Cy5-labeled mRNA showed that DLin/mRNA lipoplexes fuse directly with the plasma membrane, bypassing the endosomal-lysosomal system. We demonstrate high levels of protein expression from mRNA delivered with DLin in primary human epidermal keratinocytes, dermal fibroblasts, pluripotent stem cells (iPS cells), PBMCs, dendritic cells, human lung adenocarcinoma cells, and rat embryonic cortical neurons. DLin transfection was equally effective in proliferating cells and contact-inhibited confluent cell monolayers. We also show high-efficiency gene editing of primary human cells using DLin complexed with mRNA encoding both TALENs and NoveSlice, a novel gene-editing endonuclease. Administration of nebulized lipoplexes to rats resulted in protein expression in lung epithelial cells. Intradermal injection of DLin complexed with mRNA encoding a reporter protein in rats and in a human subject yielded localized expression in the dermal layer. In conclusion, we present a novel serum-insensitive nucleic acid delivery system and demonstrate delivery of mRNA *in vitro* and *in vivo,* including to the skin and lung.



1328. Safe and Effective Delivery of Nucleic Acids Using LNPs Formulated with Fusion-Associated Small Transmembrane Proteins

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Despite the promise of genetic medicines for the treatment of a wide range of diseases, their success has been limited by challenges in the effective and tolerable systemic delivery of nucleic acids. To address this, we developed a lipid nanoparticle (LNP) platform that incorporates fusion associated small transmembrane (FAST) proteins derived from fusogenic orthoreoviruses. FAST-LNPs effectively deliver a wide variety of nucleic acid cargoes (plasmid DNA, mRNA, or siRNA) by directly fusing with the plasma membrane of target cells. Utilization of FAST proteins for intracellular delivery allows LNPs to be formulated without poorly-tolerated fusogenic lipids, significantly improving tolerability and efficacy compared to other LNPs. We first assessed the transfection of nucleic acids using a wide range of cell types including immortalized normal and cancer cells as well as primary cell lines. FAST-LNPs were significantly less toxic and demonstrated improved efficacy when compared to cationic lipid formulations such as Lipofectamine 2000 or ionizable lipids such as DLin-MC3-DMA. When FAST-LNPs encapsulating plasmid DNA or mRNA encoding luciferase were administered by intravenous injection of into immune competent C57Bl/6 mice, widespread whole-body luminescent signal was observed over a period of several days. When organs were examined by e*x vivo* luminescence and tissue sectioning (IF and IHC), robust expression was observed in all major organ systems and tissues. When compared to LNPs formulated with DLin-MC3-DMA, *in vivo* expression of luciferase was comparable, however, FAST-LNPs displayed significantly improved tolerability as determined by body weight tracking, AST/ALT levels, cytokine analysis, and H&E staining of major uptake organs. To gain a comprehensive understanding of biodistribution and toxicity in a more clinically-relevant model, non-human primates were administered FAST-LNPs containing plasmid DNA at 10 and 20 mg/kg with single and multiple dosing regimens up to 28 days. No adverse pathology was detected in the major uptake organs such as liver, spleen, or lung. A wide distribution of plasmid DNA in all organs tested was detected via qRT-PCR. We further examined the immunogenicity of FAST-LNPs through a series of repeat-dosing experiments in immune competent C57Bl/6 mice. When 5 systemic doses were administered over a 120-day period, we did not detect any anti-drug antibodies against FAST proteins or significant increases in cytokine levels. This was additionally supported by the fact that LNPs carrying mRNA-FLuc dosed over the same period did not show reduced expression, indicating that FAST-LNPs, unlike AAV for example, are well-suited for repeat dosing of nucleic acid medicines. We then evaluated FAST-LNPs for the creation of *in vivo* hepatocyte "protein factories", which has remained an elusive target in gene therapy. To achieve this, we formulated FAST-LNPs containing plasmid encoding the liver-expressed protein follistatin, which is being investigated for the treatment of sarcopenia and other conditions. Following intravenous injection, we observed a time-dependent increase in liver follistatin that was accompanied by an increase in circulating follistatin levels in C57Bl/6 mice. Taken together, we here describe a novel nucleic acid LNP platform with a unique mechanism of action that enables high efficacy and low toxicity delivery. FAST-LNPs are a well-tolerated platform to systemically deliver a wide range of genetic medicines.

1329. LOX Biphasic Gene Electrotransfer Enhances Extruded Collagen Graft Integration Subcutaneously in a Small Animal Model

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Lysyl oxidase (LOX) is a collagen cross-linking enzyme that is active during development of various connective tissues including tendons and ligaments. LOX and LOX-like enzymes are vital for the integrity of elastic and collagen fibers and are copper-dependent group of enzymes that catalyze the formation of lysine and hydroxylysine-derived cross-

links in collagens and lysine-derived crosslinks in elastin. These crosslinks contribute to the tensile strength of collagens, and by extension the mechanical properties of tendons and ligaments. Tissue engineering approaches of collagen-based grafts for tendon and ligament repair can benefit from strategies mimicking native crosslinking approaches for improved graft integration and strength. In this study we examined a gene therapy approach for LOX delivery and expression at the site of a collagen graft implantation for ligament repair. We have developed a biphasic electrotransfer gene delivery protocol for optimized delivery to the skin using reporter genes. The biphasic approach allows minimizing heating and twitching at the electrode/skin interface, while enhancing gene delivery to the epidermis, dermis and underlying muscle. Bioluminescence imaging was utilized to quantify gene expression, while immunofluorescence staining of skin samples 48 hours after gene delivery was used to determine the location of expression within the skin layers. Gene expression with biphasic electrotransfer was enhanced over ten-fold compared to plasmid DNA injection only, without any skin damage visible macroscopically or histologically. Once pulsing parameters were optimized, intradermal gene delivery of plasmid DNA encoding human lysyl oxidase was performed and expression was quantified using ELISA. Extruded collagen scaffolds were implanted subcutaneously in a rat model, with and without LOX gene delivery to the skin overlaying the scaffolds. Samples were collected for histological evaluation on days 7, 14 and 21. LOX expression was confirmed with immunofluorescence staining. Histological evaluation revealed improved graft integration, with minimized inflammatory response, and enhanced collagen fiber structure. Future direction will focus on tendon repair in an Achilles rupture model, with LOX gene delivery for graft crosslinking, remodeling, integration and tendon repair.

1330. Abstract Withdrawn

1331. Engineering Exosomes with Altered Cellular Tropism for Targeted Payload Delivery *In Vivo*

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Exosomes are natural and abundant extracellular vesicles capable of transferring complex molecules between neighboring and distant cell types. Translational research efforts have focused on co-opting this communication mechanism to deliver exogenous payloads to treat a variety of diseases. Important strategies to maximize the therapeutic potential of exosomes therefore include payload loading, functionalization of the exosome surface with pharmacologically active proteins, and delivery to target cells of interest. Through comparative proteomic analysis (LC/MS) of purified exosomes, we identified several highly enriched and exosome specific proteins, including a transmembrane glycoprotein (PTGFRN) belonging to the immunoglobulin superfamily. Leveraging PTGFRN as a scaffold for exosome surface display, we developed our engEx™ platform to generate engineered exosomes functionalized with a variety of structurally and biologically diverse proteins. When administered systemically, exosomes are primarily taken up by macrophages in the liver and spleen. To redirect exosome uptake to other cell types, we employed our engineering platform to display functional targeting ligands, including single domain antibodies (sdAbs), single chain variable fragments (scFvs), single chain Fabs (scFabs), and receptor ligands, on the exosome surface at high density. To demonstrate that exosome surface modifications can alter cellular tropism, we generated exosomes displaying anti-Clec9A scFabs to target conventional type 1 dendritic cells (cDC1s), anti-CD3 scFabs to target T cells, and CD40 ligand to target B cells. Compared to untargeted controls, the engineered exosomes exhibited functional antigen binding that led to greater association with the cell types expressing the cognate receptor both *in vitro* and *in vivo*. In mice, systemic administration of exosomes engineered to display scFabs targeting Clec9A resulted in a 75% increase in the percentage of cDC1 cells that had taken up exosomes over controls. Exosomes targeting T cells through a displayed anti-CD3 scFab resulted in both an increase in the percentage of exosome positive cells (3.75 and 3 fold for CD4+ and CD8+) and the amount of exosome per cell (15 and 7 fold for CD4+ and CD8+) in the blood. Moreover, we showed that compared to untargeted exosomes, those with altered tropism achieved increased functional payload delivery to the target cell of interest. In primary mouse dendritic cells, anti-Clec9A exosomes loaded with STING agonist achieved a 15-fold greater pathway induction as measured by IL-12 production compared to the untargeted control. Preliminary *in vivo* data suggest that anti-Clec9A exosomes reduce the required STING agonist dose 10-fold to achieve efficacy and induce responses against tumor-associated antigen, compared to control exosomes. These results therefore demonstrate the potential of our engEx™ platform to generate novel exosome therapeutics targeted to cell types of interest for pharmacologic payload delivery.

1332. A Versatile Platform for Precision Exosome Engineering

Kevin Dooley, Russell McConnell, Ke Xu, Sonya Haupt, Madeleine Youniss, Shelly Martin, Nuruddeen Lewis, Christine McCoy, Chang Ling Sia, Su Chul Jang, Jorge Sanchez-Salazar, Bryan Choi, Rane Harrison, Sriram Sathyanarayanan, Jonathan Finn

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Exosomes are an important intercellular communication system capable of transferring complex macromolecules between neighboring and distant cell types. Translational research efforts have focused on co-opting this natural messaging system to selectively deliver exogeneous cargo to cell types of interest. Exosome engineering strategies to display these cargoes on the exosome surface or package them inside the lumen at high density are currently lacking. We reasoned that efficient loading could be achieved by tethering therapeutically relevant macromolecules to proteins that preferentially partition into exosomes during cellular biogenesis. Proteomic analysis of highly purified exosomes led to the identification of two potential "scaffold" proteins: prostaglandin F2 receptor negative regulator (PTGFRN), a single-pass transmembrane glycoprotein, and brain acid soluble protein 1 (BASP1), a myristoylated protein sequestered inside the exosome lumen. Overexpression of these scaffolds in an exosome producer cell resulted in multi-log

enrichment on and in the secreted exosomes. Using GFP as a surrogate cargo, we thoroughly characterized these scaffolds and compared the loading efficiency to other proteins commonly used for exosome engineering including LAMP2B, pDisplay, and the tetraspanins CD9, CD63, and CD81. PTGFRN and BASP1 peptide sequences were then used to display or package a variety of complex cargoes on and in exosomes, including antibody fragments, heterodimeric cytokines, TNFSF ligands, vaccine antigens, and Cas9. We demonstrate high-density cargo loading and concomitant pharmacodynamic activity in a variety of *in vitro* cell-based assays and syngeneic tumor models using PTGFRN and BASP1 engineered exosomes. Furthermore, this process for scaffold discovery was successfully applied to other exosome producer cell types, including mesenchymal stem cells, which led to the identification of additional scaffolds for exosome engineering. The exosome engineering platform described allows for high-density functionalization of the surface and lumen of exosomes with structurally and biologically diverse protein cargo to generate novel exosome therapeutics.

Cardiovascular and Pulmonary Diseases

1333. Lineage-Tracing Glandular Stem Cell Compartments in the Ferret Lung

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The domestic ferret (*Mustela putorius furo*) has served as an excellent model of cystic fibrosis (CF) due to its high degree of conservation in lung anatomy and cell biology with humans. Because CF mice do not develop lung disease, CF ferret models have proven to be a robust tool for interrogating pathobiology and accelerating the development of genetic therapies for CF. Gene editing of airway stem cells offers a promising strategy for treatment of CF lung disease, however, the animal models required to study stem cell compartments that are not conserved in mice have yet to be created. One such stem cell compartment includes airway submucosal glands (SMG), a secretory unit of pathophysiologic importance in CF lung disease that exists throughout the cartilaginous airways in humans and ferrets, but not mice. Here we report the creation of two lineage-tracing ferret models capable of genetically labeling two stem cell compartments within SMGs. Mice have SMGs restricted to the proximal trachea and research on this species has suggested that glandular myoepithelial cells (MECs) and gland ductal cells are reserve stem cell compartments for dedicated basal stem cells (BSCs) in the surface airway epithelium. Utilizing CRISPR/Cas9-mediated homologous directed repair (HDR) and homology-independent non-homologous end joining (NHEJ) knock-in strategies, we created transgenic ferret models harboring an *ACTA2*-IRES-CreERT2, *KRT7*-IRES-CreERT2 and *ROSA26*-mTmG Cre-reporter insertions using Cas9/gRNA RNP zygote injections, respectively. *ACTA2*-IRES-CreERT2 and *KRT7*-IRES-CreERT2 ferrets enable cell-specific Cre-driver expression through an IRES-CreERT2 insertion into the 3' UTR of the *ACTA2* and *KRT7* genes, and the *ROSA26*-mTmG ferrets contain a CAG-LoxP-tdTomato-stop-LoxP-EGFP cassette inserted into intron 1 of the *ROSA26* locus. Southern blotting and sequencing of the genomic flanking segments confirmed the integrity of the integration events. Double transgenic offspring (*ACTA2*-IRES-CreERT2:*ROSA26*-TG and *ROSA26*-TG:*KRT7*-IRES-CreERT2) were used to validate lineage-tracing following the induction of ferrets with tamoxifen. Lineage-tracing of *ACTA2*-IRES-CreERT2:*ROSA26*-TG ferrets at 3- and 8-weeks of ages demonstrated labeling of MECs in SMGs of the airway and other glandular organs, as well as ACTA2-expressing smooth muscle and vascular smooth muscle. Similarly, the induction of *KRT7*-IRES-CreERT2:*ROSA26*-TG ferret at 5-weeks of age demonstrated labeling of KRT7-positive lineage cells restricted to the ducts of airway SMGs. We are currently breeding these two double transgenic lines onto CFTR-G551D backgrounds to generate triple transgenic ferrets for studying the biology of these glandular stem cell compartments in the context of CF airway disease. To our knowledge, these ferrets are the first non-rodent lineage-tracing models to be generated. These models may also be useful in the directed evolution of viruses that efficiently transduce these stem cell compartments for gene editing in CF.

1334. Insertion of the CFTR cDNA in the Endogenous Locus in Airway Stem Cells Using CRISPR/Cas9 Restores CFTR Function to Wild-Type Levels in Differentiated Epithelia

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Cystic fibrosis (CF) affects about 30,000 Americans and 75,000 people globally. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride channel cause CF. CF results in chronic lung infections that cause lung failure and can only be treated by lung transplantation. Although CFTR modulators have benefited many patients, there is a wide variability in patient responses and some patients still cannot be treated using these drugs. A gene therapy that corrects CF causing mutations in airway stem cells and generates a durable layer of corrected airway cells is ideal for CF. *In vivo* viral and non-viral gene therapy strategies have been attempted but success has been limited due to the thick mucus, immune response to viral vectors, the inflamed nature of the epithelial surface and rarity of basal stem cells. Recognizing these challenges, we chose to develop an ex-vivo gene corrected autologous airway stem cell therapy for CF. In addition, we identified the upper airways (nose and sinus) as a readily accessible source of upper airway basal stem cells (UABCs). CF sinusitis is an unmet medical need. This approach enables us to treat CF sinusitis while optimizing transplantation in the sinus while minimizing safety concerns to patients. We recently reported the use of this approach to correct the F508del mutation that affects >70% of CF patients. Here, we extend these methods to insert the full coding sequence of CFTR in the endogenous locus. One technical difficulty in inserting a large sequence such as the CFTR cDNA (~4500 bp) is the packaging limit of the AAV vector (~4700 bp). To overcome this challenge, we use a dual insertion technique to insert two halves of the CFTR cDNA along with truncated CD19 (an enrichment tag). In experiments with non-CF UABCs, we have inserted CFTR cDNA with tCD19 in 5-10% primary

cytokeratin 5 positive (KRT5+) airway basal stem cells and further purified the edited cells using flow cytometry to obtain a 50-80% edited population expressing tCD19 (n = 6 independent donors). We then tested the platform in primary KRT5+ UABCs and bronchial basal stem cells (HBEC) from 10 different CF patients. The corrected and enriched CF samples were successfully differentiated in air-liquid interface cultures. CFTR function was assessed by measuring the response of the epithelial sheets to forskolin (CFTR activator) and CFTRinh-172 (CFTR inhibitor) by Ussing chamber analysis. On average, we observed >80% restoration of CFTR function (CFTRinh-172 sensitive currents) relative to non-CF controls as measured by Ussing chamber analysis. These experiments are an important first step for the autologous transplantation of edited airway stem cells to treat CF. Subsequent studies will investigate transplantation of corrected cells seeded in scaffolds approved for sinonasal repair into the airways of animal models.

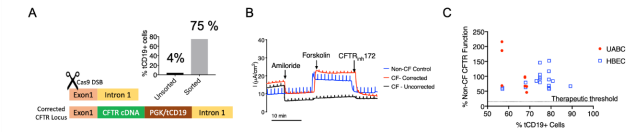

Figure 1: A. *In preliminary studies,* CFTR cDNA with tCD19 was inserted into exon 1 of primary F508del homozygous UABCs. Edited cells were sorted by flow cytometry (FACS) to obtain 75% corrected cells. B. FACS sorted cells displayed forskolin and $CFTR_{inh}$-172 responses similar to non-CF samples in Ussing chamber assays C. In UABC and HBEC samples obtained from 10 different CF patients, we observed restoration of CFTR function close to non-CF levels.

1335. A Novel AAV Variant that Exclusively Targets the Heart via a Potential Post-Entry Mechanism

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Recent technological advances have facilitated the generation of novel AAV vectors that target specific tissues and cell types. However, our understanding of AAV vector biology, in particular how each amino acid residue of the capsid protein determines the biological properties of the vector, remains largely incomplete. This paucity of knowledge has hindered the rational design of next generation AAV vectors that are optimized for broader clinical applications. In this regard, our AAV DNA/RNA Barcode-Seq approach has enabled us to comprehensively analyze the relationships between AAV capsid amino acid sequences and phenotypes throughout the entire region of the AAV capsid protein in a high-throughput manner. We have so far carried out double-alanine (AA) scanning mutagenesis of the entire AAV9 capsid protein and collected multifaced biological phenotypes in vitro and in vivo using AAV Barcode-Seq. One of our projects focuses on the biological functions of each amino acid in the N-terminal half of AAV9 capsid, including the entire VP1 unique (VP1u) region consisting of 137 amino acids that are buried inside the viral capsid. Due to its topological location, the VP1u region is not likely to be responsible for viral attachment to cells, the critical first step toward establishing viral infection and vector transduction. Here, we report the discovery of a novel AAV9 variant with a single amino acid substitution in the AAV9 VP1u region that exhibits exclusive transduction of the heart at a level that is comparable to AAV9 while reducing transduction of other major organs by 91-99%. In this study, we injected mice intravenously with a dsAAV-U6-BC library that contains 122 AAV9-AA mutants covering the entire N-terminal half of AAV9 capsid protein. In this experimental scheme, viral capsid-specific RNA barcodes can be expressed in transduced cells by the human U6 snRNA promoter and used as a measure to quantify the transduction of each AAV variant contained in the library. Eleven major organs including heart and liver were collected at 6 weeks post-injection. Total DNA and RNA were extracted from each tissue and subjected to AAV Barcode-Seq analysis. We found that one AAV9 mutant harboring an AA substitution in two highly evolutionarily conserved amino acids transduced the heart at a level comparable to AAV9 while globally detargeting other organs with a 99% reduction in liver transduction. A subsequent validation study using AAV-CMV-LacZ vectors individually packaged with capsids derived from the parental AAV9, the AAV9-AA mutant, and each AAV9 single alanine mutant confirmed the AAV barcode-Seq data and importantly revealed that the observed phenotype is ascribed to a single amino acid near the N-terminus of the AAV9 VP1u region. Thus, our study offers a novel, highly heart-specific AAV9 variant for robust in vivo cardiac gene transfer. In addition, our observations provide an important insight into the mechanisms of cell type specificity mediated by AAV capsid proteins. That is, the post-attachment process leading to transduction by AAV9 vectors differ between cardiomyocytes and other cell types, and determines cardiomyocyte specificity. We are actively investigating this post-entry mechanism of cell type specificity.

1336. Directed Evolution of AAV Targeting Lung Epithelia Using Aerosol Delivery Identifies 4D-A101, a Variant Demonstrating Robust Gene Delivery in Non-Human Primates

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Introduction To unlock the full potential of gene therapy, there is a need to identify novel vectors with enhanced tropism for target tissues when delivered by clinically relevant routes of administration at commercially feasible doses. Gene therapy for lung tissue, including for cystic fibrosis, would benefit from adeno-associated virus (AAV) vectors capable of more efficient and specific transduction of lung tissue following aerosol delivery. **Methods** An industrialized directed evolution approach ("Therapeutic Vector Evolution") was employed in non-human primates (NHP) to discover novel adeno-associated virus (AAV) variants capable of efficient and preferential gene delivery to, and robust gene expression in, lung epithelia following a single aerosol administration. Initially, aerosol delivery was evaluated using the clinically-utilized AeroEclipse II nebulizer (Trudell Medical International) adapted for use with anesthetized NHPs in a study delivering Evans blue dye (n=2). We next used our libraries of AAV capsid variants (comprising up to one billion individual capsid sequences), to identify the best-performing variant capable of transducing lung cells via aerosol administration in two

separate vector discovery experiments. The lead candidate identified, named 4D-A101, was then further characterized *in vivo* in NHPs using a reporter gene, EGFP. Animals (n=3) received approximately 1 x 10^{13} vg of variant 4D-A101 encoding EGFP by a single aerosol administration. After eight weeks, lung and other systemic tissues were harvested for analyses, including viral genome localization, protein expression, immunofluorescence, and histopathology. **Results** AeroEclipse II nebulized delivery to NHPs of Evans Blue marker dye demonstrated distribution to the trachea, bronchial tree and alveoli in all lung lobes. Similar aerosolized delivery of 4D-A101 carrying the EGFP transgene resulted in similar biodistribution of viral genomes, together with widespread transgene expression in all regions of the airway and lung lobes (48 of 48 samples). Systemic biodistribution analysis showed that over 99% of viral genomes were retained within lung tissue. GFP protein expression was also widespread within lung tissue (48 of 48 samples positive). Immunofluorescent imaging showed EGFP expression in cells of the ciliated epithelial layer within the trachea and bronchial tree, as well as broad EGFP expression within the alveoli. 4D-A101 delivery was well-tolerated, and no test article-related adverse histopathology or clinical pathology findings were identified. Interestingly, 4D-A101 also exhibited resistance to pre-existing human antibodies in IVIG *in vitro*, even at high titers (1:50), compared to wild-type serotypes. **Conclusions** These results indicate we have identified, by directed evolution, an AAV vector (4D-A101) that when delivered by aerosolization is capable of robust and widespread transduction and transgene expression throughout the primate lung with minimal systemic exposure. The tropism profile of this novel AAV vector after aerosol delivery in NHP, coupled with its resistance to human antibodies, represents a significant advance over existing AAV serotypes for the development of gene therapies for lung disease, including cystic fibrosis.

1337. Development of Receptor-Targeted Nanocomplexes for *In Vivo* Delivery of CRISPR/Cas9 as a Potential Therapy for Cystic Fibrosis

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Cystic Fibrosis (CF) is recessively inherited, multi-organ disease, however morbidity and mortality is caused mostly by progressive respiratory impairment due to mucus retention and chronic bacterial infection in the lungs. Gene therapy is an attractive therapeutic option for CF as it could target the underlying cause of the disease, rather than treating symptoms. We aim to develop a novel gene therapy for the respiratory manifestations of the disease based on gene editing with CRISPR/Cas9. This allows for the precise introduction of double strand breaks in the DNA followed, in the absence of a DNA template, by non-homologous end joining (NHEJ), an error-prone but relatively efficient DNA repair pathway that functions in both dividing and terminally differentiated cells. In order to exploit the NHEJ repair pathways for treatment of CF, CRISPR/Cas9 must be delivered with sufficient efficiency to the lung. Our approach is to deliver Cas9/gRNA ribonucleoprotein (RNP) complexes with a non-viral, receptor-targeted nanoparticle (RTN), previously described for *in vivo* DNA and siRNA delivery to the lung. These nanoparticles comprise a peptide component that mediates cell targeting of epithelial cells, and lipid components, which enable endosomal escape. Gene editing of airway epithelial cells is permanent and so repeated delivery with these non-immunogenic nanoparticles could be performed until a sufficient level of genetic correction is achieved. To provide a model, we first engineered primary, human bronchial epithelial cells to stably express GFP by lentiviral transduction and used this model to compare nanoparticle formulations for knockout of GFP by CRISPR/Cas9. Using optimal formulations, we were able to achieve levels of up to 75% GFP knockout, as measured by flow cytometry. Complexes were biophysically characterized and found to have desirable size (90 nm), charge + 40 mV and polydispersity index (~0.2) appropriate for delivery to the CF lung, where mucus accumulation will prevent the penetration of larger particles. We next used RTNs to deliver RNP formulations to primary CF basal epithelial cells to delete a deep intronic CFTR mutation that creates a cryptic splice site (3849+10kb C>T). Following repeat delivery of RTNs, the indel frequency was more than 80% by ICE analysis of DNA sequencing, while correct splicing of the CFTR gene was restored by transcript analysis. In a cell culture model of the airway epithelium, chloride ion transport was shown to be at least partially restored in Ussing chamber measurements. Finally, to evaluate the efficiency of *in* vivo editing, we delivered the RNP nanoparticles to the lungs of Ai9 TdTomato reporter mice. Ai9 is a Cre reporter allele that has a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven tdTomato. By creating targeted DSBs using two gRNAs targeting upstream and downstream of the STOP cassette, we aimed to delete the cassette, restoring TdTomato fluorescence. Following by oropharyngeal instillation of RTNs, TdTomato expression was evident in the airway epithelium, indicating successful CRISPR/Cas9 editing. We observed a widespread distribution of reporter gene expression in the respiratory tract, with no evidence of an inflammatory response to the treatment.

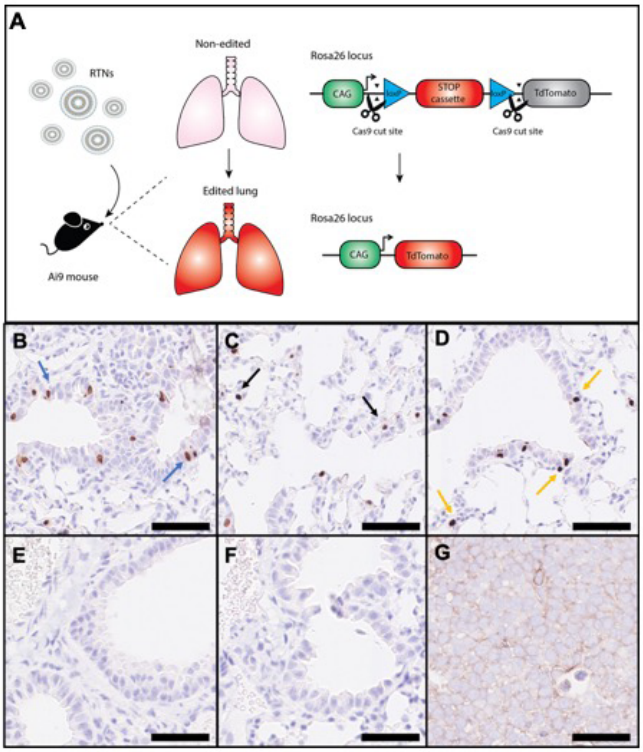

Figure 1A. Ai9 Cre reporter system. Ai9 mice have a loxP-flanked STOP cassette preventing transcription of a CAG promoter-driven tdTomato. RTN delivery of 2 gRNAs targeting upstream and downstream of the STOP cassette as a pre-formed RNP complex will result in release of the cassette, allowing for tdTomato fluorescence. **1B-G. Immunohistochemistry of Ai9 mice treated with RNP nanocomplexes.** TdTomato +ve cells were identified using an anti-RFP antibody, and are observable in the trachea (blue arrows) (**B**), bronchioles (black arrows) (**C**) and alveoli (yellow arrows) (**D**). **E + F** show control-treated mice. **G** shows IHC staining of a TdTomato +ve mouse embryo (day 9.5) as a positive control, with cytoplasmic TdTomato expression. Images are representative of the overall staining of the sections. Scale bar = 50 uM.

1338. Liver-Directed Gene Therapy for Lung Disease in Alpha1 Antitrypsin Deficiency

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Emphysema is one of the primary life-limiting obstructive lung diseases and the leading genetic cause is α-1 antitrypsin (A1AT) deficiency. Hepatocyte-derived A1AT is the major circulating serum serine protease. It largely function to inhibit elastolytic enzymes and alpha-defensins released by activated neutrophils, thus protecting the pulmonary interstitium and capillaries from degradation. Previously we have reported that an A1AT-null mouse model generated in our lab closely recapitulates the clinical characteristics of A1AT related emphysema. Moreover, these genetically predisposed mice also develop accelerated airway remodeling when they are exposed to tobacco smoke, that also closely mimics the lung phenotype and functional consequences found in smokers or ex-smokers patients. In the current studies we aimed to determine whether systemic delivery of an optimized AAV vector encoding human A1AT protein leads to beneficial anti-remodeling treatment effect in these mice: (1) Mice were treated at 20 weeks of age and were subsequently aged for 35 weeks, or (2) mice were treated vector or control at 12 weeks and were either tobacco-smoked or exposed to room air for 8 weeks. To establish preventive and therapeutic threshold of hA1AT serum levels, A1AT-null mice in both studies received single intravenous injection at doses that ranged from 1.0E+14 vg/kg to 2.0E+10 vg/kg. To assess dose-dependant progression of the lung remodeling and physiopathology as well as to identify specific diagnostic and therapeutic biomarkers, we measured pulmonary mechanics, alveolar diameters, analyzed bronchoalveolar lavage for cellular composition and markers of neutrophilic inflammation as well as measured activity of elastolytic enzymes. Treated A1AT-null mice were bled biweekly (smoking cohort) or monthly (aging cohort). A single injection with the AAV8 vector led to life-long expression of detectable levels of normal human A1AT in the serum of A1AT-null mice as determined by ELISA. At optimal AAV doses, the key respiratory parameters, i.e., the total compliance, and elastance did not change in aged and smoked treated animals, indicating preserved elastic properties of the respiratory system. Accordingly, the mean alveolar diameter was significantly increased in untreated compare to treated animals. Importantly, the biochemical activity of circulating hA1AT in AAV treated mice exhibited a high inhibitory capacity in vitro, indicating that presence of serum hAAT translates to a gain of its physiological antielastase function. In conclusion, these experiments are the first to show that AAV mediated A1AT protein augmentation is able to restore protease - antiprotease imbalance and prevent the progression of emphysema associated with A1AT deficiency and we can safely deliver AAV vectors systemically driving the production of fully-functional normal human A1AT protein.

1339. Improved Cardiac AAV Gene Transfer Efficacy in Pigs Using Temporal Mechanical Support

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Purpose: The potential breakthrough in heart failure therapy using adeno associated viral (AAV) gene therapy is becoming increasingly obvious based on successful correction of heart failure in numbers of recent small animal studies. However, efficient cardiac AAV gene transduction seems to be a challenging task in advanced mammals. We hypothesized that prolonging AAV dwell time in the coronary system using coronary balloon occlusion with an aid of mechanical cardiac support device to maintain hemodynamics during delivery can increase cardiac gene expression in pigs. Methods: AAV6 encoding luciferase gene (5.0x 10E13) was injected into the coronary artery one week after myocardial infarction in Yorkshire pigs. A catheter-based cardiac support device, Impella, was used to temporarily support hemodynamics during coronary balloon occlusion to prevent hemodynamic instability. A total of twelve pigs were allocated to one of the following groups: slow antegrade delivery (n=3), slow antegrade delivery with Impella support (n=3), delivery during coronary artery occlusion with Impella support (n=3), delivery during coronary artery

and sinus occlusions with Impella support (CA + CS, n=3), Four weeks later, tissues were harvested from various heart regions and other organs to assess luciferase expression and viral uptake. Results: Impella support offered safe vector delivery during 1-2 minutes of CA and CS occlusion. Impella + coronary artery block enhanced luciferase expression globally in the heart up to 20-fold, but not in non-cardiac tissues, such as liver, as compared to injection without coronary artery block. Impella with CA + CS block even further increased luciferase expression by increasing viral uptake, as evidenced by increased vector genome in the tissues detected by PCR. At the infarct border zone, and the remote myocardium, CA + CS occlusion led to greater than 300-fold increase in luciferase expression compared to viral injection alone. Conclusion: CA + CS block delivery during Impella support dramatically improved AAV gene expression in the heart without compromising hemodynamics or increasing off-target expression. This method offers clinically applicable and efficient cardiac gene delivery and offers new therapeutic option for patients with heart failure.

Cancer Immunotherapy: Clinical Results

1340. KTE-X19, an Anti-CD19 Chimeric Antigen Receptor (CAR) T Cell Therapy, in Patients (Pts) with Relapsed/Refractory Mantle Cell Lymphoma (R/R MCL): Results from Phase 2 of ZUMA-2

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Background: Outcomes with salvage regimens in pts with MCL who progress after Bruton tyrosine kinase inhibitor (BTKi) therapy are poor. Here, we present interim results from ZUMA-2, the first Phase 2, registrational, multicenter study evaluating KTE-X19 autologous anti-CD19 CAR T cell therapy in pts with R/R MCL. **Methods:** Eligible pts (≥ 18 years) with R/R MCL, an ECOG of 0 - 1, and ≤ 5 prior therapies (including chemotherapy, an anti-CD20 antibody, and a BTKi) underwent leukapheresis and conditioning chemotherapy followed by KTE-X19 infusion at 2×10^6 cells/kg. Bridging chemotherapy was permitted. The primary endpoint was objective response rate (ORR [complete response (CR) + partial response]), assessed by an Independent Review Committee per Lugano Classification (Cheson, et al. 2014). Interim efficacy endpoints were investigator-assessed using the revised International Working Group Response Criteria for Malignant Lymphoma (Cheson, et al. 2007). Key secondary endpoints included duration of response (DOR), progression-free survival (PFS), overall survival (OS), frequency of adverse events (AEs), and blood levels of CAR T cells. Sixty pts received KTE-X19; here, we present results in pts with ≥ 1 year of follow-up. Updated results for all 60 pts will be reported in the presentation. **Results:** As of May 30, 2018, 28 pts received KTE-X19 with ≥ 1 year of follow-up (median, 13.2 months). The median age was 65 years; 43% of pts had an ECOG 1; 21% had blastoid morphology; 82% had stage IV disease; 50% had intermediate/high-risk MIPI; 86% received a median of 4 prior therapies; 57% were refractory to last prior therapy. Eight pts received bridging therapy; all had disease present post-bridging. Investigator-assessed ORR was 86% (95% CI, 67% - 96%), with a CR rate of 57%; 75% of responders remained in response, and 64% of treated pts had ongoing responses. The 12-month estimates of DOR, PFS, and OS were 83%, 71%, and 86%, respectively; medians were not reached. The most common Grade ≥ 3 AEs were anemia (54%), platelet count decrease (39%), and neutropenia (36%). Grade 3/4 cytokine release syndrome (CRS; by Lee et al. 2014) and Grade 3/4 neurologic events (NEs) were reported in 18% and 46% of pts, respectively (no Grade 5 CRS or NEs). All CRS events and most NEs (15/17 pts) were reversible. There was 1 Grade 5 AE of organizing pneumonia, considered related to conditioning chemotherapy. Median CAR T cell levels as measured by peak and area under the curve were 99 cells/μL (range, 0.4 - 2589) and 1542 cells/μL (range, 5.5 - 27239), respectively. Peak CAR T cell expansion was observed between Days 8 and 15 and levels declined over time. **Conclusions:** With ≥ 1 year of follow-up, KTE-X19 demonstrated significant and durable clinical benefit and a manageable safety profile in pts with R/R MCL for whom there are no curative treatment options.

1341. Exhaustion and Activation Status in Apheresed T Cells Correlate with Response to Anti-BCMA CAR T Cell Therapy in Myeloma

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We recently demonstrated that 12 out of 25 heavily pretreated myeloma patients achieved a partial response or better after anti-BCMA CAR-T cell treatment. To better understand the biological basis of this therapy, we aimed to identify key correlates of response using the pre-manufacturing apheresed T cells, the infusion product, and post-infusion T cells from the 25 patients in this cohort.CAR T-cell expansion, measured by the area under the curve of CAR qPCR in the first 21 days (AUC[0-21]), was highest in responding (RE), lowest in non-responding (NR) patients (JT=38, p=1.8x10^-6). Soluble BCMA, a biomarker of disease burden, shows a similar

trend with response (JT=54, p=1.2x10^-4). Furthermore, AUC[0-21] for CAR T cell expansion and soluble BCMA decline also strongly correlated (Spearman's rank correlation test, rho=0.82; p=2.41x10^-6), underscoring the quantitative relationship between CAR T cell expansion and tumor reduction.We immunophenotyped apheresed (APH) T-cells and infusion product from the 25 patients to determine if T-cell subsets correlate with response. Phenotypically distinct T-cell subpopulations were identified using shared-nearest-neighbor clustering method (PMID: 31178118) and their correlation with response to CAR T-cell treatment was evaluated. This analysis revealed that among APH CD4+ and CD8+ T-cells, subpopulations representing naive and central memory cells were enriched in T-cells from RE individuals, while NR displayed a distinctly activated effector phenotype at baseline. Additional analyses showed that APH T-cells from RE patients were non-cycling, granzyme B-negative, CTLA4[low] but otherwise largely immune checkpoint inhibitor-negative. Apheresed CD8+ T-cells from NR exhibited high expression levels of TIM3 or LAG3, and/or granzyme B, but not PD1, CTLA4, CD45RO or CD27. These data confirm the high activation, potential exhaustion and end-stage differentiation state of APH T-cells in this group. Similar analyses of infusion product CAR T cells did not reveal subpopulations associated with response.Clustering analysis of CAR T-cell phenotypes within 20 days after infusion uncovered a non-cycling, negatively regulated, Eomes-expressing central memory CD8+ CAR T cell subset in RE patients. NR patients CAR T-cells displayed high levels of granzyme B and PD1 expression but were otherwise devoid of signs of activation. Four patients with a sufficiently high proportion of CAR expressing cells were phenotyped up to 125 days post-infusion. This analysis showed that the highly activated CAR T-cell clusters dominated at early phases post-infusion but were rapidly replaced by non-cycling CAR T-cells with downregulated CTLA4 and LAG3 but maintained expression of PD1 and TIM3. Patient 27 with VGPR had a prominent effector population four months after infusion. BCMA-redirected CD4+ CAR T-cells showed an enrichment of central memory phenotype CAR T-cells in responding patients early after infusion, with high expression of Eomes, TIM3, and other immune checkpoint inhibitor molecules. This cluster also dominated the CD4 T-cell repertoire in the first four months after infusion in the four responding patients.In conclusion, our data suggest that strategies to promote expression of Eomes and central memory function and reduce exhaustion in BCMA CAR T-cells will enhance clinical activity. Further, these results underscore the "self-sustaining" feature of successful CAR T-cell therapies in myeloma.

1342. Identification and Validation of Predictive Biomarkers to CD19- and BCMA-Specific CAR T-Cell Responses in Apheresed T-Cells

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Chimeric antigen receptor (CAR) T cell therapies have been highly effective against B cell malignancies. We here tested the hypothesis that the composition of apheresed T cells determines clinical efficacy in adult and pediatric Acute Lymphoblastic Leukemia (ALL), Non-Hodgkin's Lymphoma (NHL), Multiple Myeloma (MM), and Chronic Lymphocytic Leukemia (CLL). Apheresed T cells were engineered to express a CD19 or B cell maturation antigen (BCMA)-targeting CAR. The same 9-day manufacturing process was used for all trials. CAR T cell kinetics following infusion were monitored using a transgene-specific quantitative PCR assay. Standard clinical response assessments were performed. Apheresed T cells from 36 CLL, 30 adult ALL, 58 pediatric ALL, 33 NHL, and 25 MM patients were immunophenotyped by flow cytometry. The CLL cohort was used to discover phenotypically distinct subpopulations associated with the two main response groups; these associations were validated in the remaining patient cohorts. Eight CD8+ T cell populations or clusters were identified using the shared-nearest-neighbor clustering method (PMID: 31178118) in the CLL cohort. T cell subsets exhibiting naive (cluster 6) or early memory (cluster 4) features were significantly enriched in responding patients, whereas an effector memory CD8 subpopulation (cluster 2) marked the non-responding patients. Mapping these clusters onto apheresed CD8+ T cells from the other four diseases showed that cluster 4 predicted response to CAR T cell therapy in NHL and myeloma but not in adult and pediatric ALL. We also examined the expression of activation-regulated molecules including HLA-DR, Ki67, and exhaustion-related molecules PD1, CTLA4, TIM3, and LAG3. A CD27+ CD8+ population expressing low level CTLA4 but none of the activation or negative regulatory molecules was significantly enriched in responding CLL patients; this cluster validated in NHL and myeloma. A separate analysis of checkpoint inhibitory receptors and activation markers in memory CD4 T cell subsets confirmed the early memory, non-activated state of this population in CLL and was validated in myeloma but none of the other diseases. In vivo activation was a shared theme in CD4+ T cells for non-responding patients as well, though these CLL-defined CD4+ apheresed T cells clusters did not significantly validate in other diseases.In summary, our data confirm and extend our predictive biomarker profile in CLL to mature B cell and

plasma cell malignancies by showing that a non-cycling, non-activated early memory CD8+ T cell population in pre-manufacturing cells was validated as a biomarker in myeloma, and NHL. We also showed that responder-associated apheresed CD4+ T cells with early memory features identified in CLL after CD19 CAR T infusions are validated in myeloma after BCMA CAR T. Thus, differentiation state and in vivo activation, and potentially exhaustion, separate response groups. Our findings inform next-generation CAR T-cell manufacturing using the populations identified herein as a starting population.

1343. Correlative Analysis from CRB-402: An Ongoing Phase 1 Clinical Study of bb21217 Anti-BCMA CAR T Cell Therapy

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B-cell maturation antigen (BCMA) is primarily expressed by malignant and normal plasma cells, making it an attractive target for the treatment of multiple myeloma (MM). In a phase 1 clinical study of the BCMA directed CAR T cell therapy idecabtagene vicleucel (ide-cel, bb2121) for the treatment of relapsed/refractory MM, an overall response rate of 85% was reported. While the 11.8 month median progression-free survival reported with ide-cel in this heavily pretreated population compares favorably with available therapies (Chari et al., 2019; Lonial et al., 2016; Raje et al., 2019; Richardson et al., 2014; Siegel et al., 2012), we aimed to determine if we could extend duration of response to a BCMA directed CAR T therapy using bb21217. bb21217 uses the ide-cel CAR molecule but the manufacturing is optimized to include a PI3K inhibitor (bb007) intended to increase the in vivo persistence of functional CAR T cells based upon preliminary studies in preclinical tumor models. Peripheral blood mononuclear cells (PBMCs) from 5 MM donors were cultured with or without the addition of bb007 for 7 or 10 days. We demonstrated that the additive effect of manufacturing in the presence of bb007 with a shorter culture time leads to a unique composition of CAR T cells with a phenotype associated with improved T cell fitness. An ongoing phase I clinical study (CRB-402; NCT03274219) is assessing safety and efficacy of bb21217 in relapsed/refractory MM patients. Using mass cytometry and RNA-sequencing, we examined drug product (DP) from 18 patients enrolled in the CRB-402 study for biomarkers associated with response and durability of response. Consistent with findings in other CAR T cell clinical studies, CAR T cell peak expansion post-infusion is associated with initial clinical response (Berdeja, 2019). Phenotypic markers of early memory T cells in bb21217 drug products (e.g. LEF1, CD27 and CCR7) correlated positively with peak expansion. In contrast, markers of terminally differentiated effector cells (e.g. CD57, GZMA, GZMB) correlated negatively. To assess the relationship between T cell memory markers in drug product and durability of response, we compared DP from patients with or without disease progression by month 6 (M6). Using unsupervised clustering of all CD3+ markers, patients clustered into two groups. One cluster was enriched for patients without disease progression by M6 (5/6 patients), these patients had DP enriched for early memory markers including LEF1, CD27 and CCR7 and demonstrated low expression of effector markers CD57, GZMA, GZMB and EOMES. The second cluster enriched for patients with disease progression by month 6 (5/12 patients). In contrast, patients in this cluster had DP enriched for effector T cell markers including CD57, GZMA, and GZMB and demonstrated low expression of early memory markers including LEF1, CD27 and CCR7. The correlation of early memory markers to responses was confirmed by RNAseq, using both unsupervised clustering and gene set enrichment analysis on the differentially expressed genes across patient populations. Immunophenotyping data from ex vivo clinical samples, including memory phenotypes, and their relationship to DP phenotype and clinical outcome, will also be presented. These early data suggest that bb21217 DP enriched for early memory phenotypes is associated with more robust peak expansion and more durable disease control than bb21217 DP enriched for effector phenotypes.

1344. A Comparison of 2-Year Outcomes in ZUMA-1 (Axicabtagene Ciloleucel; Axi-Cel) and SCHOLAR-1 in Patients (Pts) with Refractory Large B Cell Lymphoma (LBCL)

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Background: In the SCHOLAR-1 retrospective analysis of outcomes in pts with refractory LBCL, the objective response rate (ORR) was 26% (7% complete response [CR] rate), serving as a benchmark for assessing novel therapies (Crump, et al. 2017). In the ZUMA-1 Phase 1/2 study evaluating axi-cel, an autologous anti-CD19 chimeric antigen receptor (CAR) T cell therapy in pts with refractory LBCL, the ORR was 83% (58% CR rate; 27.1 mo median follow-up; Locke et al. 2019). Here we compare outcomes in ZUMA-1 and SCHOLAR-1 after adjusting for potential imbalances in refractory status between studies. **Methods:** Pts in both studies had stable disease of ≤ 6 mo with ≥ 4 cycles of frontline or ≥ 2 cycles of later-line therapy, progressive disease as best response, or relapse ≤ 12 mo after autologous stem cell transplant (SCT). Standardized analyses were performed that equally weighted

the proportions of pts by refractory categorization (primary refractory, refractory to ≥ second-line therapy, or relapse after SCT) and presence of post-refractory SCT. Stratified Cochran-Mantel-Haenszel (CMH) tests and Cox models were used to compare the odds ratio for response and hazard ratio (HR) for survival between ZUMA-1 and SCHOLAR-1. *P* values were descriptive and were not adjusted for multiplicity. **Results:** Axi-cel was administered to 101 pts in Phase 2 of ZUMA-1 (median follow-up of 2.3 y). In SCHOLAR-1, 508 and 497 pts were evaluable for response and survival, respectively (median follow-up of 7.6 to 14.8 y across cohorts). In general, ZUMA-1 pts were more heavily pretreated than SCHOLAR-1 pts, with more ZUMA-1 pts receiving ≥ 3 lines of therapy (69% vs 23%). ZUMA-1 also had more pts refractory to ≥ second-line therapy vs SCHOLAR-1 (76% vs 62%). However, fewer pts in ZUMA-1 than SCHOLAR-1 were classified as primary refractory (26% vs 45%). A similar proportion of pts between studies relapsed within 1 y of SCT (21% vs 18%). After standardization, the ORR and CR rate in ZUMA-1 vs SCHOLAR-1 were 72% and 54% vs 22% and 7%, respectively. The odds ratios for ORR and CR rate were 7.2-fold and 11.5-fold higher, respectively, in ZUMA-1 than SCHOLAR-1 (CMH test; $P < .0001$ for both ORR and CR rate). The 2-year survival rate after standardization was 50% in ZUMA-1 and 12% in SCHOLAR-1, a 73% reduction in the risk of death in ZUMA-1 vs SCHOLAR-1 (HR, 0.27; $P < .0001$). **Conclusions:** This standardized analysis of ZUMA-1 and SCHOLAR-1 indicates that axi-cel treatment in a selected population increased the odds of CR and reduced the risk of death vs standard salvage regimens in an unselected population. Although limited by retrospective evaluation and cross-study comparisons, these results support literature indicating that axi-cel is a highly effective treatment option for pts with refractory LBCL.

1345. Autologous CD34+-Enriched Hematopoietic Progenitor Cells Genetically Modified for Human Interferon-Alpha2 (Temferon), Rapidly Engraft and Mature in Patients with Glioblastoma Multiforme (TEM-GBM_001 Study)

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We are conducting a Phase I/IIa clinical study in Milan (Part A: Phase I, 3x3x3 dose escalation; Part B: Phase II) to modify the tumor microenvironment with a novel cell-based therapy (Temferon) in 21 patients with glioblastoma multiforme (GBM) & unmethylated MGMT promoter (NCT03866109). Temferon consists of autologous human hematopoietic stem & progenitor cells (HSPCs) transduced *ex vivo* with a 3rd generation lentiviral vector encoding a human interferon alpha-2 (IFN) gene controlled by a human Tie2/TEK enhancer/promoter sequence & a post-transcriptional regulation layer of miRNA-126 target sequences restricting IFN transgene expression to a subtype of M2-macrophages which express receptors for the angiopoietin receptor Tie2 (TEMs). TEMs are a suitable carrier for the local & tumor restricted release of IFN thus enabling efficacy combined with reduced systemic toxicity. In animal models Temferon modifies the tumor microenvironment & induces de novo CD8+ antigen-specific T cells. To date, 6 patients have been successfully screened & recruited (4 women, 2 men, mean age 57.3 years). Autologous HSPCs were collected by single apheresis after G-CSF/Plerixafor stimulation. Temferon production was successful, with a median vector copy number (VCN) of 0.6 & transduction efficiency 54% (most HSPCs carry a single integrant). Conditioning occurred with carmustine 400mg/m^2 at D-6 & thiotepa 5mg/kg given twice on D-5. At Day 0 prior to Temferon, 3x10^6 CD34+/kg non-manipulated HSPCs were infused as support. 3 patients (Cohort 1) received Temferon 0.5x10^6 cells/kg & 1 patient from cohort 2 had an intermediate dose of 1x10^6 cells/kg. 2 patients will receive an intermediate dose shortly. Neutropenia & thrombocytopenia occurred as expected following conditioning, & in a consistent manner across all the patients with a nadir occurring by approximately D+8 & hematological recovery by D+13. Transduced peripheral blood mononuclear cells were identified by VCN on myeloid cell populations at hematological recovery & at later timepoints. 2 patients in Cohort 1 achieved a VCN in CD14+ monocytes at D+30 of 0.155 and 0.116, respectively. The measured VCN is aligned with the expected level of chimerism of approx. 9%. The 3rd patient had a low level of engraftment with a VCN of 0.011 in BM CD33+ cells at D+30. A dose ordered increase in CD14+ VCN was achieved in the 1st patient of cohort 2 at D+30 (0.185). In the 2 patients with high VCN at D+30 in Cohort 1, although a reduction in VCN in CD14+ cells was observed between D+30 & +60, the VCN remained stable up to the most recently analyzed timepoints (Pat#1: D+120, 0.055; Pat#3, D+90, 0.066). Analyses of IFN expression & gene signatures in blood & tissues are ongoing. Tumor & clinical status is monitored. In summary, these preliminary results indicate feasibility of engrafting a pre-determined fraction of Temferon cells in the BM of GBM patients, which stabilizes after D+60 & remains detectable until the latest follow up. Updated results will be presented. *Co-senior authors.

1346. Earlier Steroid Use with Axicabtagene Ciloleucel (Axi-Cel) in Patients (Pts) with Relapsed/Refractory Large B Cell Lymphoma (R/R LBCL)

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Background: In the ZUMA-1 study evaluating axi-cel, an autologous anti-CD19 chimeric antigen receptor (CAR) T cell therapy, 11% and 32% of pts in Cohorts 1+2 (C1+2; N=101) experienced Grade ≥ 3 cytokine release syndrome (CRS) and neurologic events (NEs), respectively (Locke et al. 2019). With 39.1 mo median follow-up, the median overall survival (OS) was 25.8 mo, and the 3-y OS rate was 47%. A safety expansion cohort (Cohort 4 [C4]) evaluated the impact of earlier steroid use on adverse events (AEs). Here we present the primary analysis of ZUMA-1 C4 with more pts and longer follow-up. **Methods:** Eligible pts with R/R LBCL were leukapheresed, could receive bridging chemotherapy (not allowed in C1+2), and received conditioning chemotherapy before axi-cel infusion at 2×10^6 cells/kg. C4 pts received steroid intervention at Grade 1 NEs and Grade 1 CRS after 3 days of supportive care. Primary endpoints were incidence and severity of CRS and NEs. Additional endpoints included efficacy and biomarker analyses. Objective response rate (ORR) and CAR T cell levels were compared by tumor burden. **Results:** As of May 6, 2019, 41 pts in C4 received axi-cel (8.7 mo median follow-up). Pts who received bridging therapy (68%) all had evidence of disease post-bridging. Median age was 61 y; most pts (63%) had DLBCL; 49% had an ECOG 1; 70% had disease stage III/IV; 68% were refractory to ≥ second-line therapy; 63% had ≥ 3 prior therapy lines; and 20% relapsed after autologous stem cell transplant. C4 pts generally had lower median tumor burden by sum of product diameters (SPDs; C4, 2100 mm^2; C1+2, 3723 mm^2). A greater proportion of C4 pts received steroids and tocilizumab than in C1+2 (73% and 76% vs 27% and 43%). Fewer pts in C4 experienced Grade ≥ 3 CRS (2%) and NEs (17%) than in C1+2. The ORR in C4 was 73%, with a complete response rate of 51%. With ≥ 6 mo follow-up, response was ongoing in 54% of C4 pts vs 44% of C1+2 pts. Although most pts in C4 had lower SPD than in C1+2, responses were comparable by tumor burden. Median duration of response was 8.9 mo, consistent with C1+2 (8.1 mo; Locke F. AACR 2017). Median progression-free survival was 11.7 mo (median OS not reached). Peak CAR T cell levels were 42 cells/μL blood in C1+2 vs 59 cells/μL in C4. C1+2 had a median CAR AUC of 462 cells/μL × days versus 512 cells/μL × days in C4. CAR T cell expansion by tumor burden was comparable between cohorts. Levels of NE-associated biomarkers including ferritin (pre- and post-treatment) and IL-2 (post-treatment) appeared lower in C4 than in C1+2. **Conclusions:** Earlier steroid use may reduce rates of CAR T cell treatment-related CRS and NEs without affecting efficacy. Conclusions are limited by the nonrandomized study design and differences in population sizes and baseline characteristics between cohorts. Optimizing AE management is important to improve the benefit-risk profile of CAR T cell therapy.

Gene Therapy for Inborn Errors of Metabolism: New Approaches

1347. ImmTOR™ Tolerogenic Nanoparticles Enable Gene Therapy of Mice with Maternally-Transferred Anti-AAV Antibodies in a Mouse Model of Methylmalonic Acidemia

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Tolerogenic nanoparticles encapsulating rapamycin (ImmTOR) have been shown to mitigate the formation of antibodies against AAV, allowing for vector re-dosing. Moreover, co-administration of liver-tropic AAV vectors and ImmTOR augments transgene expression even after the first dose. We tested the safety and therapeutic efficacy of an admixed ImmTOR and AAV vector combination in a mouse model of methylmalonic acidemia (MMA) using Anc80, a synthetic AAV capsid. Repeated co-administration of Anc80 and ImmTOR was well-tolerated and led to complete inhibition of IgG antibodies to Anc80 vectors. Several expression cassettes were tested with human *MMUT* gene driven by a liver-specific promoter showing the highest therapeutic potential. A more profound decrease of serum methylmalonic acid after initial and repeat injections was observed in mice treated with the combination of ImmTOR and Anc80-MMUT, which correlated with higher viral genome copy number per liver cell (vg/cell) as well as higher hepatic *MMUT* mRNA expression levels. Similarly, the levels of FGF21, an important biomarker of hepatic mitochondrial dysfunction in MMA, were further decreased after the repeat administration of Anc80-MMUT combined with ImmTOR, but not by Anc80-MMUT alone. These effects were dose-dependent, with higher doses of ImmTOR providing for higher vg/cell levels and lower plasma methylmalonic acid levels. Moreover, therapeutic treatment of juvenile mice with maternally-transferred neutralizing antibodies (Nabs) to Anc80 using Anc80-MMUT alone was completely ineffective, if not counterproductive. In contrast, the combination of high-dose ImmTOR and Anc80-

MMUT was well-tolerated by mice with maternally-transferred Nabs. Furthermore, this combination treatment prevented the formation of de novo anti-AAV antibodies, and enabled multiple redosings, which led to therapeutic benefit as indicated by a decrease in serum levels of MMA and FGF21, weight gain and survival. Therefore, the admix of ImmTOR and AAV gene therapy vector is a promising approach to mitigate the detrimental impact of maternally-transferred Nabs on gene therapy for MMA and may also provide a benefit in enhancing transgene expression at the initial dose.

1348. The First Viable Mouse Model of Combined Methylmalonic Acidemia and Homocystinuria, *cblC* Type Displays a Sustained Response to a Single Dose of AAV Gene Therapy and is Comparable to Chronic Hydroxocobalamin Administration

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Combined methylmalonic acidemia and homocystinuria, *cblC* type (*cblC*) is the most common inborn error of intracellular cobalamin (vitamin B12) metabolism caused by pathogenic variants in *MMACHC*. The protein encoded by *MMACHC* is known to transport and process cobalamin into its two bioactive cofactors, 5´-adenosylcobalamin and methylcobalamin, necessary for the enzymatic reactions of methylmalonyl-CoA mutase, MMUT, and methionine synthase, MTR, respectively. Early onset disease can be severe with symptoms including growth failure, anemia, heart defects, neurocognitive impairment, and progressive vision loss. In order to further characterize manifestations of the disease and investigate therapeutic modalities, we used genome editing to create pathogenic mutations in *Mmachc* and studied two alleles including c.163_164delAC p.Pro56Cysfs*4 (del2) and c.162_164delCAC p.Ser54_Thr55delinsArg (del3). *Mmachc* mutant embryos were present in Mendelian ratios at embryonic day 18.5, but a decreased number of homozygous mutant pups were observed at birth. Mutant mice had a median survival of five days (*Mmachc*$^{del2/del2}$ N = 14; P < 0.0001, *Mmachc*$^{del3/del3}$ N = 66; P < 0.0001). At two weeks, *Mmachc*$^{del3/del3}$ mutant mice weighed 36% less than littermate controls (N = 12; P < 0.0001) and displayed biochemical features characteristic of *cblC* including significantly elevated plasma methylmalonic acid (MMA) and homocysteine (HCYS) and decreased methionine (MET) as compared to littermate controls. Pathological examination at one month revealed hydrocephalus and hypoplasia of the corpus callosum in *Mmachc*$^{del3/del3}$ mutants. Two AAV gene therapy vectors were developed for the treatment of the *cblC* mice, including rAAVrh10-CBA-*Mmachc* (rAAVrh10) and rAAV9-CBA-co*MMACHC* (rAAV9). The AAVs (dose = 1 x 10^{11} GC/pup) were delivered by a single intrahepatic injection on the first or second day of life and compared to weekly hydroxocobalamin (OHCbl) administration. Increased survival was observed in *Mmachc*$^{del3/del3}$ mutant mice treated with rAAVrh10 (N = 9; P < 0.0001), rAAV9 (N = 11; P < 0.0044), prenatal and chronic OHCbl (N = 16; P < 0.0001), and prenatal OHCbl and rAAV9 (N = 11; P < 0.0001). Long term weights between the different treatment groups were comparable and MMA was greatly reduced six to thirteen months post treatment withprenatal OHCbl and rAAVrh10 (*Mmachc*$^{del3/del3}$ 159.7 ± 7.478 μM; N = 3) and prenatal OHCbl and rAAV9 treatments (*Mmachc*$^{del3/del3}$ 136.9 ± 47.52 μM; N = 3), but not with prenatal and chronic OHCbl (*Mmachc*$^{del3/del3}$ 1517 ± 515.1 μM; N = 4). Pathological examination of *Mmachc*$^{del3/del3}$ mutant mice treated with rAAV9, one year after vector administration, established a mitigation of neuropathology. These Mmachc mouse models recapitulate key phenotypic and metabolic disease manifestations common among patients living with *cblC*. A single intrahepatic injection of AAV gene therapy, expressing either the mouse *Mmachc* or human *MMACHC* gene, provides a superior clinical and biochemical response as compared to chronic OHCbl administration. Our results demonstrate that AAV gene therapy could be a promising therapeutic modality for the treatment of both younger and older patients with *cblC*, the most common disorder of intracellular vitamin B12 metabolism.

1349. Developing and Evaluating Second-Generation AAV Vectors for Liver-Directed Gene Therapy of Familial Hypercholesterolemia in Nonhuman Primates

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Familial hypercholesterolemia (FH) is a life-threatening genetic disorder caused by loss-of-function mutations in the low-density lipoprotein receptor (LDLR) gene. Without treatment, plasma cholesterol levels in homozygous FH (hoFH) patients typically exceed 500 mg/dl, resulting in premature and aggressive atherosclerosis. Consequently, this can lead to cardiovascular disease (CVD) before age 20 and death before age 30. Combination treatment with statins and ezetimibe (a cholesterol absorption inhibitor) can reduce low-density lipoprotein cholesterol (LDL-C) levels and CVD. Despite the implementation of such an aggressive multi-drug therapy approach, however, the LDL-C levels of HoFH patients often remain elevated and their mean life expectancy is only 32 years. While liver transplantation is currently the best treatment option for these patients, liver-directed gene therapy may represent a viable alternative. We previously developed an AAV8 vector containing the native human LDLR (hLDLR) cDNA under the control of a liver-specific promoter. In the current study, we took a systematic approach to optimize three key components of the vector, namely the AAV capsid, the LDLR cDNA codons, and the intron. We first evaluated vector constructs in LDLR-deficient mice and selected the most efficient construct for evaluation in nonhuman primates (NHPs). Concurrently, we used a scorecard approach to assess the initial diversity on the AAV3B hyper-variable region (HVR) VIII. After two rounds of selection in human hepatocyte-xenografted Fah-/-/Rag2-/-/Il2rg-/- (FRG) mice, we selected the top 16 variants for further evaluation in NHPs with

a validated barcode system. We used two AAV3B variants along with AAV3B to package the optimized hLDLR construct. Detailed evaluation followed administration of this construct at two vector doses to NHPs under transient immune suppression with prednisolone. We achieved significant reduction of LDL levels as early as three days post-dosing, with this reduction lasting for about four weeks. Conversely, the control vector (AAV8.hLDLR-native) only showed mild LDL reduction in one out of four treated animals. We detected hLDLR expression via *in situ* hybridization and immunohistochemical staining on liver biopsies collected on day 18 post-vector administration. However, expression levels were significantly lower at the time of necropsy (day 120). As we detected a positive T-cell response via ELISPOT assays to hLDLR and, to a lesser degree, to the AAV capsid in liver lymphocytes isolated at the time of necropsy in 50% of the NHPs, an immune response to the human transgene product may have reduced transgene expression at this later time point. We also observed elevated liver enzymes, especially among animals treated with AAV3B and one of the AAV3B variants. Overall, this study demonstrates that we generated an improved and more efficient AAV vector for gene therapy of FH.

1350. Intermittent mRNA Therapy for Arginase Deficiency Results in Prevention of Dysmyelination of the Corticospinal Tract

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Arginase deficiency, caused by inactivating mutations in the ARG1 gene, results in hyperammonemia, hyperargininemia and the presence of circulating guanidino compounds. The disorder is associated with prominent neuromotor features including spastic diplegia/tetraplegia, clonus, and hyperreflexia; loss of ambulation, intellectual disability, and progressive neurological decline are other signs. There is currently no completely effective medical treatment available. We recently, using a constitutive murine model of the disorder, described leukodystrophy as a significant component of the CNS features of arginase deficiency: utilizing electron microscopy (EM), marked dysmyelination was detected in 2-week-old homozygous *Arg1* knockout mice with the corticospinal tract (CST) being adversely affected. Thus, dysmyelination appears to be a prominent cause of unique neuromotor features. In this present study, we sought to examine if the administration of a lipid nanoparticle carrying the human *Arg1* mRNA, to constitutive knockout mice, could prevent this abnormality of myelination in the CST. Methods: Three genotype groups of mice were included in this study: a wild type group, an untreated biallelic *Arg1* knockout group, and an mRNA/lipid nanoparticle (LNP)-treated knockout group. Recent studies by our group have demonstrated that murine myelination in the CST begins around postnatal day (P) 8. Therefore, beginning at P8, knockout mice were administered human codon-optimized mRNA in an LNP by intraperitoneal route until P14; at that time mice were euthanized and studied. Cervical segments of spinal cord containing the corticospinal tract were processed for EM and myelinated axons (defined as axon caliber covered by at least one layer of electron-dense myelin sheath) were identified with about 15-20 images taken from the CST region for each animal. The myelinated axon G-ratio analysis was also determined and performed using EM images taken at 8,000X. Results: Comparing to wild types, low magnification electron micrographs of cervical segments in the untreated knockout mice demonstrated a drastic reduction of myelinated axons in CST, with many being small-sized axons. Signs of degenerating axons in CST of untreated KO mice were also present with thin myelin layers containing electron-dense debris. With daily Arg1 mRNA/LNP administration, electron microscopy demonstrated dramatic recovery of myelination in the CSTof P14 treated knockout mice when compared with age-matched wild type controls; oligodendrocytes were seen to be extending processes to wrap many axons. Quantitatively, myelinated axon density in the cervical spinal cord was greatly reduced in the untreated KO group. However, with intermittent LNP/Arg1 mRNA administration, the CST axon density was substantially increased. Myelin thickness was then assessed by calculating the G-ratio of the CST. While the G-ratio is elevated in the untreated KOmice (i.e. thinner myelin sheath, when myelination was present) compared to the wild type, the G-ratio is reduced with intermittent hARG mRNA/LNP administration, indicative of an increase in the thickness of the myelin sheath with Arg1 mRNA/LNP administration. Conclusion: mRNA/lipid nanoparticle administration in arginase deficient mice prevents dysmyelination of the corticospinal tract and may have important implications for the prevention of spastic diplegia in this condition.

1351. AAV Gene Therapy in Mucopolysaccharidosis IVA Murine Models

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Mucopolysaccharidosis type IVA (MPS IVA) is due to the deficiency of N-acetylgalactosamine 6-sulfate sulfatase (GALNS) and characterized by systemic skeletal dysplasia. The current standard of care is enzyme replacement therapy, which does not address the skeletal impairment and valvular heart disease. We have evaluated AAV8 vectors expressing different forms of human native GALNS or GALNS with an aspartic acid octapeptide (D8) under liver-specific thyroxin-binding globulin (TBG) or CMV early enhancer/chicken β actin (CAG) promoters. The four different vectors (AAV8-TBG-hGALNS, AAV8-TBG-D8-hGALNS, AAV8-CAG-hGALNS, AAV8 CAG-D8-HGALNS) were delivered intravenously into 4-week-old MPS IVA knockout (KO) and/or immune tolerant (MTOL) mice at a dose of $5x10^{13}$ and/or $2x10^{14}$ GC/kg (n = 4-8 per experiment). These mice were monitored for 12 weeks post-injection. GALNS enzyme activity was elevated significantly in plasma of all treated mice two weeks post-injection. The activity observed was 6-60-fold higher than that in WT mice and was maintained throughout the monitoring period. The highest plasma enzyme activity on average was observed in MTOL mice treated with AAV8-CAG-D8-GALNS. In tissues post-autopsy, KO mice treated with either of four different vectors showed the highest activity in the liver (25-46 folds higher than those in wild-type mice; $p < 0.02$). The enzyme activities in heart and bone treated with AAV8-TBG-hGALNS and AAV8-TBG-D8-hGALNS were similar to those in wild-type mice. Meanwhile, the delivery of AAV8-CAG-hGALNS and AAV8 CAG-

D8-HGALNS resulted in higher activities in heart (5.6-5.8 folds of enzyme activity in wild-type mice; $p < 0.01$) and bone (2.7-4.6 folds of enzyme activity in wild-type mice; $p < 0.05$ - 0.001). Treatment with AAV vectors resulted in the reduction of plasma KS levels to normal levels two weeks post-injection, which were maintained until necropsy (at 16 weeks old). Micro CT analysis of femur from these experimental mice showed that AAV8 CAG-D8-HGALNS and AAV8-CAG-hGALNS suppressed abnormal proliferation of trabecular bone observed in untreated KO mice more than AAV8-TBG-hGALNS and AAV8-TBG-D8-hGALNS and that skeletal phenotype in micro CT was ameliorated, suggesting more impact on the bone. Both AAV8-TBG-hGALNS and AAV8-TBG-D8-hGALNS vectors reduced the storage in articular cartilage, ligaments, and meniscus surrounding articular cartilage and growth plate region as well as heart muscle and valves. Pathological findings in AAV8 CAG-D8-HGALNS and AAV8-CAG-hGALNS vectors are under investigation. Our results suggest that the continuous presence of high levels of circulating enzyme could potentially increase the penetration into bone and heart, reduce KS levels, and improve storage in these regions. The current data support a strategy for developing a novel treatment to address the bone and heart disease in MPS IVA using AAV gene therapy.

1352. Base Editing of an Allosteric Site within Serpina1 Yields Improved Phenotypes in Models of Alpha-1 Antitrypsin Deficiency

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Alpha-1 Antitrypsin Deficiency (A1AD) is a rare genetic disease most commonly caused by a single point mutation that promotes misfolding and polymerization of the resulting PiZ protein. The toxic build-up of aggregates within hepatocytes often leads to fibrosis and in certain patients may progress to clinical liver disease. The insufficient Alpha-1 Antitrypsin (A1AT) in circulation induces a protease-antiprotease imbalance in the lungs of affected individuals resulting in loss of elastin and progressive pulmonary disease. Base editing is a gene-editing technology that enables programmable conversion of one base to another without double-stranded break intermediates. Previously published studies have identified point mutations within the A1AT protein that are capable of stabilizing PiZ A1AT. Based upon this precedent, we hypothesized that a cytosine base editor (BE4) could be used to introduce a C>T transition mutation within SERPINA1 to generate an allosteric compensatory mutation (M374I) within the A1AT protein. In cellular secretion assays and elastase inhibition assays we have verified that the PiZ+M374I protein exhibits a phenotype intermediate to that of PiZ and wild-type A1AT. In cultures of primary human hepatocytes from unaffected individuals we have demonstrated robust base editing of the compensatory mutation. In patient-derived iPSC models we have observed an improvement in cellular secretion of A1AT after transfection with base editing reagents. Furthermore, we have employed lipid nanoparticles to deliver both guide RNA (gRNA) and mRNA encoding base editor to the liver of PiZ transgenic mice yielding high rates of base editing. Importantly, this base editing generates a significant increase in serum A1AT concentration and serum elastase inhibitory capacity, as well as a decrease in A1AT globule burden as assessed by PAS-D staining. Based upon the results in various biochemical, cellular and in vivo models, the described allosteric base editing strategy has the potential to address both A1AD liver and lung disease. Dr. V. Chaudhary and Dr. M. Packer are co-First authors; Dr. F. Gregoire and Dr. C.Mueller are corresponding authors

1353. In Vivo Base Editing to Correct a Murine Model of Mucopolysaccharidosis Type IH

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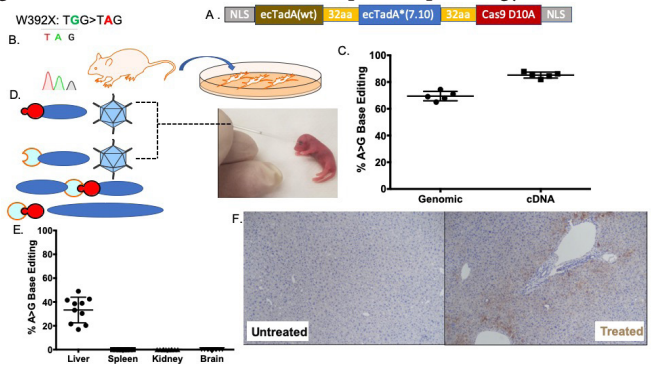
Mucopolysaccharidosis type IH (MPS IH) is a severe lysosomal storage disease caused by loss-of-function mutations to the α-*L*-iduronidase *(IDUA)* gene. IDUA is a lysosomal hydrolase that catabolizes glycosaminoglycans (GAG). Impaired IDUA activity results in progressive GAG accumulation leading to multi-organ system pathology. *In vivo* genome editing represents a promising strategy to correct *IDUA* mutations that cause MPS IH, and has the potential to permanently restore IDUA function over the lifespan of the recipient. Using programmable nucleases to create double-stranded DNA breaks (DSBs) that initiate *IDUA* gene correction or incorporation of wild-type *IDUA* cDNA via homology-directed repair (HDR) suffers from undesired insertions and deletions (indels) and low efficiency in many cell types. Here, we used base editing to directly convert A>G (T<u>A</u>G>T<u>G</u>G) in a murine W392X *IDUA* gene MPS IH model (Figs. 1A and 1B). Base editing does not lead to significant DSBs or indels, obviates the need for a repair template, and typically results in higher editing efficiencies than nuclease-initiated HDR. Electroporation of mRNA encoding an optimized A>G base editor (ABEmax) into primary fibroblasts isolated from W392X *IDUA* mice resulted in correction of the mutation in >60% of genomic DNA amplicons and >80% of cDNA amplicons (Fig. 1C), suggesting stabilization of the corrected mRNA transcript through avoidance of nonsense-mediated decay. Using the split-intein dual-AAV *in vivo* base editing delivery system (Levy *et al. Nat. Biomed. Eng.* 2020), ABEmax and the targeting sgRNA were encoded in two AAV9 vectors (Fig. 1D). Facial-vein co-injection of both AAV9 particles *in vivo* to one day old W392X mice (n=11 animals) resulted in ABE-mediated W392X correction in 33% (± 10%) of genomic DNA amplicons from the liver (Fig. 1E) and durable enzyme expression over the seven month course of the study. Concomitant IDUA protein expression was observed in the liver following immunohistochemistry (brown staining in Fig. 1F), along with decreased GAG storage material in peripheral organs (liver, spleen, and kidney). The absence of evidence of editing in the spleen and kidney (Fig. 1E) suggests that GAG burden was reduced from IDUA enzyme produced in the liver. Neither was editing observed in the central nervous system (CNS). As such, we have initiated studies with CNS- and visceral organ-tropic AAV

variants to define the most efficacious AAV delivery system that results in widespread BE to facilitate global pathology reduction. Using the W392X *IDUA* mouse that recapitulates the human condition and is analogous to the highly prevalent human W402X mutation, we observed base editing in the liver and clearance of GAGs following BE AAV delivery in neonates *in vivo*. Collectively, these data show the promise of a base editing approach to precisely correct a common genetic cause of MPS IH *in vivo* and prevent pathology.



Technical Advances in Cell Therapies

1354. Genetically Corrected iPSC-Derived Neural Stem Cell Grafts Deliver Naglu-IGFII Fusion Protein to Affect CNS Disease in Sanfilippo B Mice

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Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB, MPS IIIB) is a recessive genetic disorder that severely affects the brain and is caused by a deficiency in the enzyme α-N-acetylglucosaminidase (NAGLU), leading to intralysosomal accumulation of partially degraded heparan sulfate. There are no effective treatments for this disorder. We carried out *ex vivo*, lentiviral correction of neural stem cells derived from *Naglu*$^{-/-}$ mice (iNSCs) using a modified NAGLU, consisting of this enzyme fused to a receptor binding peptide of IGFII to improve NAGLU cross-correction. Following long-term transplantation of these corrected iNSCs into *Naglu*$^{-/-}$ mice, we detected NAGLU-IGFII activity in all engrafted animals. Transplanted *Naglu*$^{-/-}$ mice showed rescue of NAGLU activity, a decrease in storage material accumulation, reduced astrocytosis and microglial activation, with beneficial effects achieved throughout the brain. We also identified novel neuropathological phenotypes in untreated mutant mice involving a decrease in MAP2 protein and the accumulation of synaptophysin-positive aggregates. Following transplantation, MAP2 expression was restored in *Naglu*$^{-/-}$ mice and significantly fewer synaptophysin-positive aggregates were observed. Our results demonstrate long-term engraftment of virally corrected iNSCs in the brain that are capable of cross-correcting disease-associated pathology in *Naglu*$^{-/-}$ mice. Our findings suggest that genetically engineered iNSCs could potentially be used to deliver modified enzymes and treat MPS IIIB.

1355. Sleeping Beauty IDUA Transposed Human Plasma Cells for Long-Term Treatment of an Immunodeficient Murine Model of Mucopolysaccharidosis Type I

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Background: Mucopolysaccharidosis type I (MPS I) is a recessive lysosomal storage disease caused by deficiency of iduronidase (IDUA), resulting in systemic accumulation of glycosaminoglycans (GAGs). The most severe form (Hurler syndrome) accounts for over 50% of cases and is associated with severe skeletal deformities (dysostosis multiplex), leading to short stature and spinal cord compression, cardiac and pulmonary complications and developmental delay. Untreated, children with Hurler syndrome die before the age of 10. Enzyme replacement therapy and hematopoietic stem cell transplantation are current therapies for MPS I. Immusoft's B cell engineering technology provides a cell based approach for IDUA delivery in the treatment of MPS I. The product consists of *ex vivo* expanded human B cells that have been genetically engineered using the Sleeping Beauty (SB) transposon system to express and secrete IDUA. After infusion these cells can migrate to and occupy diverse tissues and may survive for years *in vivo*. Secretion of IDUA from these adoptively transferred cells has the potential to provide a considerable increase in the delivery of enzyme to affected tissues and relief from metabolic storage disease for MPS I patients.Materials: We developed an immunodeficient murine mouse model to study the effect of human B cells engineered to secrete IDUA. NSG MPS I mice were generated by crossing previously established NODSCID IDUA deficient mice with NSG mice, for introduction of the IL2gamma deficiency allele. These mice show no endogenous IDUA expression with progressive accumulation of GAGs in all tissues. The animals demonstrate significant skeletal dysplasias characteristic of MPS I, including thickened and widened long bones, and widened zygomatic arches, also observed in other mouse models of MPS I. Experimental design: Human B cells were isolated and underwent SB-mediated IDUA transposition with subsequent *in vitro* differentiation towards plasma cells. NSG MPS I mice (n=16) were infused with CD4 positive T cells (preconditioning) at day -7, followed by two infusions with the pKT2/EEK IDUA transposed B cells on day 0 and 36. Weekly blood samples were assayed for plasma IDUA and human IgG out to 32 weeks post-infusion. Groups of animals (n=4) were terminated at 3, 6, and 6.5 months postinfusion and tissues collected. Results: Plasma IDUA levels surged to 2.5 nmol/hr/ml at 5 weeks after infusion and subsided to low but persistent levels in the circulation. Presence of human IgG in plasma throughout the study duration indicated successful engraftment of human plasma cells. Tissue GAGs were reduced significantly in all tissues (except for brain) at all measured time points. Conclusions: Transplantation with IDUA secreting human plasma cells resulted in longterm expression of IDUA and reduction of tissue GAGs in NSG-MPSI mice. Studies of the effect of IDUA-secreting plasma cells on skeletal manifestations of the disease are underway.

1356. Allotransplantation of Functional Retinal Ganglion Cells for Glaucoma

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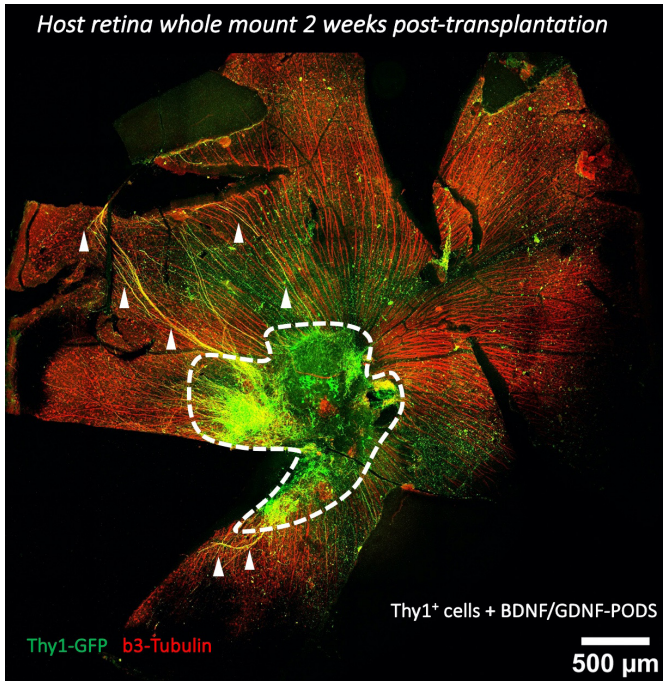
Neurodegenerative diseases of the retina and optic nerve including Glaucoma result in progressive and irreversible vision loss and blindness. Since patients initially remain unaware of the disease, their condition is often only diagnosed once significant proportions of the affected retinal neurons have been lost. To date, the majority of strategies to address retinal degeneration are centered around the treatment of immediate symptoms, including the reduction of intraocular pressure and the neuroprotection of damaged neurons. Cell replacement, however, promises to restore previously lost vision for patients at advanced disease stage. To enable successful cell replacement, retinal neurons need to be manufactured on a clinically relevant scale from renewable cell sources. Over the past years, our laboratory and others have shown that retinal neurons can be differentiated in-vitro from ES/iPS cells within 3-dimensional retinal organoids. Our work highlights that retinal ganglion cells (RGCs) derived from Thy1-GFP miPSCs, mature to exhibit functional, morphological and molecular characteristics reflective of RGC diversity observed in-vivo, underlining the potential of organoid-derived neurons to one day replace intrinsic RGCs within hosts. Within 3 weeks of culture, Thy1-GFP+ RGCs express markers including HoxD10, Fstl4, CART, Cdh6, Mmp17 (for direction-sensitive RGCs), Spp1, Kcng4 (α-RGCs) as well as Melanopsin (Opn4) characteristic for intrinsically photosensitive RGCs, as confirmed by Immunohistochemistry, Flow Cytometry and RT-PCR. Aided by the delivery of growth factors BDNF/GDNF the yield of RBPMS+ RGCs in organoids could be increased by 46%, allowing for RGC yields of up to 5%, illustrating the protocols potential for translation to a clinical manufacture scale. Following isolation with magnetic microbeads targeting Thy1, RGCs were formulated at 20,000 cells in 2ul and transplanted into the vitreous of healthy mouse pups, adults as well as mouse models of NMDA and microbead-induced RGC loss, mimicking glaucomatous RGC degeneration. Across all conditions, transplant success exceeded 50%, with healthy hosts retaining donor RGCs in 80-100% of cases. Post-transplantation, donor RGCs were confirmed to survive up to 12 months within host retinas and were observed to extend axonal projections into the host optic nerve, arguing for the eventual ability of these donor RGCs to rewire the retina-brain connection.



1357. Human Transcription Factor Library Based Cell Type Engineering

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Human pluripotent stem cell-derived cells and tissues serve as model systems for human development and for investigating cellular functions in health and disease. Furthermore, these differentiated cells can be utilized as cell replacement therapies. Many techniques exist to differentiate stem cells into cell types of interest of which overexpressing transcription factors (TFs) represents an particularly elegant approach. Here, we systematically explored the TF programming landscape of stem cell differentiation by assembling and applying a comprehensive human TF library containing 1,564 TFs. We identified 290 single TFs that drove stem cells into diverse lineages with up to 99% efficiency in just four days in standard culturing conditions. We generated and characterized neuronal-like, fibroblast-like, oligodendrocyte-like and splice-isoform-specific programmed vascular endothelial-like cells. These cells showed molecular profiles and transcriptomic signatures similar to primary cells, and acquire functional activity *in vitro*. Programmed oligodendrocyte-like vascular endothelial-like cells engraft and function *in vivo*. Due to the uniform culturing conditions, we succeeded in parallel programming of stem cells into multiple cell types simultaneously. Within developing cerebral organoids, orthogonal programming of oligodendrocyte-like cells resulted in accelerated myelination.We exploited the cell-autonomous nature of our approach for parallel programming of diverse cell types by single TFs per cell. By employing single cell sequencing, we are also exploring the combinatorial space of stem cell programming.

Altogether, our parallel and orthogonal programming approach is efficient to produce tissues of increasingly complexity and fidelity to their natural counterparts.

1358. Prenatal Therapy with Placental Cells Transduced with a Lentiviral Vector Encoding a Bioengineered fVIII Transgene Results in Sustained Therapeutic fVIII Levels and in Product Specific Tolerance

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Prenatal therapy (PNT) using an ultrasound-guided approach has been performed in more than 50 patients, proving that this procedure poses minimal risk to both the fetus and the mother. Hemophilia A (HA), is an ideal disease to treat by PNT, since 75% of HA cases can be diagnosed through prenatal screening. The type of mutation and family history can predict the severity of the disease and the patient's likelihood of developing FVIII inhibitors, thus allowing intervention in those who could most benefit from this approach. We reported that PNT of sheep fetuses (n=11) with (PLC-mcoET3) human placental cells (PLCs) transduced with a lentiviral vector encoding a bioengineered fVIII transgene (mcoET3), at a dose of 10^7-10^8 cells/kg, increased plasma fVIII activity levels by 41±14% (n=6) for at least 2 years after PNT. During this time period, all PNT recipients were devoid of mcoET3-specific T cells, and antibodies, demonstrating they remained tolerant to the therapy. To understand the nature of this state of tolerance, here we investigated whether re-administration of PLC-mcoET3 post-natally (i.v. at a cell dose producing 20 IU/kg/24h), in the absence of danger signals, would alter the animals' tolerant state towards the mcoET3 secreted by the cell/gene therapy product received prenatally. As a control, we administered purified ET3 protein (i.v. at 20IU/kg) to prenatally treated animals. To determine whether PNT recipients' plasma contained anti-mcoET3 IgG antibodies, an mcoET3-specific ELISA was performed using plasma collected at Week 0 (W0) before the first infusion, and at W1, W2, W3, W4, and W5 after boosting. To determine whether ET3-specific T effector cells developed, ELISpot assays for T_{h1} cells were performed using PBMC collected at W0 through W5 of the boosting regimen. A positive response was defined by: 1) the ratio between the number of spot-forming units (SFU) in the presence and absence of ET3 $\geq$ 2; and 2) a threshold minimum of 10 SFU/2x10^5 PBMC. The PNT recipient that received a boost of PLC-mcoET3 postnatally had a progressive increase in FVIII activity over time, as determined by aPTT, with FVIII levels at W5 of 240% above W0 levels, potentially as a result of PLC engraftment and sustained secretion of mcoET3. ELISpot assays were negative, demonstrating that postnatal PLC-mcoET3 boost did not induce a T_{h1} response, and suggesting T cell tolerance had been induced to PLC-mcoET3 as a result of PNT. An anti-mcoET3 IgG was detected in these animals at a very low (1:20) titer, beginning at W3 and persisting through W5. Because the levels of FVIII activity in plasma continued to increase from W0-W5, it is highly likely that the IgG detected was a non-inhibitory antibody. In contrast, in PNT recipients receiving a postnatal boost of purified ET3 protein, a drastic decline in plasma FVIII activity occurred over time, which coincided with a strong anti-ET3 IgG response (titer of 1:245 to 1:857) that appeared at W3 and persisted through to W5. Moreover, a positive anti-ET3 T_{h1} cell response was detected at W5 in these animals. In conclusion, PNT of fetal sheep with an optimized human cell/gene therapy platform for FVIII production resulted in sustained FVIII plasma levels >40% for at least 2 years and induction of mcoET3-specific immune tolerance when delivered via a PLC-based cell platform.

1359. Combined Gene and Cell Therapy for the Treatment of Hemophilia A within an Implantable Therapeutic Device

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Hemophilia A (HA) is a rare bleeding disorder caused by absence or dysfunction of FVIII protein. New regenerative medicine approaches to treat haemophilia A require insights into cell compartments capable of producing FVIII. We and others previously demonstrated that FVIII is produced specifically in endothelial cells. The aim of our work is to develop a novel *ex vivo* cell-based therapy using a medical device (Cell Pouch™, Sernova Corp.) leading to an improvement in patient quality of life. We isolated blood outgrowth endothelial cells (BOECs) from healthy and patients' blood. BOECs express the endothelial markers (e.g. CD31, KDR, Tie-2, VEC, vWF) both at RNA and protein levels and were able to form tubules network when cultured in matrigel. HA BOECs were transduced with a lentiviral vector (LV) carrying the B domain deleted form of FVIII under the Vascular Endothelial Cadherin promoter (LV-VEC.hFVIII) and were characterized for endothelial phenotype and for the number of integrated LV copies/cell (~3). We observed that FVIII was expressed by 80% of LV-VEC.hFVIII transduced cells and was efficiently secreted in the supernatant. ELISA assay showed that LV-VEC.hFVIII BOECs secreted higher amount of FVIII (24 ng/ml) compared to not transduced (4.5 ng/ml). Ten million LV-VEC.hFVIII-BOECs were transplanted intraperitoneally in association with cytodex® 3 microcarrier beads in NOD/SCID γ-null HA mice (NSG-HA, n=6). BOECs survived and secreted FVIII at therapeutic levels (12%) up to 18 weeks and

ameliorate the bleeding phenotype of HA mice. Subsequently, a large-scale expansion of BOECs was performed prior transplantation in Cell Pouch™. The large scale expanded cells were investigated for the presence of chromosomal aberration, senescence markers and tumorigenic potential showing a safe profile suggesting that BOECs can be expanded without negative consequences. Finally, LV-transduced HA BOECs were transplanted into an implanted prevascularized, scalable medical device (Cell Pouch™) under the skin, optimized for sustained secretion of therapeutic FVIII in the NSG-HA mice, showing BOECs engraftment up to 12 weeks. Moreover, genomic integration profile of LV transduced BOECs was analyzed at different expansion passages or procedure timepoints. The molecular analysis showed the classic integration pattern of LVs, with a marked tendency to integrate within transcription units and no preference for promoter or regulatory elements. Therefore, no enrichment for oncogenes was observed, as well as expansion of clones with integration in common integration sites or biases towards gene classes related to cancer genes, but it is necessary to produce a longer time points for safety endpoints. Furthermore, a transcriptomic approach by RNA-seq analysis has been used to assess the comprehensive changes in mRNA expression in healthy, HA and LV-VEC.hFVIII transduced BOECs and data are in process to be analyzed. Overall, these results pave the way for future clinical testing in HA patients by transplantation of GMP produced autologous gene corrected BOECs within implanted device.

1360. Privileged Cell-Material Interactions Through Engineered Synthetic Receptors

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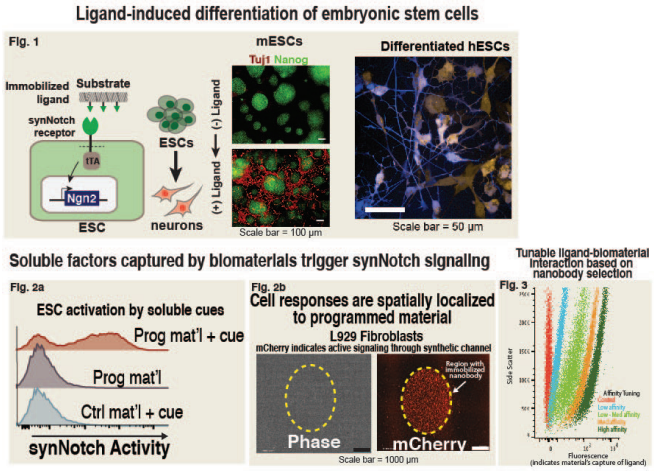
Introduction: Cells integrate diverse inputs from their environments and coordinate responses with selectivity, and thus hold promise as therapeutic agents. However, attempts to produce cell-based therapies have encountered barriers that arise from a lack of the ability to govern cell functions. One solution entails programming cell control modules into biomaterials to support cell retention, organization and differentiation at transplant sites while allowing cells to react to dynamic features of their microenvironments. Here, we build on advances in cellular and materials engineering to develop a platform that enables orthogonal, dynamic communication between cells and biomaterials and demonstrate utility toward controlling stem cell behaviors for tissue repair. **Methods:** We have developed a synthetic sense and response platform based on the juxtacrine Notch receptor. As with native Notch, activation of our synthetic Notch (synNotch) platform only occurs when cognate ligand is expressed on a cell or immobilized to a surface; free, soluble ligand is not able to generate the mechanical strain required to induce signal transduction. This opens the intriguing possibility to spatially pattern cellular behaviors based on synthetic, engineered signaling networks and programmable biomaterials. In the experiments outlined here, gene circuits encoding synNotch platform elements were delivered to target cells (murine and human embryonic stem cells and murine L929 fibroblasts) by lentiviral transduction. SynNotch ligand or anti-ligand nanobodies capable of immobilizing soluble ligand in culture medium were conjugated to biomaterials via primary amine chemistry. SynNotch activation was monitored by mCherry expression, and the ability of synNotch to drive differentiation of stem cells was assessed through immunolabeling, assayed via flow cytometry or confocal microscopy. **Results:**Biomaterials programmed with synNotch ligand induce neuronal differentiation of ESCs engineered to express Neurogenin2 upon synNotch activation, as observed by loss of pluripotency, neurite projection and neuron-specific tubulin (Tuj1) expression (Fig. 1). In separate experiments, materials activate synNotch to induce BMP4 expression in mESCs, leading to mesodermal differentiation as indicated by activation of Flk1 and Pdgfra expression. Programming biomaterials with an anti-ligand nanobody converts the juxtacrine synNotch platform to a paracrine platform capable of detecting soluble cues (Fig. 2a). Further, by selectively programming specific regions of materials to bind ligand, we spatially coordinate responses to globally present, soluble factors (Fig. 2b). Finally, we demonstrate the ability to tune biomaterial binding to soluble factors, a feature that will likely lead to dynamic readouts of the cellular microenvironment based on engineered features of cell-biomaterial interactions (Fig. 3). **Conclusions:** By combining cellular and biomaterial design, we demonstrate a platform that integrates an engineered detection and response system into regenerative medicine strategies. This approach enables prescription of stem cell behaviors with a high degree of specificity. We envision that this platform will enable the coordination of cell-cell and cell-material interactions for a variety of regenerative medicine goals.



Vector and Cell Engineering, Production or Manufacturing V

1361. Single Molecule Real-Time Sequencing Reveals Higher Genome Heterogeneity in AAV Vectors Produced from Insect Cells Compared to Mammalian Cells

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Great strides have been made to increase the yield, safety, and potency of adeno-associated virus (AAV) vectors. In the era of vector mass production for supporting clinical trials and commercialization, manufacturing recombinant AAV (rAAV) with *Spodoptera frugiperda* (Sf9) insect cells using baculovirus expression vector (BEV) infection has been a leading platform for scalable production. While this technology is safe and very effective in generating rAAVs, recent studies have identified biological differences between vectors produced in insect cells and mammalian cells. In our study, the integrity of encapsidated vector genomes packaged by Sf9 and HEK293 cells-based systems was directly compared. Alkaline agarose gel electrophoresis of the encapsidated DNA revealed that single-stranded rAAV genomes are more heterogeneous in Sf9-derived AAV vectors than in HEK293-packaged vectors. In order to determine the composition of these truncated populations, we employed single molecule, real-time (SMRT) sequencing and AAV-GPseq analysis to profile the sequences and structures of encapsidated genomes. We previously employed AAV-GPseq to accurately describe the heterogeneity of AAV genomes caused by siRNA cassettes. A highlight of that study was our ability to sequence-verify that truncated genomes are indeed self-complementary (sc) and similar to scAAVs generated by mutating one ITR to disrupt terminal resolution. In this study, we observed that the majority of the truncated species from the Sf9/BEV produced vectors were atypical of the truncated forms produced from HEK293 cells. We found that a substantial population of Sf9/BEV truncated genomes also resemble scAAV genomes, but bear only a single ITR. Interestingly, the ITRs of these specific genomes remain unresolved. Upon further inspection of ITR integrity among single-ITR, truncated genomes, we observed an enrichment of a variety of mutated ITRs. Interestingly, all of these unresolved ITRs have intact AA' and D regions, which house the rep-binding element (RBE) and terminal resolution site (TRS) motif. However, these ITRs exhibited deletions of the BB' or CC' arms, or harbor an extra arm to form "trident-shaped" hairpin structures. Our data collectively suggest a potential defect during viral genome replication, resolution, and/or encapsidation. The origins of these conspicuously mutated ITRs are currently under investigation. Based on these new findings, we investigated the relationship between Rep protein expression and vector genome heterogeneity. The wild-type AAV2 genome expresses four proteins critical for replication and packaging (Rep78, 68, 52, and 40). In the dual-baculovirus/Sf9 cells system used here, only Rep78 and Rep52 proteins are expressed from the very late baculovirus polyhedrin promoter. The absence of Rep68 and Rep40 may result in a defect during the genome replication and/or resolution. We have generated new baculovirus constructs to allow expression of all four Rep proteins in order to reduce the abundance of unresolved genomes. Since truncated genomes are likely less efficient for transgene expression, it will be necessary to conduct further experiments to decipher and solve the underlying mechanisms causing genome heterogeneity of rAAV vectors produced in the Sf9/BEV system.

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1362. SSV-Seq 2.0, an Optimized PCR-Free Method for the High-Throughput Sequencing of Adeno Associated Viral Vector Genomes

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The adeno-associated virus (AAV) is very popular as a viral vector to deliver therapeutic DNA. With the success of clinical trials using these vectors, the regulatory bodies increased the level of requirements regarding the quality control (QC) of these new drugs. In particular, the presence of residual DNA in the final product is of significant concern due to the potential risk of oncogenicity, immunogenicity and decrease in the potency. We have previously published a high-throughput method, named Single-Stranded Virus Sequencing (SSV-Seq), for the analysis of residual DNA in AAV vector stocks produced either in HEK293 or Sf9 insect cells. Based on Illumina sequencing technology, SSV-Seq allows a comprehensive characterization and quantification of DNA impurities, and can also provide information regarding the AAV vector genome identity with a computational analysis of the single nucleotide variants (SNV), and the visualization of the sequencing coverage along the therapeutic DNA. However, this sequencing method could be biased over GC-rich regions due to the PCR amplification step as part of the library preparation workflow. In this study, we show that not only high GC content, but also the presence of nucleotide stretches can lead to a defect in PCR amplification, and consequently to a drastic drop in the sequencing coverage. A novel PCR-free protocol has been developed for the library preparation to circumvent this problem. The SSV-Seq 2.0 experimental procedure has been tested for the analysis of an AAV8 vector harboring a CAG promoter. Composed of 69% of GC on average and multiple homopolymers, the optimized protocol allows to restore a high sequencing coverage over this region. The PCR-free method represents an alternative for the analysis of rAAV genome that contain difficult sequences. HTS-based assays represent the most exhaustive way to control the AAV vector genome quality and identity, and at the same time, they provide exhaustive data of nucleic acid impurities to fulfill the regulatory requirements. This is an example of how continuous improvement must be undertaken to adapt the existing sequencing-based methods and develop novel ones, to increase the quality control standards of AAV vectors.

1363. Characterizing Next-Generation Baculovirus Transduction Processes - A Quality by Design-Based Approach for AAV Manufacturing

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Introduction: Increased demand for robust gene therapy manufacturing processes and cost of goods reduction has been driving the development of processes that yield higher product titers and tighter control of quality attributes. To accommodate this need, a proprietary DuoBac expression system has been developed. This technology enables expression of therapeutic AAV in insect cells induced by two baculoviruses. As conventional baculovirus-driven AAV expression systems require three baculoviruses, the transition to a DuoBac system can significantly reduce bioprocess complexity. Due to its novelty, the aim of this study was to use a scale-down multivariate approach to characterize this expression system in an upstream bioprocessing context. **Methods:** By means of a Central Composite Design (CCD), experimental variance was introduced during DuoBac-mediated transduction of Sf+ cells for the following parameters: (1) the amount of baculovirus encoding both capsid and replication proteins (Rep/Cap); (2) the amount of baculovirus encoding transgene (TG); and (3) the Viable Cell Density (VCD) at the Time of Infection (TOI). Two analyses were performed on purified samples for each experimental condition: qPCR was used to determine the vector genomes concentration (gc), while SEC-HPLC was used to determine the total amount of particles (TP) regardless of content. These two metrics were subsequently used to calculate the TP/gc ratio, representing the proportion of total AAV capsids relative to full capsids. **Results:** Two response models were developed to study the effects of parameter variance on (1) full capsids and (2) the TP/gc ratio - providing a representation of process yield and product quality, respectively. For both models, all investigated parameters were found to be statistically highly significant ($p<0.01$). The full capsid model (r^2 pred.=0.95) described a local optimum at maximum VCD at TOI; and low TG levels. Similar optimal conditions were described for the TP/gc ratio model (r^2 pred.=0.85), where a local minimum was established. It was found that, despite using a relatively wide design space, the TP/gc ratio response yielded low variance (CV=21%, n=17). Furthermore, this study shows an approximate two-fold reduction in TP/gc ratio variance when switching from a conventional (triple) baculovirus expression system (CV=26%, n=13) to a DuoBac expression system (CV=14%, n=9) in comparable design spaces. **Discussion:** The results of this study show that the investigated novel DuoBac-based AAV expression system can tolerate high parameter variance with little effect on TP/gc ratios. Furthermore, preliminary process comparison implies that a DuoBac system yields lower variance in TP/gc ratios than conventional triple-baculovirus AAV expression systems. Although the role of empty particles in gene therapies are still under debate, DuoBac systems hold great potential for improving process robustness and product quality of AAV-based gene therapies.

1364. Measuring the Concentration of Adeno-Associated Virus (AAV) with Multi-Angle Dynamic Light Scattering (MADLS) Using the New Zetasizer™ Ultra

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New Improvements to Dynamic Light Scattering (DLS)

Recent improvements to Dynamic Light Scattering (DLS) have enabled the ability to measure particle concentration or viral capsid titer for viral vectors. The particle concentration measurement is enabled by two key innovations. The first is the implementation of Multi Angle Dynamic Light Scattering (MADLS). MADLS collects DLS scattering information at three separate angles, to generate three correlation functions, and then with Mie-theory, are combined into a single higher quality particle size distribution. The second is adaptive correlation (AC), which enables the DLS measurement to separate the transient results from steady state results, to enable faster and more repeatable sizing measurements on the primary particles of interest. Combined, these improvements to DLS enable the Zetasizer Ultra to rapidly measure the viral titer of AAV samples in compact, benchtop instrument, that is cuvette-based, non destructive, label free, and requires only 20 microliters of sample.

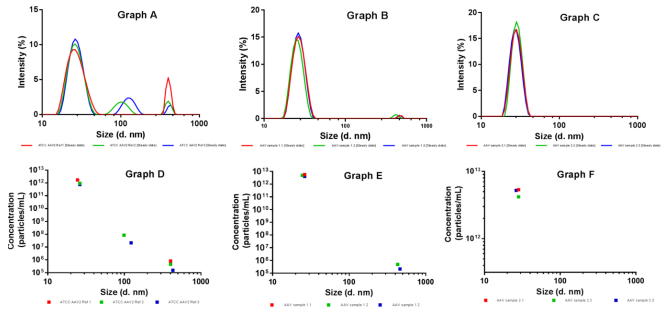
Methods:

Zetasizer Ultra

Three AAV samples were provided by Allergan. A Zetasizer Ultra from MalvernPanalytical was used, in combination with the ZS Xplorer version 1.1 software. All measurements were performed in triplicate, at 25 ^{O}C, and in the quartz cuvette. 20 microliters of sample was transferred to the cuvette prior to the measurement. Background subtraction was performed with vehicle buffer in same cuvette measured on on attenuator setting 11. Material RI of 1.45 was used. Dispersant RI of 1.33 and viscosity of 0.887 cP at 25 ^{O}C. **ELISA:** All measurements were performed by Allergan using optimized ELISA conditions. **Results:** In this study, Allergan has kindly shared data from their evaluation of the Zetasizer Ultra[3]. Examples of three adeno-associated virus (AAV) samples are shown. The concentration results are compared to results from capsid ELISA based virus titre assays. Data was collected using Multi-Angle Dynamic Light Scattering (MADLS) to Measure Size, Aggregation State and Particle Concentration.

AAV Size and Concentration Measurements

By using the MADLS measurement on the Zetasizer Ultra, particle size distributions for three AAV samples were measured in triplicate. Low levels of aggregated species were observed, more so in the standard (A) than in their own AAV samples (B and C). By using the new Particle Concentration measurement, unique to the Zetasizer Ultra and calculated alongside the MADLS measurement, the concentration of AAV and each aggregated species was also measured (D-F) in minutes.



Concentrations were compared to those recorded using Allergan's established capsid ELISA method (Table 1), showing less than 15% RSD for Allergan's samples and 45% RSD for the standard. It should be noted that the ATCC AAV-2 reference standard was subjected to one free/thaw cycle, which was the likely cause of increased aggregation and variability.

Sample	Capsid ELISA	MADLS Based Particle Concentration					
		Virus peak (n=3)	%CV	Aggregate peak 1	%CV	Aggregate peak 2	%CV
ATCC AAV2 reference sample	0.92 x 10^{12}	1.14 x 10^{12}	45	5.30 x 10^{7} (n=2)	84.6	4.73 x 10^{5} (n=3)	69.3
Allergan sample 1	6.14 x 10^{12}	4.82 x 10^{12}	15	3.52 x 10^{5} (n=3)	56	n/a	n/a
Allergan sample 2	4.29 x 10^{12} 2.83 x 10^{12} (assay ran twice)	4.92 x 10^{12}	13	n/a		n/a	

Conclusion: The Zetasizer Ultra was successfully able to measure the size and viral capsid titer of three AAV samples. The results were in strong agreement with ELISA.

1365. An International Collaboration on the Establishment of the First World Health Organization (WHO) International Standard for Gene Therapy

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Assuring the quality and safety of gene therapy products using integrating vectors, e.g. CAR-T products Kymriah and Yescarta for cancer therapies and *ex vivo* therapies Strimvelis and Zolgensma for monogenic immunodeficiency, presents a great challenge because they are cell-based multi-gene products. Although the safety level of lentiviral vectors has been improved by partial deletion of 3' long terminal repeat (LTR), uncontrolled integration can still cause insertional mutagenesis and lead to overexpression or disruption of adjacent genes at the site of integration. Therefore, the efficacy and safety of LV-based products are critically determined by the average vector copy number (VCN) in the transduced and limiting the integration copy number in transduced cells is, at current status, an effective measure to reduce the genotoxic and tumorigenic potential. In addition, changes in production sites and manufacturing processes have become increasingly common, posing challenges to developers regarding reproducibility and comparability of results. An International standard can circumvent the issues derived from method-variations and measurement-uncertainty and thus improve data quality, consistency and comparability. An International collaborative study has been completed in 2019 with the participation of 31 laboratories from 13 countries to establish the 1st World Health Organization (WHO) International Reference panel for Lentiviral vector (LV) integration copy quantitation and the 1st WHO International Reference Reagent for LV integration site analysis. The study results will be presented and the issues identified during the International collaborative study will be discussed. This study highlights the significance of the global available Standard in enabling data comparability cross-trials and cross-manufacturing processes and demonstrates the importance of International collaborations towards the standardization of gene therapy

1366. Stability Under Stress of AT132, an AAV-Based Gene Therapy Drug Product for Systemic Administration to Patients Affected by X-Linked Myotubular Myopathy

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Accurate characterization of stability under physicochemical stress has an important role in the manufacturing of gene therapy products. AT132 is an Audentes Therapeutics AAV8-based vector for the treatment of X-linked myotubular myopathy, a severe neuromuscular disorder. Proper characterization of AAV-based products requires evaluation of viral particle, genomic DNA and protein stability. The present study focuses on all three components using biophysical, biochemical and biological assays to evaluate the stability of AT132 and help establishing appropriate evaluation methods. Forced degradation studies were conducted to test the stability of the AT132 Drug Product (DP) in a closed container (CZ vial) system under different stress conditions, including freeze-thawing (10-cycles), acid treatment (0.01 M for 24h), base treatment (0.01 M for 24h), H_2O_2 treatment (1% for 24h), temperature stress (53 °C for 30min) and mechanical stress (vibration, 300 RPM for 24h). Physico-chemical properties and biological activities of AT132 DP were measured using different assays, including appearance test, light obscuration (LO), dynamic light scattering (DLS), size exclusion chromatography (SEC), vector genome (vg) titer, AAV8 capsid titer, alkaline gel, SDS-PAGE, infectivity and protein expression assay. Results were compared between untreated (control) and stressed (treated) samples. The biophysical and biochemical characteristics of AT132 treated samples didn't change relative to the untreated controls except for base-treated DP. The biological activity (infectivity and protein expression) of AT132 DP after freeze-thaw stress, heat stress and mechanical stress (vibration) showed no difference compared to unstressed samples. On the contrary, chemical (acid, base and H_2O_2) treatment ablated either partially or completely its biological activity. In conclusion, AT132 DP was stable under severe freeze-thaw (10-cyles), heat and mechanical stress, while its quality was severely affected by acid treatment and its biological activity completely lost under base and H_2O_2 treatment. Cell-based assays are necessary for the evaluation of AT132 product quality attributes along with biophysical and biochemical assays.

1367. Process Analytical Technology Strategy for Lentiviral Manufacture

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With the rapid advancement of cell and gene therapies into the clinical and industrial landscape, concepts such as Quality-by-Design, Process Analytical Technologies (PAT) and real-time control are considered achievable mechanisms to support the development of next generation manufacturing platforms. However, delivering on these concepts requires a detailed understanding of cell behavior and the ability to identify biomarkers correlating to the critical quality attributes of the product, which thus could be used to support a PAT strategy. In this paper, we will show an analytical strategy and data-driven modelling approaches used to capture, analyse and interpret multivariate omics datasets, to identify markers from which a dynamic biochemical fingerprint was derived and used for real-time monitoring during lentiviral manufacture using the Oxford Biomedica platform process. To generate a detailed map of cell behavior during viral vector manufacture, we analysed time-matched samples for their metabolomic (using LCMS) and transcriptomic (using RNAseq) profiles. These complex datasets were structured to permit multivariate analysis. A combination of Principal Component Analysis, variable importance, network analysis and differential expression were used to identify key metabolomic markers in significant pathways. Self-Organising Map neural networks were used to identify key inflexion timepoints, when cell metabolism changed in response to the manufacturing process. We finally applied regularized Canonical Correlation Analysis and pathway analysis to integrate metabolic with gene expression profiles, identifying a list of key markers used as a dynamic fingerprint, diagnostic of culture quality. To enable a PAT strategy, we also collected spectral data *in situ* during viral vector manufacture using an in-line immersion probe Raman spectrometer (Kaiser Optical Systems Inc.). We developed an automated Partial Least Square analysis platform to construct chemometric models for each of the markers identified during the omics analysis (over 80 potential markers). We finally applied these models during lentiviral manufacture, tracking the fingerprint markers and monitoring product quality in real-time. This approach demonstrates how PAT can be used as an exciting tool to monitor and control complex manufacturing processes. Going forward, it could also enable the generation of data with greater mechanistic and biological depth, for instance in the context of Design-of-Experiments. This could link key molecular pathways, from transcription to metabolism to end-product, all in relation to the culture environment. It could also be used to monitor, in real-time, the effects of pathway manipulation and allow the design of "digital twins" which in turn could dramatically shorten the development pipeline for high quality lentiviral products.

Cancer - Oncolytic Viruses

1368. A Measles Virus Vector Pseudotyped with CD46-Retargeted Canine Distemper Envelope Achieves Oncolysis in the Presence of Measles-Immune Human Serum

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Wild type Measles Virus (MeV) entry is mediated via its interaction with two pathogenicity determining receptors, SLAMF1 and Nectin-4. Vaccine lineage MeVs have an additional tropism for CD46 which is overexpressed on many human tumors, providing a basis to use them as an oncolytic virotherapy platform. However, widespread measles seropositivity severely limits the deployment of MeV-derived oncolytic viruses as a systemic therapy. Here we engineered an attenuated vaccine strain of MeV by replacing its hemagglutinin (H) and fusion (F) envelope glycoproteins with corresponding glycoproteins from wild-type canine distemper virus (CDV), a homologous but serologically distinct morbillivirus. Since the substituted CDV-H protein was found to interact with human NECTIN-4 (but not with human SLAMF1) this unwanted tropism was ablated via a single amino acid mutation. Single chain antibody fragments against human CD46 were then genetically fused to the extraviral C-terminus of the CDV-H protein and, after further substitution of the CDV-F protein signal peptide, the fully-retargeted CDV H/F complex was shown to interact efficiently with CD46, triggering CD46-dependent intercellular fusion at levels comparable to the original vaccine lineage MeV H/F complex. When we assessed the oncolytic properties in tumor-bearing mice, both viruses were demonstrated to be similarly effective. However, when tumor-bearing mice mice were passively immunized with measles-immune human serum, only those treated with the new CD46-specific virus responded favorably. Our results show that a measles virus pseudotyped with CD46-retargeted canine distemper envelope represents a strong alternative to current vaccine linage MeVs for treatment of measles-immune cancer patients.

1369. ONCR-177, an Oncolytic HSV-1 Designed to Potently Activate Systemic Anti-Tumor Immunity

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ONCR-177 is a highly modified recombinant oncolytic Herpes Simplex Virus (oHSV) designed to be a safe and efficacious therapy for the treatment of solid tumors. ONCR-177 retains one copy of the ICP34.5 gene for oncolytic potency and resistance to type 1 interferon. Tumor selective replication and inhibition of neural pathology and latency are provided though redundant tissue specific microRNA attenuation and UL37 mutagenesis. In addition, ONCR-177 is armed with five transgenes selected using an in vivo screen for strong abscopal activity:

IL-12 for NK and T cell activation, CCL4 and FLT3LG extracellular domain for expansion and recruitment of classical dendritic cells (cDC), and antagonists to two clinically validated immune checkpoint targets, PD-1 and CTLA-4, to counter T cell exhaustion. Intra-tumoral administration of ONCR-177 mouse surrogate, mONCR-171, resulted in durable local and distant complete tumor regression. A survival benefit was observed across several syngeneic bilateral tumor models representing various degrees of HSV permissivity and baseline T cell infiltration, including models reported to be resistant to other oHSV therapies. Re-challenge studies demonstrated that curative mONCR-171 therapy elicited specific and long-lasting protective immunity. mONCR-171 activity required the presence of both T cells and NK cells and was associated with the generation of tumor antigen specific multifunctional T cells. Abscopal anti-tumor activity could not be explained by systemic propagation of the virus as viral DNA and payloads were largely confined to the injected tumor, with little to none detected in plasma or contralateral tumor. There was no indication of cytokine release syndrome, even at high intratumoral doses of mONCR-171. In conclusion, ONCR-177/mONCR-171 potently activates local and systemic anti-tumor immune responses that result in durable responses, extended survival, and the elicitation of protective immunity. These encouraging preclinical data warrant the clinical investigation of ONCR-177 for the treatment of patients with metastatic cancer.

1370. Phase I/II Trial of Adipose Tissue Derived Mesenchymal Stem Cell Delivery of a Measles Virus Strain Engineered to Express the Sodium Iodide Symporter in Ovarian Cancer Patients

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Ovarian cancer (OvCa) is the most lethal gynecologic malignancy, and there is an urgent need for development of novel therapeutics. Overexpression of the measles virus (MV) receptors CD46 and nectin-4 in OvCa the latter an ideal target for MV based therapeutics. We conducted first in human trials of intraperitoneal (IP) administration of the MV-CEA (expressing the human carcinoembryonic antigen) and MV-NIS strains and we demonstrated excellent safety and evidence of biologic activity as indicated by decreases in tumor marker CA125, and promising median overall survival of 26.6 mo (vs expected survival of only 6-12 mos) in a heavily pretreated patient population (Galanis et al, Cancer Res). We hypothesized that MV delivery using mesenchymal stem cells (MSC) as carriers can further improve efficacy by facilitating viral homing in tumor sites and protecting the virus from antibody neutralization. Following proof-of-principle studies supporting efficacy in orthotopic OvCa models (Mader et al, Clin Cancer Res) and FDA guided toxicology studies, we activated a phase I trial with primary endpoint to determine the maximum tolerated dose of MV infected MSC and characterize safety. A total of 11 pts were enrolled in the phase I trial, 4 at dose level 0 (10^7 adipose tissue MSC infected with 10^9 TCID50 MV-NIS) and 7 pts at dose level 1 (10^8 MSC infected with 10^9 TCID50 MV-NIS). Abdominal fat was harvested at the time of IP catheter implantation and MSC were produced by the Mayo Clinic's Cell Therapeutics Laboratory. Pts received 10^9 TCID50 of MV-NIS IP in cycle 1; MSC at the corresponding dose level were thawed and infected with MV prior to IP dosing in cycles 2-5: a 94% feasibility success rate on the intent to manufacture the intended MSC dose was accomplished. Eligible OvCa pts had platinum resistant or refractory disease, were immune to MV and had acceptable hematologic, liver and renal function. No dose-limiting toxicity was observed and no patient experienced ≥ grade 3 toxicity. Only mild side effects were observed with grade 1 abdominal pain (2 pts in dose level 0, 4 pts in dose level 1), fever/chills (2 pts in dose level 0, 2 pts in dose level 1), grade 1 flatulence (5 patient in dose level 1), and grade 1 diarrhea (3 pts in dose level 1) being the most common. Efficacy appeared to be MSC dose dependent with a median time to progression of 2.4 mo (range 1.24 - 4.12 mo) in dose level 0 vs 6 mo (0.8 mos - 25.3 mo) in dose level 1. To further investigate the ability of this approach to elicit an antitumor immune response, we tested for the development of humoral and cellular immunity (using IFN-γ, IL-4 and IL-17 ELISPOTs) against the ovarian cancer antigens folate receptor alpha, HER2/neu and IGFBP2. Initial analysis included extreme responders, i.e., 4 pts who progressed after 2 treatment cycles and 4 pts who remained progression free after 6 treatment cycles: humoral and strong cellular immune responses against all three ovarian cancer antigens were developed among responders but not observed in non-responders. Conclusions: Adipose tissue derived MSC can safely deliver MV strains without dose limiting toxicity being encountered. This form of cell based delivery resulted in cell dose dependent improvement in clinical outcome and development of antitumor cellular and humoral immune responses. A phase II trial of this approach is ongoing with 9 pts having enrolled todate; updated data will be presented at the meeting.

1371. A Novel ADP-Deleted, NIS-Expressing Adenovirus: Theranostic Applications and Clinical Development

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Oncolytic virus therapy provides a unique platform for cancer therapy. Their ability to both directly kill tumor cells and act as vehicles for transgene delivery make them particularly attractive therapeutic agents. Inclusion of the sodium iodide symporter (NIS)

transgene allows for both enhanced therapeutic effects through viroradiotherapy and for non-invasive imaging of infected tumors which facilitates real-time monitoring of antitumor effects. Our lab has been focused on the development, optimization, and clinical translation of NIS-expressing oncolytic adenoviruses (OAd-NIS). Our adenoviral vectors express NIS from the E3 region and initial constructs overexpressed the adenoviral death protein (ADP) which results in enhanced oncolysis (OAd5/3-Cox2-E3-NIS-ADP(+)). Although this virus did enable radiotracer uptake in prostate and lung cancer models, we hypothesized that ADP overexpression may negatively affect NIS expression and subsequent radiotracer uptake. A counterpart virus was cloned which was identical except for the deletion of ADP within the E3 region (OAd5/3-Cox2-E3-NIS-ADP(-)). Initial in vitro data confirmed improved NIS expression after infection with ADP(-) compared to ADP(+) in melanoma, lung, and pancreatic cancer cell lines. Viral performance was assessed in vivo in lung and pancreatic cancer models. SPECT/CT imaging was used to evaluate technetium uptake in patient derived pancreatic cancer xenografts. ADP(-) vectors outperformed both ADP(+) and Ad-CMV-NIS control exhibiting superior radiotracer uptake and longer duration of imaging capabilities. The therapeutic effect of combination therapy with ^{131}I administration after virus infection was then analyzed. We observed improved tumor control in individuals treated with virus and radioiodine when compared to monotherapy alone. Tumors infected with ADP(-) viruses showed the highest levels of ^{131}I uptake. We injected high titers of ADP(-) systemically into immunocompetent pigs and no toxicity was observed. We are now moving into the clinical translation of OAd5/3-Cox2-E3-NIS-ADP(-) for patients with locally advanced pancreatic ductal adenocarcinoma (PDAC). Our near-term goal is to initiate a phase I clinical trial investigating radiotherapy after intratumoral injection of virus into PDAC patients. This process has highlighted the limitations of current pre-clinical models that both resemble the disease and allow oncolytic adenovirus replication. In preparation for a phase I clinical trial, we have met with the FDA who approved our request to conduct IND-enabling toxicology studies in healthy farm pigs alone. Furthermore, we are developing a swine model of PDAC using transgenic KRAS$^{G12D/+}$/TP53$^{R167H/+}$ miniature pigs with Cre-inducible KRAS from Exemplar Genetics. We have designed and tested Cre-expressing adenoviruses (Ad-Cre), developed the necessary surgical procedures to deliver Ad-Cre to the pancreas, and refined MRI sequences and protocols to non-invasively monitor tumor formation. We anticipate this model will help us achieve our ultimate goal of systemic delivery of OAd-NIS viruses for imaging and treatment of metastatic disease.

1372. Immunostimulatory Bacterial Antigen-Armed Oncolytic Measles Virotherapy Significantly Increases the Potency of aPD1 Checkpoint Therapy to Eradicate Glioblastoma

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Introduction: Glioblastoma (GBM) is one of the most common primary malignant brain tumors in adults. Immune checkpoint inhibitor therapy in GBM lacks clinical activity. Early-phase clinical trials with derivatives of the Edmonston measles virus (MV) vaccine strain support the feasibility of MV virotherapy approaches to revert immunosuppression in GBM. Helicobacter pylori neutrophil-activating protein (NAP) is a major virulence factor of Helicobacter pylori bacterial infection and potent Th1 type response immunomodulator capable of boosting immunogenicity. In this study, we sought to tackle local and systemic GBM induced immunosuppression using bacterial antigen-armed oncolytic MV strains and systemic immunomodulation. We hypothesized that intratumoral administration of MV expressing secretory NAP (MV-s-NAP) will further enhance the viral immunostimulatory properties and antitumor activity and will result in synergy when combined with anti-PD1 immune checkpoint blockade. **Methods:** Novel measles virus (MV-s-NAP-uPA) strains were engineered to encode the secretory NAP transgene and retargeted to allow viral entry in murine cells through the urokinase- type plasminogen activator receptor (uPAR). Syngeneic GL261 and CT-2A orthotopic glioblastoma mouse models and GBM6 and GBM12 orthotopic *glioblastoma* PDXs, selected for their difference in permissiveness in MV infection were used to evaluate immunotherapeutic and antitumor responses. The remodelling of tumor microenvironment and immune cell signatures involved at the treatment response were assessed using histologic and flow cytometry phenotypic analyses. **Results:** MV-s-NAP-uPA demonstrated superior cytopathic effect i*n* vitro against all GBM tumor cell lines tested compared to control virus (MV-GFP-uPA). Infection with uPA retargeted MV stimulated immunogenic death cascade in glioma cells with extracellular release of damage-associated molecular patterns (DAMP) molecules. MV-s-NAP infection led to significant PD-L1upregulation and increase of MHC class I molecules by tumor cells supporting priming of the tumor microenvironment to immune checkpoint inhibitor therapy. While repeat *in situ* immunization with MV-s-NAP provided modest survival benefit, combination treatment with intraperitoneal injection of anti-PD1 blockade significantly prolonged survival with 80% of the mice bearing intracranial GL261 (P= 0.0431) and 72% of mice with CT-2A tumors (P=*0.0077*) remaining alive over the course of 180-day experiment compared to 30% and 16% respectively with single aPD1 immunotherapy or virus alone. Combination immunovirotherapy induced massive influx of lymphoid cells in the brain, with CD8$^+$ T cell predominance. Survival

benefits were lost in mice following CD8+ T cells depletion, but not CD4+, NK1.1+ and granulocyte depletions, demonstrating that the antitumor effect is primarily CD8+ T cell dependent. Combination treatment also inhibited distant, nontreated primary intracranial CT-2A glioma growth in ~35% of the mice (*P= 0.0347*). **Conclusions:** Our findings suggest that combination of the oncolytic immunostimulatory MV-s-NAP virotherapy with aPD1 immune checkpoint blockade is an effective strategy to overcome the limited efficacy of immune checkpoint inhibitors in GBM, therefore representing a very promising therapeutic avenue for this lethal brain tumor.

1373. Single-Cell Transcriptomics Analysis of a Complete Response Phenotype in Mice with Disseminated Lung Cancer after Systemic Therapy with Oncolytic Adenovirus

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The efficacy of treatment for many types of cancer has significantly advanced due to the clinical success of immunotherapies. However, the effectiveness of immunotherapy heavily relies on stimulating of the effector function of tumor antigen-specific CD8+ T cells, the adaptive arm of the immune system. To study the potential role of innate immunity in anti-tumor response, we developed a disseminated lung cancer model in mice lacking functional T cells and treated the tumor-bearing mice with oncolytic adenovirus AVID-317. In the absence of the functional T cells, tumor-bearing mice had significant extension of survival after AVID-317 therapy: the median survival was 101 days for AVID-317-treated group vs 49 days for buffer-treated group, HR (logrank)= 0.3114 (95% CI 0.11, 0.83, p=0.0029). Strikingly, treatment with AVID-317 lead to complete regression of all metastatic nodules in ~25% of mice. This group of mice we classified as responders. The remaining mice, whilst had longer median survival time than buffer group, eventually succumbed to the disease progression and were classified as non-responders. To understand the signatures that drive a complete tumor regression after oncolytic adenovirus treatment in the absence of fully functioning adaptive immunity, we comprehensively analyzed the transcriptional profiles and the identity of tumor-infiltrating cells in responder and non-responder mice. We performed a single-cell RNA sequencing analysis and examined 16,297 single cells from 4 mice: two mice from both the non-responders and responder cohorts. These analyses showed that all cell types from the non-responder mice had a distinct signature of unresolved stress. All hematopoietic cells in non-responder mice had lower-level expression of ribosomal protein 26 (RPS26) and other RPs, indicating a shift of the global cellular translational machinery toward preferential translation of stress response genes. Next, we identified the phenotypic differences between each cell type present in the lungs of responder and non-responder mice after virotherapy. In non-responder mice, macrophage and dendritic cell populations had very pronounced signatures of activation of pro-inflammatory pathways, most notably the IFN-I/II and IL-1. In responders, the oncolytic virus treatment triggered significant influx of B cells, which expressed high levels of CD24. Furthermore, NK and NKT cells in responder mice expressed very high levels of Klrg1 and Klrc1, demonstrating their full functional maturation. Taken together, we found that treatment of tumor-bearing mice with oncolytic adenovirus AVID-317 can effectively alter the tumor microenvironment to facilitate a robust and durable innate immune anti-tumor response. Our data also suggests that non-productive response to virotherapy may be mechanistically underlined by the exuberant Th1-polarized inflammation.

1374. Fusogenic Oncolytic Vaccinia Virus Changes the Tumor Immune Microenvironment by Cell Fusion and Enhances Systemic Anti-Tumor Immunity

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Oncolytic virotherapy is a novel cancer treatment and oncolytic virus shows clinical benefits against refractory cancers. Oncolytic virus has two therapeutic functions, inducing direct oncolysis and anti-tumor immunity. Currently, oncolytic viruses are mostly treated via direct intratumoral route. This approach provides powerful therapeutic effects against the injected tumors via anti-tumor immune response following viral oncolysis. However, in the non-injected tumors such as metastatic tumor region, viral therapeutic potentials are reduced because it depends on indirect function through inducing anti-tumor immunity. In this study, we present more powerful oncolytic agent of novel phenotypic vaccinia virus, which has higher potentials of both oncolysis and anti-tumor immunity. Vaccinia virus, once widely used for smallpox vaccine, is recently used for oncolytic virotherapy. We previously developed MAPK-dependent recombinant vaccinia virus (MDRVV), which achieved tumor specific viral replication by deletion of two viral growth factors, VGF and O1L. We recently have isolated a mutant clone which induces cytopathic fusion between infected cells from MDRVV showing non-fusogenic phenotype. Whole-genome sequencing of the fusogenic vaccinia (FUVAC) identified silent mutation in viral K2L gene encoding serine protease inhibitor which inhibits cell-cell fusion, although the deletion of VGF and O1L was maintained in FUVAC. FUVAC enhanced the viral oncolytic effect against various types of human and murine cancer cell lines *in vitro*, compared with MDRVV. Furthermore, FUVAC caused more efficient apoptosis, necrosis and immunogenic cell death than MDRVV. The oncolytic activity of FUVAC was also examined in syngeneic mice model, which having bilateral subcutaneous CT26 tumors. FUVAC had higher replication efficacy and stronger anti-cancer effect against the injected tumors than MDRVV. Importantly, FUVAC significantly inhibited the growth of non-injected tumors, regardless of the virus absence. Tumors were collected, and tumor-infiltrating lymphocytes were analyzed by flow cytometry. FUVAC more efficiently induced CD8+ T cell infiltration in the non-injected tumors than MDRVV. Furthermore, FUVAC reduced tumor-associated immune suppressive cells such as regulatory T cells, myeloid-derived suppressor cells and tumor-associated macrophages in the injected tumor. In accordance with these symptoms, the anti-cancer effects of FUVAC in both injected and non-injected tumors were completely suppressed by depletion of CD8+ T cells, but not CD4+ T cells. In conclusion, FUVAC has higher

therapeutic potential for cancer virotherapy. Our study demonstrates that FUVAC changes the tumor immune microenvironment by cell fusion and enhances systemic anti-tumor immunity. Therefore, FUVAC would be better therapeutic platform as an oncolytic vaccinia virus and be suitable for combination with immunotherapy.