Clinical Trials Spotlight

1. LentiGlobin Gene Therapy in Patients with Sickle Cell Disease: Updated Interim Results from HGB-206

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β-globin gene transfer may reduce or eliminate complications in patients with sickle cell disease (SCD). LentiGlobin gene therapy (GT) comprises drug product (DP) made from autologous hematopoietic stem cells (HSCs) transduced with the BB305 lentiviral vector (LVV) encoding β-globin with an anti-sickling T87Q substitution (HbA^{T87Q}). The safety and efficacy of LentiGlobin GT in SCD is being evaluated in a phase 1 study, HGB-206 (NCT02140554). Patients were initially treated with DP made from bone marrow harvested (BMH) HSCs (Group A, fully enrolled), then from DP made from BMH HSCs but using a refined manufacturing process (Group B, fully enrolled), and subsequently from plerixafor-mobilized HSCs (Group C, currently enrolling). Adults with severe SCD (history of recurrent vaso-occlusive crisis, acute chest syndrome, stroke, or tricuspid regurgitant jet velocity of >2.5 m/s) were enrolled. Autologous CD34+ cells, collected by BMH or apheresis following mobilization with plerixafor, were transduced with BB305 LVV. After myeloablative busulfan conditioning, patients were infused with the transduced cells and monitored for safety and efficacy. Summary statistics are median (min-max). As of May 15, 2018, 22 patients had HSCs collected, 18 patients had DP manufactured and 15 patients were treated. Eleven patients (9 in Group A, 2 in Group B) underwent BMH and 12 patients (1 in Group B [who also had BMH], 11 in Group C) underwent mobilization/apheresis. Median of 4.3 (0.1—10.8) x 10^6 and 10.4 (3.8—21.6) x 10^6 CD34+ cells/kg were collected per BMH (N=26) and per mobilization cycle (N=17), respectively. DP and treatment characteristics are shown in Table 1. DP characteristics were improved in Group B and Group C vs Group A. The safety profile post-DP infusion was consistent with myeloablative conditioning and underlying SCD; most common non-hematologic grade ≥3 AEs were stomatitis, febrile neutropenia, and vaso-occlusive pain. No grade ≥3 DP-related AEs, graft failure, veno-occlusive liver disease, replication competent lentivirus detection or clonal dominance were reported. At last visit (Table 1), HbA^{T87Q} levels were higher in Group B (3.2—7.2 g/dL) vs Group A (0.5—1.2 g/dL). In 4 Group C patients at the 3-month visit, HbA^{T87Q} (4.1 [3.2—6.0] g/dL) levels were equal to or exceeded HbS levels (3.3 [2.8—3.8] g/dL). In 1 Group C patient at the 6-month visit, HbA^{T87Q} was 8.8 g/dL and total Hb was 14.2 g/dL. These data support the safety and feasibility of plerixafor-mediated HSC collection in patients with SCD. HGB-206 protocol changes have improved LentiGlobin DP characteristics yielding higher HbA^{T87Q} levels. Additional data will determine the clinical effect of increased HbA^{T87Q}/HbS ratios.

Table 1. DP and Treatment Characteristics

	Group A (N=7)	Group B (N=2)	Group C (N=6)
Bone marrow harvests (number)	2 (1—4)	2.5 (2—3)	NA
Mobilization cycles (number)	NA	1*	1 (1—2)
Cell dose (x10^6 CD34+ cells/kg)	2.1 (1.6—5.1)	2.7 (2.2—3.2)	7.1 (3.0—8.0)
DP vector copy number (copies/diploid genome)	0.6 (0.3—1.3)	3.1 (1.4—5.0)	4.0 (2.8—5.6)
Transduced cells (%)	25 (8—42)	87 (46—95)	81 (78—88)
Neutrophil engraftment (days)	22 (17—29)	26 (23—28)	19 (18—20)
Platelet engraftment (days)	56 (29—63)	46 (31—61)	28 (12—64)†
Hospitalization time from conditioning to discharge (days)	37 (29—54)	41 (36—46)	34 (30—65)
Follow-up (months)	24.2 (22.8—32.9)	11.4 (8.5—14.3)	3.0 (1.2—6.0)

Values are presented as median (min—max); *For research purposes; †n=4 Group C patients; platelet engraftment was pending in 2 Group C patients as of data cut; NA = not applicable

2. Ex-Vivo Gene Therapy for Hurler Disease: Initial Results from a Phase I/II Clinical Study

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Transplantation of genetically-engineered autologous hematopoietic stem and progenitor cells (HSPC) is an attractive treatment option for inherited metabolic disorders, due to the potential to obtain supra-normal expression of the corrective enzyme in the hematopoietic progeny that may durably cross-correct tissues including the CNS. We recently started a phase I/II clinical trial (NCT03488394) in children affected by Hurler Disease, the severe form of Mucopolysaccharidosis type I (MPSI-H). MPSI-H is caused by alpha-L-Iduronidase (IDUA) deficiency leading to systemic glycosaminoglycan (GAG) accumulation, skeletal abnormalities, neurodevelopmental decline and death within the first decade of life. Two patients (14 and 24 months of age) have been treated as of Jan 15, 2019, and others are currently under evaluation. HSPC harvest by Lenograstim and Plerixafor mobilization was uneventful and effective resulting in cryopreserved drug products of 15 and 24 million CD34+ cells/kg, respectively, and transduction efficiencies above 80% using a shortened transduction protocol and

Prostaglandin E2 (PGE2) as transduction enhancer. Follow up in patient 1 reached 6 months as of Jan 15, 2019. His hematologic recovery after Flu/Bu-based myeloablative conditioning was fast (ANC<100/mcl: 5 days; last transfusion on day+10) and uneventful. Importantly, the patient reached normal blood IDUA activity by day+11 and supra-normal activity by day+14 that stabilized around 5 fold above the upper limit of normal, along with an in vivo vector copy number around 2 that was sustained in multiple hematopoietic lineages. IDUA activity was also demonstrated in the cerebrospinal fluid, and urinary GAGs were strongly reduced to near-normal levels early after gene therapy (d+90). Clinical updates on the first 2 treated patients will be presented. We also performed ancillary xenotransplantation studies comparing the engraftment capacity of the drug product with non-cultured CD34+ cells from patient 1. Both produced similarly-sized, multi-lineage grafts in primary and secondary NSG mouse recipients, confirming a minimal functional impact of the ex vivo procedure on HSPC. Transduction efficiency of the drug product remained above 90% throughout secondary transplantation, with a vector copy number around 3 integrations per cell. Taken together, our shortened, PGE2-based transduction protocol allows efficient HSC transduction without compromising their engraftment potential, thus supporting rapid patient recovery from transplantation and metabolic correction of the MPSI-H enzyme defect in critical target tissues. These initial clinical results strongly support benchmarking of our autologous ex vivo gene therapy approach against allogeneic transplantation, the current standard of care for MPSI-H patients.

3. Insertion Site Analyses on Liquid Biopsies Reveal the Clonal Repertoire and Early Premalignant Expansions Hidden in Solid Tissues

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In hematopoietic stem cell (HSC) gene therapy (GT) applications, vector integration site (IS) studies carried on blood and Bone Marrow (BM) cells allow to unravel the safety and efficacy of gene transfer. However, these analyses do not convey information of vector-marked clones residing in other solid organs, unless invasive biopsies are taken. This limitation hampers the description of the whole clonal repertoire in HSC-GT patients, prevents safety and efficacy monitoring of in vivo GT applications and limits the early identification of pre/malignant clones expanding in solid tissues. Here, we developed Liquid-Biopsy-Insertion Site-Sequencing (LiBIS-Seq), a PCR technique allowing the retrieval of IS from cell-free circulating DNA (circDNA) purified from blood plasma (BP) of GT-treated patients. CircDNA are DNA fragments released in BP by apoptotic and necrotic cells and, in our rationale, it should contain, at least in part, vector/genome junctions (circIS) derived from vector-marked cells residing in solid tissues. LiBIS-Seq was initially performed on BP-circDNA from 7 patients treated by Lentiviral Vector (LV)-based HSC-GT for Metachromatic Leukodistrophy (MLD) and retrieved >10,900 unique circIS. CircIS had the typical LV integration profile observed in HSC and recapitulated the safety and polyclonal pattern previously reported in this trial. Since only 40% of the circIS shared with IS retrieved from cell-DNA, we hypothesize that a fraction of IS could derive from vector-marked clones embedded in solid organs. To confirm this, we analyzed the BP circDNA from 3 dogs and 6 Non-Human Primates treated by LV-mediated liver directed GT and retrieved >11000 and >5000 circIS, respectively. CircIS analyses indicated a highly polyclonal pattern without persisting dominant clones, in agreement with the IS results observed from liver genomic DNA. Finally, we tested if LiBIS-Seq enables the early detection of pre/malignant T-cell Acute Lymphoblastic Leukemia (T-ALL) clones triggered by LMO2 insertions in g-retroviral vector-based clinical trials for Severe-Combined-Immunodeficiencies-linked to X chromosome (SCID-X1) and Whiskott-Aldrich-Syndrome (WAS). Our preliminary results, based on the analysis of cellular and circ-DNA collected before, at and after T-ALL diagnosis in a SCID-X1 patient, showed that in circulating blood cells the IS targeting LMO2 of the leukemic clone reached the maximum level of abundance of 1.4% at the time of diagnose, while in circIS this integration had a level of abundance higher than 90% even 4 months before the T-ALL and become undetectable post-chemotherapy. Analyses on the remaining SCID-X1 and on WAS patients are ongoing. Hence, LiBIS-Seq technology enables the study of the clonal repertoire at the whole organismal level in HSC-GT patients and for the first time opens the door to longitudinal clonal tracking studies in in vivo GT applications without the need of invasive biopsies. Finally, LiBIS-Seq allows the early and sensitive detection of non-circulating premalignant clones triggered by insertional mutagenesis residing in solid organs and undetectable by conventional IS analysis.

4. Preliminary Results from Cohorts 1 and 2 of CAPtivate: A Phase 1/2 Clinical Trial of AAV8-Mediated Liver-Directed Gene Therapy in Adults with Late-Onset OTC Deficiency

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***Background*:** Ornithine transcarbamylase (OTC) deficiency is an X-linked disorder that results in impaired flux of ammonia through the urea cycle and consequently hyperammonemia. Ammonia is a potent neurotoxin and elevated levels of ammonia can result in recurrent vomiting, neurobehavioral changes, seizures, and cause irreversible neurocognitive damage. OTC deficiency has high morbidity and mortality if untreated. Current standard of care includes limiting dietary protein intake, supplementation with a high-energy source

(glucose), and administration of nitrogen scavenging agents. Although this lowers the incidence of hyperammonemic episodes, risk of life-threatening hyperammonemic crises remains and neurocognitive outcomes are often suboptimal. Gene therapy aimed at restoring liver OTC activity presents a potential novel therapeutic strategy. DTX301 is a self-complementary recombinant AAV8 vector that expresses a codon-optimized human OTC cDNA under transcriptional control of a liver-specific promoter, and is currently under investigation as a possible therapy for patients with OTC deficiency. ***Methods*:** CAPtivate is a global, multi-center, open-label Phase 1/2 dose-escalation trial evaluating the safety and preliminary efficacy of a single IV infusion of DTX301 in adults with symptomatic late-onset OTC deficiency. Three subjects have been enrolled in cohorts 1 and 2, and 3 subjects will be enrolled in each subsequent planned cohort. The primary endpoint is the incidence of adverse events (AEs), treatment-emergent AEs, and serious AEs. Secondary endpoints are changes from baseline in the rate of ureagenesis and 24-hour AUC for plasma ammonia levels. Following an initial 52-week study assessment period, subjects are eligible to enroll in a 4-year extension study to evaluate the long-term safety and efficacy of DTX301. ***Results*:** Dosing of 3 patients each in cohort 1 at $2x10^{12}$ GC/kg and cohort 2 at $6x10^{12}$ GC/kg, respectively, has been completed. No infusion-related or serious AEs have been reported to date; all AEs have been grade 1-2. Two patients had mild, asymptomatic ALT increases that were resolved with corticosteroids, per protocol. Two patients have achieved a normalized ureagenesis rate, discontinued all ammonia scavenger medications, and liberalized dietary protein restrictions. All 3 patients from cohort 1 have completed the 52-week study period and have enrolled into the long-term extension study. Following review of cohort 2 data, 1 subject was enrolled at a dose of $1x10^{13}$ GC/kg (cohort 3) and 2 additional subjects will be enrolled in cohort 3. ***Conclusions*:** Early data from cohorts 1 and 2 of CAPtivate, an on-going Ph1/2 AAV gene transfer clinical trial are favorable and indicate that DTX301 may be a potential new therapeutic approach for patients with OTC deficiency.

5. Dominant Negative PD1 Armored CART Cells Induce Remission in Refractory Diffuse Large B Cell Lymphoma (DLBCL) Patients

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Dominant Negative PD1 Armored CART Cells Induce Remission in Refractory Diffuse Large B Cell Lymphoma (DLBCL) PatientsYan Yuan1, Liansheng Huang2, Chengfei Pu3, Tianling Ding1, Xibin Xiao2 Zhiyuan Cao3, Ting Wu1, Luying Ding2, He Sun3, Zhao Wu3, Lei Xiao3, Xiaohong Zhang2 and Tong Chen1*1 Department of Hematology, Huashan Hospital, Fudan University, Shanghai, China.2 Department of Hematology, The Second Affiliated Hospital of Zhejiang University School of Medicine, Zhejiang, China3 Innovative Cellular Therapeutics, Shanghai, China*Corresponding to: Tong chen, chentong@fudan.edu.cn The chimeric antigen receptor (CAR) T cell treatment has been demonstrated as an effective therapy to relapse/refractory B cell malignancy. However, tumor microenvironment influences and affects 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) during 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 cells including an anti-CD19 second generation (2G) CAR molecule and a dominant negative PD1 molecule (Figure A). Compared with conventional CART cells, these "armored" CART cells show the enhanced capability of tumor killing after multiple-round tumor challenging and more "memory-like" phenotypes (Figure B). These results suggest dominant negative PD1 molecules may protect CART cells from exhaustion in the tumor microenvironment.Further, we report clinical trials of two refractory diffuse large B cell lymphomas (DLBCLs) patients that were successfully treated using the armored CAR T cells described above. Both of these two patients failed to achieve response after multiple rounds of chemotherapy and radiotherapy. However, after infused with autologous CART cells at 5.23×10^6/kg and 1.97×10^6/kg, respectively, they showed significant tumor mass decrease and SUV max declines in PET/CT results and ongoing responses (From 34.48 to 3.89 at day 27, from 25.02 to 2.38 at day 31, respectively, *see* Figure C). **Conclusion:** These two clinical trials revealed the significant anti-bulky lymphoma response with respect to these armored CAR T cells and limited and tolerated cytokine release syndrome and central nervous system toxicity. Also, dominant negative PD1 molecules may augment CAR T cells persistence in patients after activation by lymphoma cells, thus enhancing efficacy of CAR T cells in treatment of hematomas. Finally, the techniques described herein are 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 recruit more patients for the clinical trials.

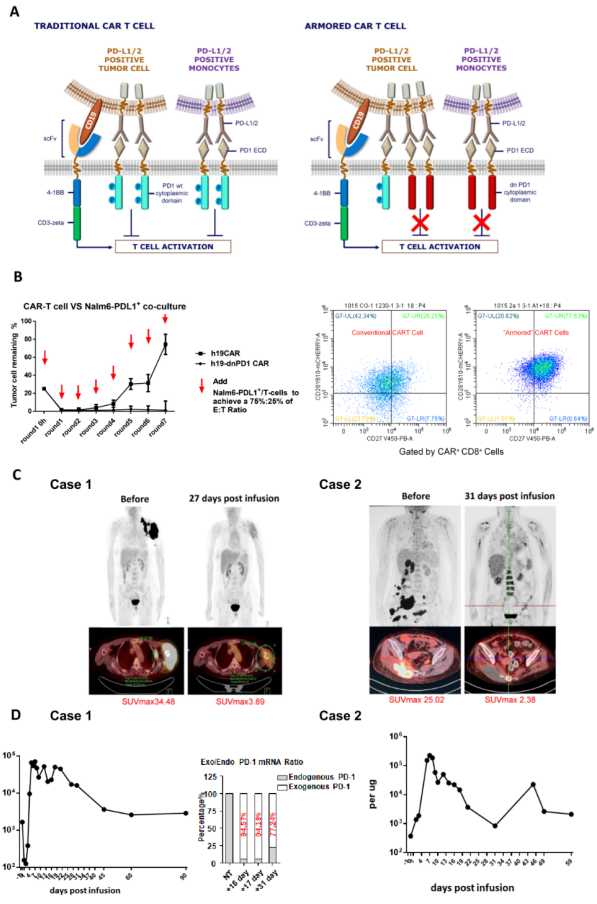

Figure. Dominant negative PD1 anti-CD19 CART cell (dnPD1-CAR19) construction,ex vivo experiment result and clinical case effect. **A:**Traditional CAR construction(left) and dnPD1 armored CAR construction(right).**B:**ex vivo anti tumor effect of dnPD1-CAR19 versus Traditional CAR19.Left:Percentage of remaining GFP-positive tumor cell (Nalm6 and Nalm6-PD1) after co-colture with CAR19 and dnPD1-CAR19 in continuous 6 rounds experiments. Right:the GFP+tumor cells after the 6th round of experiment. **C:**PET/CT image before and after CART cell infusion of these 2 cases.Left:case1;Right:case2.**D:**Persistence of CART cells in peripheral blood mersured by copy number per ug genomic DNA. RT-QPCR analysis of endogenous and exogenous PD1 expression of the case1 patient's $CD3^+$ cells .

6. Mechanism of Action of Toca 511 and 5-FC in Animal Models and Cancer Patients

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Toca 511 (vocimagene amiretrorepvec) is a replicating retrovirus encoding an optimized cytosine deaminase (CD), that in preclinical and human clinical studies selectively infects cancer cells. Toca 511 alone is non-inflammatory, and actively inhibits type 1 IFN induction. Toca 511 combined with 5-flurocytosine, which is converted to 5-FU within infected cells, leads to tumor regression in several animal models. Murine studies in subcutaneous, metastatic colon and orthotopic brain tumor models have all shown a common mechanism of action (MOA) that involves the de novo generation of systemic anti-tumor immunity; the immune activity is induced by tumor cell lysis, depletion of myeloid immune suppressive cells by diffusible 5-FU in the tumor microenvironment, and subsequent tumor T cell infiltration, with a key role for CD4 cells. In a recurrent High Grade Glioma (HGG) Phase 1 trial using an extended release formulation of 5-FC, Toca FC, (NCT01470794), 26% (6/23 pts) of a subgroup of patients are alive after 3 or more years , compared to historical numbers of around 5-10 %. This subgroup had tumors ≤ 5cm diameter and a Karnofsky score of 70 or above, were at 1st or 2nd recurrence, had received no prior bevacizumab, and were dosed at approximately 1E9 transduction units of Toca 511 or above, Five of the 6 long-lived patients have a complete clinical response with no observable tumors in MRI scans, by independent neuroradiologist review. The durable responses, with delayed onset, are supportive of an immune mechanism of action in patients. Analyses of pre-treatment tumor and of post-treatment blood samples from both responding and non-responding patients suggest: 1) that the responses may be immune mediated; 2) that a prognostic immune cell signature can be seen in tumor microenvironment of patients who respond; 3) that there is a cytokine signal in the blood plasma that correlates with response; and 4) that responders do not have an above normal level of neo-antigen load in their tumor. These studies have been followed up by a 400 patient, open-label controlled pivotal clinical trial in rHGG (NCT02414165), with a final analysis planned for the second half of 2019. We plan to further evaluate immune cell signatures in samples from the pivotal trial to determine if an association with survival can be confirmed. We also plan look for such signatures in patients with disseminated advanced solid tumors in a Phase 1 trial (NCT02576665).

Advances in Genome Editing, Hemophilia Gene Therapies

7. Barcoded Clonal Tracking of CRISPR-Cas9 and rAAV6-Mediated Gene Targeting in Human Hematopoietic Stem and Progenitor Cells

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Hematopoietic stem and progenitor cell (HSPC) transplantation has the power to cure genetic diseases of the blood and immune system. HSPC gene therapy clinical trials using retroviral vectors has proceeded with mixed results, but the methodology maintains the advantage of being able to predict and track adverse (and normal) clonal events via analysis of unique integration sites in cell populations. Recent developments of the CRISPR-Cas9 system enable targeted manipulations of the HSPC genome that have revolutionized the concept of correcting disease-causing genetic mutations in patient-derived autologous HSPCs. By combining ribonucleoprotein delivery of the CRISPR-Cas9 system and homologous DNA donors via recombinant adeno-associated virus serotype six (rAAV6), it is possible to elicit high frequencies of gene targeting in HSPCs across many clinically relevant loci. However, due to the precise nucleotide-resolution nature of gene targeting, these current methodologies do not allow for clonal tracking of gene targeted HSPCs. Here, we created barcoded rAAV6 homologous DNA donor libraries to track the clonal contribution of gene targeted HSPCs on hematopoietic reconstitution *in vivo*. The barcoded libraries can be used to introduce silent mutations within a coding sequence or to target unique DNA tags outside of the coding sequence, with sequence diversity ranging from $1x10^3$ to $1x10^6$ unique barcodes. To first establish that this method can track oligoclonal expansion of gene-targeted HSPCs *in vivo*, we created a barcoded AAV6 donor library that contained DNA templates with the SFFV promoter driving the expression of oncogenic mutant $NRAS^{G12D}$. We targeted barcoded SFFV-BFP-$NRAS^{G12D}$ into the *AAVS1* locus in HSPCs and then transplanted the targeted HSPCs into sublethally irradiated immunodeficient NSG mice to assess whether constitutive $NRAS^{G12D}$ expression was sufficient to induce a clonal hematopoietic phenotype. 100% of the control mice transplanted with SFFV-BFP targeted HSPCs survived the length of the study (week 18), while only ~50% of the mice transplanted with SFFV-$NRAS^{G12D}$ HSPCs survived until week 8 (N=8-10 mice per group). Six to eight weeks post-transplantation of SFFV-$NRAS^{G12D}$ cells, histopathological analysis (H & E staining) of the bone marrow and spleen indicated infiltration of hyperproliferative myeloid cells. Sequencing analysis of the engrafted human myeloid cells showed an oligoclonal signature (6 unique barcodes making up 50% of the total reads) that was presumably responsible for the HSPC transformation towards the myeloid lineage and ultimately reduced animal survival. To assess normal polyclonal hematopoietic reconstitution, we targeted the *HBB* gene with silent mutations (up to 36,000 DNA permutations) in HSPCs. Compared to single-sequence *HBB* targeted HSPCs, barcoded *HBB* targeted HSPCs displayed similar frequencies of gene targeting, long-term engraftment, red blood cell differentiation, and adult hemoglobin expression, confirming the barcoded library had no influence on HSPC cell biology and importantly, maintained the open reading frame following gene targeting. Initial sequencing analysis of engrafted human cells suggests >100 unique and shared barcodes (with many barcodes represented at different frequencies) in the lymphoid and myeloid lineages indicating *HBB* gene targeting in multilineage potent HSPCs capable of durable engraftment. We anticipate that this methodology has the potential to be used for HSPC clonal tracking of rAAV6-mediated gene targeting outcomes in translational and basic research settings.

8. Large-Scale CRISPR-Cas Genome-Wide Activity Profiling in Human Primary T-Cells Reveals Genetic and Epigenetic Determinants of Off-Target Effects

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CRISPR-Cas nucleases are a simple and robust technology to modify the genomes of living cells with broad utility for basic research and human medicine. While many *in vitro* and cell-based assays have been described to define CRISPR-Cas editing activity, there remains no easy way to routinely perform these types of analyses on large numbers of targets. Previously, we developed CIRCLE-seq, an *in vitro* method for defining the genome-wide activity of CRISPR-Cas9 nucleases by selective sequencing of Cas9-cleaved genomic DNA molecules. Though highly sensitive, its scalability is limited due too labor-intensive processing steps and systematic loss of material through sequential purification steps. To streamline CIRCLE-seq, we developed and optimized CIRCLE-seq High-throughput Analysis of Nuclease Genome-wide Effects by Sequencing (**CHANGE-seq**). CHANGE-seq is based on a novel enzymatic workflow leveraging Tn5 transposon-based tagmentation, optimized gap-repair and exonuclease treatments. Our new method overcomes limitations of the original by eliminating the need for specialized equipment for genomic DNA shearing, reducing genomic DNA input requirements by approximately 5-fold, and substantially minimizing processing steps, time, and cost. To directly compare CIRCLE-seq to CHANGE-seq, we generated CHANGE-seq profiles at 11 sites we had originally characterized with CIRCLE-seq. We found that CHANGE-seq and CIRCLE-seq read counts were significantly correlated in 21 of 22 experiments, and the overlap in sites detected by both CHANGE-seq and CIRCLE-seq is comparable or better than CIRCLE-seq technical replicates (>85%). To broadly test CHANGE-seq, we evaluated 110 non-repetitive*S. pyogenes* Cas9 target sites in 13 therapeutically important *loci* in human primary $CD4^+$/$CD8^+$ T-cells (AAVS1, *B2M*, *CBLB*, *CTLA4*, *CCR5*, *CXCR4*, *FAS*, *LAG3*, TRAC locus, *PDCD1*, *PTPN2*, *PTPN6*, *TRBC1*). CHANGE-seq identified a total of 202,043 genome-wide off-target cleavage sites (ranging from 20 to 60,000 per target). We analyzed the data in aggregate to identify major determinants of off-target activity and observed two obvious effects: 1) there is increased tolerance for G-to-A mismatches and 2) RNA

or DNA bulge loops in off-target sites are best tolerated in the center. Next, we sought to evaluate the impact of chromatin accessibility on cellular off-target activity. To identify cellular off-target sites, we optimized GUIDE-seq for human primary T-cells and analyzed 22 targets randomly chosen from the 110 we characterized with CHANGE-seq. The number of sites detected by CHANGE-seq and GUIDE-seq are significantly correlated (R^2=0.74, p=1.3x10^{-4}). To identify regions of open chromatin, we performed ATAC-seq on human primary T-cells from the same donor. Among the 347 sites identified by both CHANGE-seq and GUIDE-seq, we found that CHANGE-seq sites in open chromatin regions are significantly more likely to be detected by GUIDE-seq than those in closed chromatin regions (odds ratio = 2.45, p= 1.2x10^{-5}). In sum, CHANGE-seq enabled the generation of large-scale genome-wide activity datasets, revealing important genetic and epigenetic factors that affect genome-wide off-target activity. Future analyses of these large-scale data using state-of-the-art machine learning methods may yield additional insights into fundamental principles governing genome-wide genome editing activity.

9. CRISPR/Cas9-Mediated Homology Independent Targeted Integration in Human Hematopoietic Stem and Progenitor Cells

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The use of CRISPR/Cas9-based genome editing techniques to target therapeutic transgenes to their endogenous genetic loci addresses many of the limitations associated with viral vector-based gene replacement strategies, such as the potential for ectopic expression, transgene silencing, variable gene dosage, and/or insertional mutagenesis. Common methods of Cas9-mediated targeted integration utilize the cellular homology-directed DNA repair (HDR) pathway; however, these approaches are inefficient (typically ≤1% recombination) in quiescent primary cells, such as long-term repopulating hematopoietic stem and progenitor cells (HSPCs), where, due to their protracted G_0/G_1 phasing, non-homologous end joining (NHEJ) is the primary DNA repair mechanism. Recently, a novel NHEJ-based approach to Cas9-mediated transgene knock-in, known as homology independent targeted integration (HITI), has demonstrated improved site-specific integration frequencies in both dividing and non-dividing cells. We have utilized a HITI-based approach to achieve robust site-specific transgene integration within mobilized human CD34+ HSPCs. As proof-of-concept, a copGFP expression cassette was targeted to a clinically relevant genetic locus, the human *ITGB2* gene encoding the beta-2 integrin subunit CD18. Mutations in CD18 are associated with leukocyte adhesion deficiency type 1, an inherited immunodeficiency. First, a HITI donor template bearing a CMV promoter-driven copGFP reporter gene flanked by 20-nt *ITGB2*-specific sgRNA target sequences (designated *ITGB2-ts*) was constructed and packaged within recombinant adeno-associated virus serotype 6 (rAAV6) capsids. Mobilized human CD34+ HSPCs isolated from healthy donors were transduced with rAAV6-copGFP (multiplicity of infection = 10e5 particles/cell) and electroporated with pre-formed, *ITGB2*-targeted Cas9/sgRNA ribonucleoprotein (RNP) complexes at a 1.25 micromolar final concentration. Upon nuclear entry, the *ITGB2-ts*-flanked reporter cassette and the endogenous *ITGB2* target gene are concomitantly cleaved by Cas9, thus promoting NHEJ-mediated transgene insertion at the site of the Cas9-induced chromosomal double-strand break. To determine the optimal time of rAAV transduction, HSPCs were transduced with rAAV6-copGFP at either 48 hr or 36 hr pre- or 0.5 hr post-RNP electroporation. Gene edited cells were cultured under hypoxic conditions (5% O_2) at 37°C for up to 28 days post-electroporation and periodically sampled for flow cytometry and genomic DNA (gDNA) extraction. Transduction of HSPCs prior to electroporation resulted in enhanced cellular viability compared to post-electroporation transduction. Flow cytometry revealed efficient rAAV transduction at 4 days post-transduction (range 18-to-46% copGFP+ cells). The percentage of cells expressing copGFP slowly decreased over the extended culture period, stabilizing at approximately 5-to-10 percent of the bulk cell population at >2 weeks post-electroporation. Site-specific transgene integration was confirmed by PCR analysis of bulk cell gDNA using reporter- and flanking gene-specific primer pairs. To estimate the frequency of transgene integration, CD34+ cells were plated in a CFU assay. At 10-to-14 days post-plating, copGFP+ colonies were enumerated and subjected to gDNA isolation for PCR analysis. Approximately 12% of colonies demonstrated copGFP expression. Integration junction-specific PCR analysis of colony gDNA confirmed that reporter gene expression was attributable to integrated donor template sequences, as opposed to rAAV-copGFP episomes or random transgene integration events. In summary, HITI-based transgene knock-in provides an effective alternative to HDR-mediated donor template recombination in human CD34+ HPSCs.

10. Human Hematopoietic Stem and Progenitor Cells that Have Undergone Nuclease-Induced Homology Directed Repair with an AAV Delivered Donor Template Exhibit Engraftment Deficiencies in Mouse Xenotransplant Studies

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Nuclease-driven gene editing of hematopoietic stem and progenitor cells (HSPCs) has the potential to deliver high impact therapies to patients with severe genetic diseases. By exploiting the cellular DNA repair machinery, selected targets can either be disrupted through the non-homologous end joining (NHEJ) repair pathway, or new DNA sequence can be precisely introduced when a homologous donor DNA is provided. To determine if edits mediated by the respective DNA-repair pathways support long-term xenotransplant engraftment in NSG mice, we used a megaTAL targeted against the Bcl11a erythroid enhancer, which is a critical regulatory element controlling the switch from fetal to adult hemoglobin. In vitro, we were able to produce human HSPCs with 77.4% indels, and we saw a high level of maintenance of indels, average 76.6%, in long-term (16 week) NSG engrafted cells. We next evaluated the capacity of the Bcl11a erythroid

enhancer megaTAL in combination with an AAV-delivered donor template to induce homology directed repair (HDR) in HSPCs. *In vitro*, we have been able to achieve >70% HDR, which is comparable to what has been reported by other groups using different nuclease technologies [Wang et al, Nat Biotech 2015; DeWitt et al, Sci Transl Med 2016; Gundry et al, Cell Rep 2016]. While we, along with others in the field, have made considerable progress increasing HSPC HDR rates *in vitro*, maintenance of HDR *in vivo* remains to be a challenge [Dever et al, Nature 2016; DeWitt et al, Sci Transl Med 2016; Hoban et al, Blood 2015]. One established method to stringently demonstrate differences in engraftment capabilities of different human stem cell populations is a limiting dilution analysis (LDA) xenotransplant model. Here we show that human HSPC that are HDR-positive have a lower NSG repopulating frequency than HSPCs from the same pool of cells that are HDR-negative. To assess this, we treated human CD34+ cells with a Bcl11a-erythroid enhancer-targeted megaTAL and an AAV2/6-BFP donor template with homology arms directed to the cut site. Two days after nuclease treatment and transduction with AAV, we sorted cells based on blue fluorescent protein (BFP) expression, with the BFP-high population representing cells that had undergone HDR. BFP-negative cells were collected from the same cell product and used as a comparator. We introduced cells into NSG mice at four doses, ranging from $3x10^5$ to $2.5x10^4$ cells per mouse. Sixteen weeks post-transplantation, 70% of mice receiving the highest dose of HDR-negative cells exceeded the study threshold for positive engraftment (1% human CD45+), with expected decreases in the number of positively engrafted mice as doses decreased. In comparison, there were no engrafted mice (human CD45 > 1%) in any of the dose groups receiving HDR-positive HSPC. Several potential explanations for the observed engraftment deficiency are plausible. HDR could be mutually exclusive with the true long-term HSC. Alternatively, the process of performing HDR (perhaps as a function of the form and/or quality of the donor DNA delivery) may drive HSPCs to a more differentiated (short term engraftment) phenotype. Regardless of mechanism, further investigation will be required to both understand and overcome the engraftment barrier of HDR-positive HSPCs to make this approach viable for the treatment of disease.

11. CRISPR/Cas9-Mediated Targeted Insertion of Human F9 Achieves Therapeutic Circulating Protein Levels in Mice and Non-Human Primates

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CRISPR/Cas9-mediated genome editing holds tremendous promise for the treatment and potential cure of human genetic diseases by addressing their underlying cause. Many genome editing studies to date have focused on knocking out genes by the error-prone repair mechanisms of non-homologous end joining (NHEJ) that predominate in most cell types. We present data showing that the delivery of lipid-nanoparticle (LNP) encapsulated CRISPR/Cas9 components in conjunction with AAV-borne donor template DNA can enable replacement of a missing genetic function, achieving robust levels of targeted gene insertion in primary mouse and human hepatocytes as well as in mouse livers *in vivo*. Furthermore, using insertion of a human *F9* template into the albumin locus as a model insertion, we demonstrate that therapeutic levels of circulating FIX can be achieved in non-human primates using this combination LNP + AAV strategy. This approach opens the way for the development of therapies for a wide range of genetic diseases which require stable gene insertion and expression.

12. B Cell Depletion with Anti-mCD20 Eliminates FVIII Memory B Cells in Inhibitor Positive Mice and Enhances AAV8-coF8 ITI When Combined with Rapamycin

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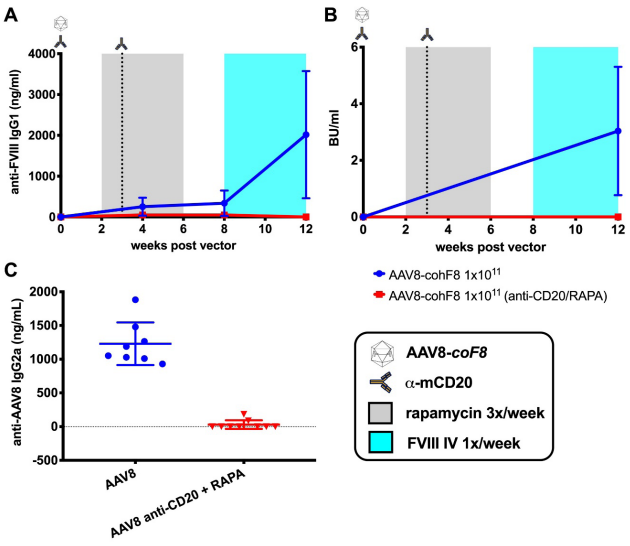
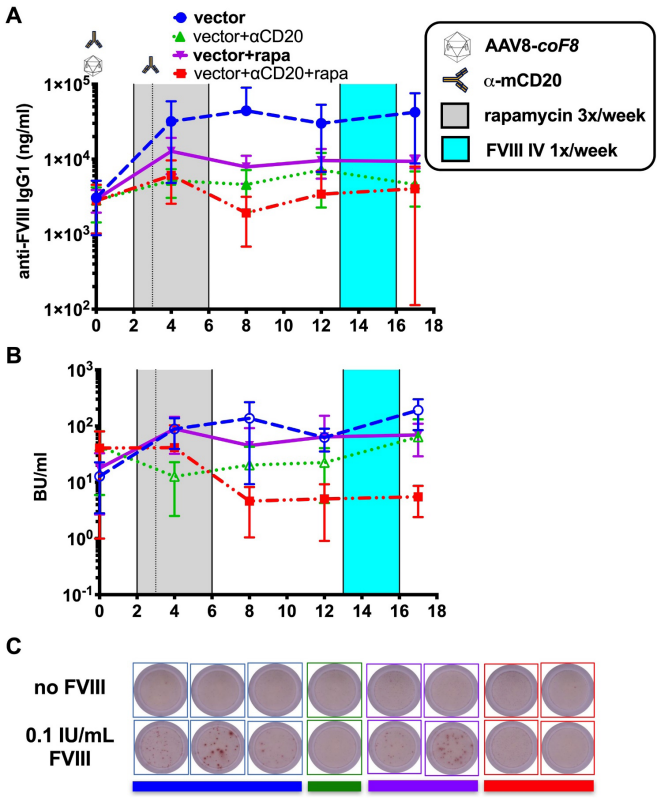
Hemophilia A is an inherited coagulation disorder resulting in the loss of functional clotting factor VIII. Presently, the most effective treatment is prophylactic enzyme replacement therapy (ERT). However, this requires frequent life-long intravenous infusion of plasma derived or recombinant clotting factors and is not a cure. A major complication of ERT is the development of inhibitory antibodies that nullify the factor replacement therapy. Inhibitor patients require treatment with bypassing agents to restore coagulation while they undergo immune tolerance induction (ITI) therapy. ITI therapy can last from months to years and require daily or every other day infusion of supraphysiological levels of FVIII and is effective in up to 70% of hemophilia A patients. ITI with bypassing agent therapy is costly and hemostasis is not as well

managed as with FVIII protein therapy. Preclinical and recent clinical studies have shown that gene replacement therapy with AAV vectors can effectively cure hemophilia A patients. However, it is unclear how hemophilia patients with high risk inhibitor F8 mutations or with established inhibitors will respond to gene therapy, as these patients have been excluded from ongoing clinical trials.We have previously reported that AAV-*F8*gene transfer in naïve BALB/c-*F8*$^{-/Y}$mice (BALB/c-HA) results in anti-FVIII IgG1 inhibitors following gene transfer despite this background having a weaker response to FVIII protein therapy. To prevent this, we used transient immune modulation with anti-mCD20 (18B12) at vector administration and three weeks later and one month of oral rapamycin beginning two-weeks after vector (Fig. 1). Next we investigated if we could improve ITI in inhibitor positive mice by combining anti-mCD20 and rapamycin with AAV8-*coF8*. Our hypothesis was that continuous expression of FVIII from gene transfer compared to transient FVIII from weekly protein therapy, would enhance regulatory T cell induction and promote deletion of FVIII reactive B cells, following reconstitution. We established four groups of mice with a similar range of inhibitor titers. All groups received the same dose of AAV8-*coF8* vector and then received either no additional treatment, anti-mCD20, rapamycin, or both anti-mCD20 and rapamycin. Mice given vector alone or vector with rapamycin had a substantial increase in inhibitors (Figs. 2a-b). However, both groups that received anti-mCD20 either stabilized or had a sharp reduction in inhibitors. Importantly, only mice receiving both rapamycin and anti-mCD20 failed to increase inhibitors following intravenous FVIII protein therapy (Figs 2a-b. Splenocytes harvested from selected mice within each group were tested for FVIIImemory B (B_{mem}) cells, which were not detected in either group receiving anti-mCD20 (Fig. 2c). Our data show that B and T cell immune modulation complement AAV-*coF8* gene therapy in naïve and inhibitor positive hemophilia A mice with a high risk for inhibitor and suggest that such protocols should be considered for AAV gene therapy in high risk or inhibitor positive hemophilia patients.





13. Non-Genotoxic Anti-CD117 Immunotoxin Conditioning Facilitates Hematopoietic Stem Cell Transplantation Gene Therapy for Hemophilia A

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Hematopoietic stem cell transplantation (HSCT) for coagulation factor VIII (fVIII) gene therapy is a potentially curative approach for the treatment of the most common severe bleeding disorder, hemophilia A (HA). Our laboratory has previously established the ability to produce stable therapeutic levels of fVIII in a mouse model of HA following irradiation or chemotherapy and transplantation of Sca-1^{+} hematopoietic stem and progenitor cells (HSPCs) genetically modified *ex vivo* using recombinant lentiviral vector encoding a bioengineered high-expression fVIII variant, termed ET3. Conventional myeloablative regimens for HSCT such as total body irradiation and myeloablative alkylating chemotherapy are associated with adverse side effects, including sterility, hormonal dysregulation and genotoxicity. These risks represent a primary barrier to clinical translation of HSCT gene therapy for HA and many other monogenic

diseases. Therefore, the development of targeted, non-genotoxic conditioning agents is favorable. We have successfully implemented the use of an immunotoxin bioconjugate comprised of a monoclonal antibody against the stem cell factor receptor c-Kit (anti-CD117) and the ribosome-inactivating protein toxin saporin (sap) for non-genotoxic HSCT conditioning. Wild-type C57BL/6J or HA mice were conditioned with 0.5 mg/kg CD117-sap with or without immune suppression using anti-thymocyte globulin (ATG). Depletion of total bone marrow (BM) cells and HSPC compartments were assessed after five days. We achieved significant reduction of total BM cells harvested from two femurs in mice conditioned using CD117-sap compared to control mice given sap-conjugated IgG isotype control or immune suppression only ($48 \pm 3.8 \times 10^6$ vs $29 \pm 3.8 \times 10^6$, $p = 0.007$, $n = 5$). Similarly, significant depletion of bone marrow LSK ($lin^- Sca\text{-}1^+ c\text{-}Kit^+$) and long-term HSC ($LSK^+ CD150^+ CD48^-$) compartments was observed (LSK: 61 ± 9.6 vs $26 \pm 7.8 \times 10^3$, $p = 0.019$, $n = 5$ to 6; LT-HSC: 6.4 ± 1.5 vs $1.2 \pm 1.1 \times 10^3$, $p = 0.019$, $n = 5$ to 6). Following CD117-sap conditioning, HA mice were transplanted with 1×10^6 ET3-transduced $Sca\text{-}1^+$ HSPCs from GFP^+ congenic mice. We observed robust mixed hematopoietic chimerism and myeloid engraftment as early as 2 weeks post-transplantation in recipients conditioned with CD117-sap + ATG ($86.4 \pm 5.3\%$ GFP^+ granulocytes) compared to CD117-sap ($0.1 \pm 0.1\%$, $p < 0.0001$) or ATG alone ($1.2 \pm 0.3\%$, $p < 0.0001$). Peripheral blood granulocyte chimerism in this group continued to increase at 4 weeks ($93.9 \pm 4.0\%$) and 6 weeks ($97.3 \pm 0.4\%$). Moreover, these mice exhibited mean therapeutic levels of circulating fVIII activity of 0.24 IU/ml as early as 2 weeks, continually increasing up to 0.55 IU/ml by 6 weeks post-transplant. Since our fVIII expression cassette is driven by a myeloid-specific promoter, we expect circulating levels of fVIII to continue to increase with the progression of tissue-based myeloid lineage differentiation, macrophage and dendritic cell maturation. One recipient from the CD117-sap + ATG group showed signs of immunological rejection and appeared to actively reject the graft by 4 weeks. Further studies are underway to investigate strategies for early immune suppression and prevention of graft rejection. Taken together, these preliminary data are a promising proof of concept demonstrating the feasibility of using non-genotoxic immunotoxin conditioning for HA gene therapy.

14. Insertional Oncogenesis in X-CGD Patient after MFGS Retroviral Vector-Mediated Gene Therapy

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Gene therapy has been developed as a highly desirable treatment option for patients with X-linked chronic granulomatous disease (X-CGD), who lack the donors for transplantation. In Japan, a gene therapy trial using MFGS-$gp91^{phox}$vector was approved in 2012, and a 27-year-old patient was treated. While he showed the improvement in the refractory infections, oxidase positive neutrophils in peripheral blood declined to the level less than 1% at months 6, and became undetectable at months 18, as in the previous trial with the MFGS vector. At 32 months after gene therapy, however, the patient showed the decrease in platelet count and the emergence of blasts with the frequency of 5 to 8% in peripheral blood and bone marrow, which was comparable to myelodysplastic syndrome (MDS). Karyotype change such as monosomy7 was not observed. LTR capture followed by NGS identified the vector integration into IVS2 of *MECOM*. Integration site-specific droplet digital PCR (ddPCR) revealed that the clone with this integration emerged at months 6 and its copy number in monocytes and neutrophils reached to almost 1 per cell at months 12. Most colonies from CFU-C assay using 12 months' bone marrow also showed the integration. These results suggested that the single clone with *MECOM* integration maintained myeloid-lineage hematopoiesis, and additional events might be required for blast transformation at month 32. In the previous trials using SFFV vector, the patients who developed MDS showed the extensive methylation of the promoter sequence in proviral LTR. Because the ectopic expression of CYBB with high oxidase activity inhibits the growth of the transduced cells, loss of CYBB expression by promoter methylation might contribute to the expansion of the blast clones. In our patient, however, the blast cells showed no methylation of CpG at promoter in LTR, but more than 100 of G to A mutations in provirus *CYBB* that caused multiple amino acid changes. All these mutations were detected at months 6, and we guessed the involvement of APOBEC3 protein that causes G to A mutations during the reverse transcription of retrovirus. Cloning of provirus sequence from transduced patient's CD34 positive cells (before transplantation) showed that about 5% of clones contained one or more G to A mutations. Mutation-specific ddPCR against some G to A mutations also showed the signals in patient's CD34 positive cells, but no signal in reverse-transcribed DNA from virus RNA. Although the occurrence of the hypermutation in provirus has rarely been reported in the gene therapy clinical trials, these results demonstrated that the G to A mutations occurred during the reverse transcription and integration, which strongly suggested the involvement of APOBEC3 protein. The result of our trial using MFGS vector indicated the potential of insertional oncogenesis of retroviral vector, regardless of the LTR-subtype and the disease backbone. Multiple G to A mutations in *CYBB* that was assumed to emerge during the transduction led to the loss of oxidase activity and facilitated the survival and proliferation of the clone with vector integration into *MECOM* locus. Patient underwent haplo-identical hematopoietic stem cell transplantation from his father and vector-marked blast cells are not detectable at 18 months after transplantation.

AAV Vectors and Disease Targets I

15. Regulatory and Exhausted T Cell Detection after Intramuscular AAV1 Delivery in Nonhuman Primates and Human

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With the recent FDA and EMA approvals for Luxturna® and Glybera® respectively, recombinant adeno-associated virus (rAAV) vectors appear, more than ever, to be efficient tools for gene transfer. However, studies on mice and large animal models, but also in humans, demonstrate that intramuscular (i.m) AAV delivery can trigger immune responses to AAV capsid and/or transgene. Intra-muscular delivery of rAAV1 in humans has also been described to induce a tolerogenic response to rAAV characterized by the presence of capsid-specific regulatory T cells (Tregs) in periphery. To understand the mechanisms responsible of tolerance and parameters involved, we tested 3 modes of delivery: the intramuscular delivery (i.m), the peripheral venous limb perfusion (VLP) and the intra-arterial double balloon catheter method (IAPD) in rhesus monkeys. These 3 methods have been well tolerated and led to transgene expression. Moreover, these administration routes appear to be safe with only one VLP-injected animal showing a low positive IFNγ response to the capsid. Interestingly, only animals injected i.m present infiltrated Tregs and exhausted T cells *in situ*. These cells have been detected at both Day 21 post-delivery and at necropsy (Day 60) at the same frequency suggesting an early tolerance towards the capsid and/or the transgene sustained over time and confirming the results obtained on alpha-1 antitrypsin-deficient (AATD) patients 5 years post vector administration. Currently we are performing an in-depth analysis of the suppressive function and exhausted nature of both of these infiltrating T-cell populations .

1. Mueller, C, Gernoux, G, Gruntman, AM, Borel, F, Reeves, EP, Calcedo, R, *et al.* (2017). 5 Year Expression and Neutrophil Defect Repair after Gene Therapy in Alpha-1 Antitrypsin Deficiency. *Molecular therapy : the journal of the American Society of Gene Therapy* **25**: 1387-1394.

16. Recombinant Adeno-Associated Virus (rAAV) Expressing a Pan-Hemagglutinin (HA) Antibody Protects Mice Against Influenza

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Every year about 3 to 5 million cases of severe illness are caused by influenza virus infection worldwide and the most effective approach to prevent the disease is vaccination. The immunocompromised and elderly populations are particularly susceptible to the infection because of their weak immune system and poor vaccine efficacy. Because of its demonstrated safety and efficacy as a gene transfer vehicle, treatment with AAV carrying the gene for neutralising antibody against influenza virus could provide long-term protection in high risk group populations. We have previously generated two monoclonal antibodies (mAb 10A9 and mAb 11H12) against a conserved region (the fusion peptide) of the HA protein of influenza (Manceur et al., 2017; PLoS ONE 12(6): e0180314). These mAbs can detect 13 HA subtypes of influenza A and B by immunohistochemistry. In this study, we investigated if the cDNA for these two mAbs can protect mice when challenged with two strains of influenza virus (H3N2-HK/8/68 and H1N1-A/Netherlands/602/2009). Mice were treated with a single dose of 2.0 X 10^{11} AAV serotype 9 vector genomes (vg) carrying the heavy and light chains of mAb 10A9 or mAb 11H12 regulated by a strong ubiquitous CAG (CMV enhancer/β-actin) promoter. Three routes of administration were tested: intravenous (IV), intramuscular (IM) and intranasal (IN). 21 days later the mice were challenged with influenza virus. We also tested the same dose of AAV9 carrying the same mAbs regulated by a strong muscle specific promoter derived from the slow troponin I gene (ΔUSEx4). As a control, mice were treated with an intraperitoneal injection of purified mAbs at a dose of 30 mg/kg two hours before challenge. Treatment with AAV9-CAG-10A9/11H12 by either of the three routes of administration provided protection, as demonstrated by the reduced weight loss and increased survival 14 days following the challenge. The best protection was achieved through intramuscular administration. The IM route proved even better than the treatment with purified mAbs injected intraperitoneally. Treatment with AAV9 carrying mAbs controlled by the muscle specific promoter was also protective, but to a lower extent, as compared to the ubiquitous promoter. There was a correlation between the protective efficacy and the amount of mAbs in the serum after gene transfer (IV and IM). In conclusion, a single dose of AAV9 can deliver enough antibody to protect mice against challenge with influenza virus. The use of a muscle specific promoter could improve the safety and prolong the efficacy of the treatment by reducing the risk of triggering a deleterious immune response against the transgene.

17. Spinal Subpial Delivery of AAV9 Produces a Potent, Long-Lasting Block of Neuraxial Degeneration and Disease Manifestation in Adult Mice by Silencing an ALS-Causing Mutant Gene

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Introduction: Central nervous system (CNS)-linked hereditary disorders such as amyotrophic lateral sclerosis (ALS), represent an important target for gene silencing as a new therapy. SOD1 gene

mutation is characterized by widespread neuronal degeneration of alpha-motoneurons, interneurons, descending motor tracts and activation/proliferation of glial cells. This ratifies the need to effectively target multiple cell types, and spinal axons in order to achieve a long-lasting therapeutic effect. Currently, the major limitation of viral-based silencing approaches is poor penetrability into the adult CNS. To address this limitation, we report the use of a novel subpial vector delivery technique which in adult animals successfully delivers adeno-associated virus (AAV) throughout whole spinal cord and brain motor centers. Our current study was designed to; 1) Study the SOD1 silencing effect and resulting treatment potency after subpial of AAV9-ShRNA-SOD1 delivery in adult ALS $SOD1^{G37R}$ mice, and 2) confirm the feasibility and safety of subpial vector delivery technique in adult non-human primates. **Methods:** $SOD1^{G37R}$ transgenic adult mice (both sexes) and their non-transgenic littermates were injected subpially at cervical and lumbar sites with AAV9-shRNA-SOD1 (10 µl; $1.2x10^{13}$ GC/ml) at an average age of ~120 days (presymptomatic stage) or 348 days (clinically symptomatic stage). After treatment, the body weight, grip test, and open field motor performance were monitored every week. On the day of sacrifice (i.e. end stage time point), muscle fibrillation and/or motor evoked potentials were recorded and tissue harvested. The level of SOD1 silencing and the neuroprotective effect was assessed by Q-PCR, WB, immunofluorescence (neuronal and glial markers), and in situ hybridization. **Results:** A combined single subpial injection of AAV9-ShRNA-SOD1 in cervical and lumbar spinal cord of adult presymptomatic $SOD1^{G37R}$ mice delays disease onset up to 85 days, produces long-term suppression of neurological signs of disease, including preservation of normal: motor performance, upper and lower limb grip strength and righting reflex up to the ~470 days of age (i.e. end of the study; Δ 81 days compared to non-treated siblings). At ~470 days all treated animals showed a continuing presence of motor evoked potentials and lack of fore and hind limbs muscle fibrillation (evidence of preserved neuromuscular junction) which corresponds with near complete preservation of alpha-motoneurons/interneurons, absence of glial activation/proliferation, silencing of mRNA of mutated human SOD1 and suppression of misfolded protein aggregation along the whole length of spinal cord. Moreover, the gene expression profile in subpially-treated $SOD1^{G37R}$ mice was normalized up to 89% when compared with non-treated and negative controls. Remarkably, subpial treatment after disease onset blocks progression of the disease and further alpha-motoneuron degeneration. Single cervical subpial injection in non-human primates produces homogeneous AAV9-mediated gene delivery with effective transduction of neuronal and no-neuronal cells, as well as axons throughout the whole cervical spinal cord (gray and white matter). **Conclusion:** This study demonstrates that spinal subpial delivery of shRNA-SOD1 silencing vector is highly potent in ameliorating mutated SOD1gene-induced ALS disease. This effect is achieved even if treatment is initiated in adult pre-symptomatic or symptomatic animals.

18. Early Diagnosis and Speed to Effect in Spinal Muscular Atrophy Type 1 (SMA1)

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Background: SMA1 is a rapidly progressing disease resulting in death or need for permanent ventilation by 2 years of age; early intervention with disease modifying treatment is critical. In the pivotal phase 3 nusinersen study (ENDEAR), ~10% of patients died/required permanent ventilation within 2 months after initiation of therapy (time required for 4 loading doses); 39% of patients died/required permanent ventilation by 6 months from dosing. This may reflect a non-immediate therapeutic impact related to the loading dose schedule. **Objectives:** Explore rapidity of therapeutic effect of onasemnogene abeparvovec (AVXS 101) gene-replacement therapy (CL-101 phase 1 study), as measured by early changes in Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) score, compared with the response to nusinersen in ENDEAR (≤5-point increase at 2 months post-dosing). **Approach:** SMA1 infants were treated with a one-time AVXS-101 dose (NCT02122952; cohort 2; N=12; 24 months follow up). Outcomes were event-free survival (EFS; CL-101: death or ≥16 hours ventilation/day for >2 weeks; ENDEAR [NCT02193074]: death, tracheostomy, or ≥16 hours ventilation/day for >21 days) and motor function improvements from baseline using CHOP-INTEND. **Results:** All 12 AVXS-101-treated patients showed EFS at study end, versus 49/80 (61%) nusinersen patients. At 1, 3 and ~10 months post-AVXS-101, CHOP-INTEND increased 9.8, 15.4, and 27 points. At 2 and ~10 months post-nusinersen initiation, CHOP-INTEND increased ≤5 and ~10 points. By 6 months, 11/12 (92%) AVXS-101-treated patients achieved CHOP-INTEND scores ≥40 versus 30/78 (38.5%) nusinersen-treated patients at last interim data-cut (day 183-394). **Conclusions:** AVXS-101 appears to improve survival and induce more rapid motor function improvements compared with nusinersen. Advances in understanding SMA underscore the need for early diagnosis and treatments with a near-immediate onset of action to maximize clinical improvements.

19. Preclinical Gene Therapy with scAAV9/AGA in Aspartylglucosaminuria Mice Provides Evidence for Clinical Translation

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Aspartylglucosaminuria (AGU) is an autosomal recessive inherited lysosomal storage disease caused by the loss of functional enzyme aspartylglucosaminidase (AGA), which results in the accumulation of AGA substrate (GlcNAc-Asn) in all tissues and body fluids. AGU patients have a slow but progressive neurodegenerative disease course characterized by intellectual disability, skeletal and motor abnormalities, and early mortality. There is no approved treatment for AGU patients. Due to the failure of bone marrow transplantation and the lack of feasibility of enzyme replacement therapy in this disease, gene therapy has become a reasonable and meaningful approach which

might provide a long term therapeutic benefit to AGU patients. To test our hypothesis that scAAV9/AGA gene therapy might provide a long-term therapeutic benefit, we carried out a comprehensive efficacy experiment in which $AGA^{-/-}$ (AGU) mice were administered intravenously (IV) or intrathecally (IT) either high ($1x10^{12}$ or $1x10^{11}$ vg/mouse for IV or IT, respectively) or low ($2x10^{11}$ or $2x10^{10}$ vg/mouse for IV or IT, respectively) doses of scAAV9/AGA at 6-months (early-symptomatic cohorts) or at 2-months (pre-symptomatic cohorts). Endpoints of the study included AGA activity and AGA substrate levels in tissues and body fluids, behavioral tests, and histopathology. In our 6-month-old early-symptomatic cohorts, scAAV9/AGA administration led to: 1) dose-dependent increase and sustained AGA activity to a supra physiological level in serum, cerebrospinal fluid (CSF), peripheral tissues (heart and liver) and central tissues (brain and cervical spinal cord); 2) dose-dependent elimination of GlcNAc-Asn substrate in serum, urine, CSF, liver and brain; 3) significantly restored movement in open field tests for high dose cohorts; 4) dose-dependent preservation of Purkinje cells in the cerebellum by HE staining; and 5) significantly reduced gliosis by glial fibrillary acidic protein (GFAP) staining. Our 2-month-old pre-symptomatic cohorts are still under analysis and accumulating data have shown very similar therapeutic benefit, further confirming the effectiveness of our scAAV9/AGA gene therapy in AGU mice. In parallel, a safety experiment in AGU mice was conducted to evaluate the toxicity of scAAV9/AGA at a supraphysiological dose ($1x10^{12}$ vg/mouse for IT, 10 times higher than our efficacious dose). Treated mice had no abnormal neurological symptoms and maintained body weight throughout the whole experiment. Taken together, these results demonstrate that treatment of AGU mice with scAAV9/AGA is both effective and safe, providing strong proof-of-concept evidence that scAAV9/AGA gene therapy should be considered for human translation.

20. Positive Cohort 1 Results from the Phase 1/2 Trial with AAV8-Mediated Liver-Directed Gene Therapy in Adults with Glycogen Storage Disease Type Ia

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Glycogen storage disease (GSD) represents a group of inherited metabolic disorders of glycogen synthesis and breakdown. GSDIa results from deficiency in the enzyme glucose 6-phosphatase (G6Pase), which is responsible for catalyzing the terminal step for both glycogenolysis and gluconeogenesis. Patients with GSDIa are unable to release glucose from glycogen stores into the circulation during periods of fasting, resulting in severe hypoglycemia. Shunting of glucose 6-phosphate into alternative pathways leads to hyperuricemia, hyperlactatemia, and hyperlipidemia. Additional complications include hepatic adenomas, hepatoceullar carcinoma, renal disease, and osteoporosis. Liver-directed gene therapy represents a novel treatment approach aimed at restoring G6Pase activity. DTX401 is an adeno-associated virus serotype 8 (AAV8) vector that expresses the human G6Pase gene (G6PC) under the transcriptional control of a liver specific promoter. The GSDIa phase 1/2 gene therapy study (NCT03517085) is a global, multi-center, open-label dose escalation trial evaluating the safety, tolerability, and efficacy of a single DTX401 IV infusion in adults with GSDIa. The study uses a continual reassessment method (CRM) to evaluate potential doses of 2.0 $x10^{12}$ Genome Copies (GC)/kg, 6.0 $x10^{12}$ GC/kg and 1.0 $x10^{13}$ GC/kg in cohorts of three patients. All three patients in Cohort 1 had null mutations and received a single IV dose of DTX401 at 2.0 $x10^{12}$ GC/kg. At week 12, all patients demonstrated a biologic response, reflected by an increase time to a hypoglycemia event (defined as glucose <60 mg/dL or onset of clinical symptoms) during a controlled-fasting challenge. Patients 1 and 2 had clinically meaningful improvements in time to hypoglycemia event of 103% and 120%, respectively, while Patient 3 showed an improvement of approximately 20%. In addition, all patients have been able to decrease their baseline total daily cornstarch use, with a mean decrease of 39% at Week 12 (**Table 1**). DTX401 was generally safe and well-tolerated. There were no infusion-related adverse events and no treatment-related serious adverse events reported. All adverse events were Grade 1 (mild) or 2 (moderate) and there were no instances of dose-limiting toxicity. Patients 1 and 2 had mild elevations in ALT, similar to previous observations in other programs using AAV-based gene therapy. Both patients were treated on an outpatient basis with a well-tolerated tapering regimen of corticosteroids. Data from Cohort 1 demonstrated that DTX401, an AAV8-mediated liver-directed vector expressing the hG6PC, had an acceptable safety profile and resulted in clear biological activity, as measured by an increase in time to hypoglycemia during a controlled fasting challenge and reduction in daily cornstarch use at week 12, compared to baseline. A Data Monitoring Committee (DMC) reviewed a minimum of 12 weeks of data for all subjects and recommended that it is safe to enroll subjects into Cohort 2 at a dose of 6.0 $x10^{12}$ GC/kg.

Key Efficacy Endpoints at Week 12 Compared to Baseline

Assessment	Time	Patient 1	Patient 2	Patient 3
Time to hypoglycemic event, hours	Baseline	3.8	4.1	5.4
	Week 12	7.7	9.0	6.5
	Percent change	+103%	+120%	+20%
Total daily cornstarch, grams	Baseline	405	171	285
	Week 12	160	165	138
	Percent change	-60%	-4%	-52%

21. Systemic AAV-Mediated Gamma-Sarcoglycan Therapy for Treatment of Muscle Deficits in LGMD2C Mice

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Background: Limb-girdle muscular dystrophy type 2C (LGMD2C) is caused by gamma-sarcoglycan (SGCG) deficiency. The sarcoglycans (α, β, γ, and δ-SG) 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). The lack of SGCG results in concomitant loss of other components of the DAPC leading to increased membrane permeability, myofiber degeneration, chronic inflammation, and elevated creatine kinase levels, among other events. Like the other sarcoglycanopathies, LGMD2C presents as a progressive muscular dystrophy starting in the girdle muscles before extending to lower and upper extremity muscles, and can also present in the diaphragm and cardiac muscles, resulting in respiratory and cardiac failure in a subset of LGMD2C patients. Presentation typically occurs in mid to late teens. To date, there is no form of therapy for LGMD2C. Given the small size of *SGCG*, we hypothesized that the *SGCG* gene would be efficiently packaged into an adeno-associated virus (AAV) vector and that gene replacement to diseased muscle would result in a therapeutic benefit in LGMD2C. The *sgcg*-/- mouse lacking SGCG has clinical-pathological features in muscle that replicate the human disease, making it an ideal model for translational studies. **Oobjective:** The goal of this study is to provide preclinical proof-of-principle for efficacy of AAV-mediated SGCG gene transfer in SGCG knock-out (*sgcg*-/-) mice. **Approach:** For treatment, we designed a self-complementary AAVrh74 vector containing a codon optimized human *SGCG* transgene driven by the muscle specific MHCK7 promoter that expresses well in cardiac and skeletal muscle (scAAVrh74.MHCK7.hSGCG). We next established efficacy of vector delivery by systemic injection through the tail vein of *sgcg*-/- mice to provide rationale for delivery in a clinical trial that would lead to clinically meaningful results. **Results:** Administration of scAAVrh74.MHCK7.hSGCG to *sgcg*-/- mice was effective in restoring SGCG expression in muscle fibers to near wild-type levels. IV delivery in all dosing cohorts resulted in widespread transgene expression in muscles throughout the hindlimbs, forelimbs, torso, and the heart. SGCG protein was correctly localized to the sarcolemma and was accompanied by a restoration of other components of the DAPC. This led to decreased creatine kinase activity along with improvements in histopathology, including reduction in central nucleation and increased fiber diameter. Finally, assessment of muscle physiology demonstrated increased specific force and resistance to contraction-induced injury, which indicates a functional benefit provided by IV delivery of scAAVrh74.MHCK7.hSGCG. **Conclusion:** Clinical delivery of a normal copy of the *sgcg* gene to diseased muscle in patients should allow for the production of functional wild-type protein, as well as, potentially, reversal of disease pathology, and an improvement in muscle function. The data from this pre-clinical study will be used in establishing a minimum effective dose for translation of AAV-mediated hSGCG gene transfer in LGMD2C patients.

22. Multiplex and Clonal Assessment of Production Yield of In Silico Designed AncAAVs

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With the continuing success of adeno-associated virus-based therapies in treating varied pathologies, both in promising preclinical models and now with accumulating in-human data, the need for reliable high-yield manufacturing has only grown. Why some projects that hit efficacy targets translate well to at-scale manufacturing, and others don't, is not well understood, and such shortfalls represent the focus of many process development groups. We know both the capsid and the genome can affect gross yield, and that some capsid serotypes are higher yielding than others. Here, we aimed at interrogating a complex library of capsid variant which were in silico designed to deconstruct the evolutionary lineage of AAV into putative evolutionary intermediates called AncAAVs. The 9 predicted nodes in the AAV phylogeny underlie AAV serotypes 1, 2, 3, 6, 7, 8 and 9, all of which are structurally and phenotypically distinct driving their various applications in the clinic. Libraries along these nodes were constructed to combinatorially vary residues on the viral capsid that discriminate these major AAV serotypes. Importantly, we were able to link the variants in these libraries to unique barcode identifiers through a method named CombiAAV and evaluate their performance using a multiplex Illumina-based readout via a methodology we name AAVSeq. We report production data on 37912 in silico AAV variants which are divergent 1-40% from known AAVs. Of these AAV designs, to date, 94% of those rationally designed AAVs, AAV production plasmids were generated using CombiAAV. Over 64% of the predicted variants were shown to generate genome-containing and DNAse protected virions with 100% of those demonstrating biological activity in vivo. For the 24524 variants that led to productive manufacturing in a HEK293-based transfection system, quantitative data was generated on yield. Furthermore, structural interference was performed mapping residues and motifs of importance to the vector yield. Analysis was further performed on viral supernatant versus overall lysate in order to determine determinants on the AAV capsid of release into the supernatant during production. Lastly, clonal validation of the multiplex screening was performed illustrating the power and limitations of AAVSeq for the interrogation of AAV yield in manufacturing processes.

Gene Therapy for Metabolic Disorders: New Approaches

23. sAAV-Mediated CPS1 Expression Rescues CPS1 Deficiency in Adult Conditional Knock Out Mice

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Throughout development and adulthood, the liver carries out a broad range of essential metabolic processes, including protein catabolism. Protein breakdown generates ammonia as a byproduct, which is detoxified primarily by the urea cycle. Carbamoyl phosphate synthetase 1 (CPS1) catalyzes the first committed, rate-limiting step of the urea cycle by condensing ammonia and bicarbonate into carbamoyl phosphate. Loss or dysfunction of CPS1 activity results in elevated plasma ammonia, aberrant serum amino acid levels, cerebral edema, and death if untreated. Current treatment for CPS1 deficiency consists primarily of dietary protein restriction, which is only marginally effective and leaves patients vulnerable to recurrent hyperammonemia and progressive, irreversible neurological decline. Liver transplantation is the only curative option but is limited by organ availability and the need for immune suppression. Gene addition of CPS1 is therefore an attractive alternative strategy for treating CPS1 deficiency but also presents unique challenges. CPS1 cDNA is 4.5kb which, when combined with other cis regulatory elements, exceeds the classical AAV genome capacity. Larger capacity lentiviruses and adenoviruses may accommodate CPS1, but issues remain with unwanted genomic integration and immunogenicity. To overcome these limitations, split AAVs (sAAVs) that divide the payload into two overlapping halves were explored. The separate halves are packaged into viruses individually and concatemerize via homologous recombination to reconstitute the transgenic payload after co-transduction of the same cells (Figure 1). To determine the ability of sAAVs to treat CPS1 deficiency, sAAVs encoding human codon optimized *CPS1* (hcoCPS1) driven by the constitutive CAG promoter were generated. These sAAVs successfully concatemerize and express hcoCPS1 mRNA and protein *in vitro*. To test sAAV efficacy *in vivo*, a conditional CPS1 knock out mouse model was used. These mice contain biallelic floxed *Cps1* which is removed by treatment with an AAV expressing Cre recombinase (AAV-Cre). Dose escalation studies showed that the minimum dose of sAAVs necessary to modestly extend lifespan in Cre-treated mice is $3x10^{14}$ gc/kg; therefore, a dose of $5x10^{14}$ gc/kg was chosen to study long-term survival. Floxed CPS1 mice injected with AAV-Cre and sAAVs showed increased lifespan (>30 days; $p<0.01$) and reduced plasma ammonia compared to controls that received AAV-Cre alone, all perishing by day 22 (treated: 339.6μM ± 94.5; untreated: 1349.9μM ± 379.6 [mean ± SD]; $p<0.01$). Over time we detected a slow decline in weight and rise in plasma ammonia, necessitating further intervention. Treatment with the small molecule n-carglumic acid, an analog of NAG, the allosteric activator of CPS1, further extended lifespan (all mice >120 days; $p<0.01$) and maintained near normal plasma ammonia (baseline: 138.87μM ± 86.5; post-treatment: 217.8μM ± 69.9; $p=0.15$). Immunohistochemical analysis demonstrated broad distribution of CPS1 throughout the liver parenchyma in sAAV-treated mice, while control mice showed only small loci of remaining expression inadequate to result in minimal necessary ureagenesis and survival. In conclusion, sAAV-mediated CPS1 expression extends lifespan, controls plasma ammonia, and maintains healthy weight and activity in a mouse model of this severe disorder of nitrogen metabolism.

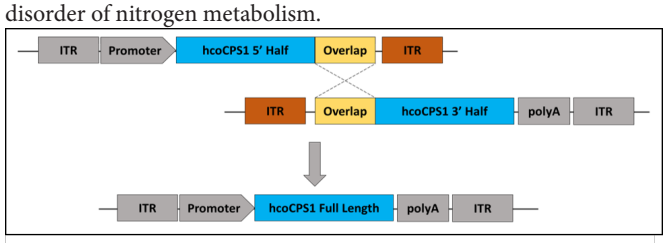

Figure 1. Schematic of sAAV concatemerization and reconstitution of full-length transgenic payload.

24. ImmTOR™ Tolerogenic Nanoparticles Enhance Transgene Expression after Both Initial and Repeat Dosing in a Mouse Model of Methylmalonic Acidemia Treated with an Anc80 AAV Vector

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One of the immunological barriers to systemic gene therapy in pediatric patients using adeno-associated virus (AAV)-based vectors stems from the inability to re-dose patients due to *de novo* formation of vector-induced neutralizing antibodies (Nabs). We have developed tolerogenic nanoparticles encapsulating rapamycin (ImmTOR™ or SVP-Rapamycin), which if co-administered with AAV-based vectors provide dose-dependent and long-term suppression of humoral and T cell responses against AAV, allowing for productive AAV vector re-dosing. Moreover, co-administration of liver-tropic AAV vectors and ImmTOR™ leads to an immediate increase in transgene expression even after the first dose. Here 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 rationally engineered AAV vector. MMA is a life-threatening autosomal recessive disorder most often caused by mutations in methylmalonyl-CoA mutase (*MUT*) gene, leading to accumulation of methylmalonic acid and resulting in life-threatening metabolic ketoacidosis. MMA is estimated to affect 1/50,000-1/100,000 children born in the US. MMA can be studied in hypomorphic $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice, which are deficient in the *Mut* gene, but are rescued from neonatal lethality by expression of the *Mut* gene in skeletal muscle under the control of muscle creatine kinase (MCK) promoter. These mice manifest key clinical and biochemical features of MMA, including growth retardation, susceptibility to dietary and environmental

stress, and highly elevated serum methylmalonic acid concentrations. Repeated co-administration of Anc80 and ImmTOR™ was well-tolerated and led to complete inhibition of IgG antibodies to Anc80 in wild-type and *Mut*$^{-/-}$;Tg$^{INS\text{-}MCK\text{-}Mut}$ mice. Separately, several Anc80-MUT expression cassettes were tested for their therapeutic activity in the *Mut*$^{-/-}$;Tg$^{INS\text{-}MCK\text{-}Mut}$ model with wild-type human *MUT* gene driven by a liver-specific promoter showing the highest therapeutic potential as indicated by weight gain and decrease in serum concentration of methylmalonic acid. A more profound and consistent decrease of serum methylmalonic acid after initial and repeat injections was observed in mice treated with the combination of ImmTOR™ and Anc80-MUT than in those treated with Anc80-MUT alone (and weight gains in the former were more consistent, especially after repeat dosing). Higher viral genome copy number per liver cell (vg/cell) as well as higher hepatic *MUT* mRNA expression levels were also seen in mice injected with the combination of ImmTOR™ and Anc80-MUT. These effects were dose-dependent, with higher doses of ImmTOR™ providing for higher vg/cell levels and lower plasma methylmalonic acid levels. In summary, the admixed combination of ImmTOR™ and Anc80-MUT is a promising approach to mitigate the detrimental impact of *de novo* formed Nabs on gene therapy for MMA, and may also provide a benefit in enhancing transgene expression at the initial dose.

25. Development of a Novel AAV-Based Therapy in Combination with Tolerogenic ImmTOR Nanoparticles for a Sustained Treatment of Ornithine Transcarbamylase Deficiency

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Ornithine TransCarbamylase deficiency (OTCd) is a monogenic, X-linked, urea cycle disease that affects 15,000-60,000 people worldwide. The disease results in accumulation of ammonia in the blood, which causes irreversible brain damage. Despite dietary restriction and ammonia scavenging drugs, many pediatric patients die early in life due to ammonia crisis and neurotoxicity.Our aim is to develop a novel therapeutic to treat OTC deficiency, which is based on Adeno-Associated virus (AAV)-based gene therapy in combination with biodegradable synthetic particles containing rapamycin (ImmTOR). ImmTOR has been recently shown to mitigate the formation of neutralizing anti-AAV antibodies and inhibit capsid-specific T cell responses in mice and non-human primates (Meliani et al., Nature Commun. 2018). This tolerogenic AAV strategy would have two potential benefits: 1) ability to treat pediatric patients with the possibility to re-dose to maintain therapeutic levels, and 2) prevent liver damage associated with cellular immune reaction to the therapeutic virus.We generated a novel therapeutic ssAAV8 vector containing a Codon-Optimized (CO) human OTC transgene under the transcriptional control of a liver specific promoter. In order to improve the safety of the therapeutic vector, potential enhancer sequences present between the ITRs and the therapeutic transgene were eliminated from the vector, which were shown to act as transcription activators in the liver. A dose finding experiments performed in the OTC-spf^{ash} mouse model demonstrated that the codon-optimized cassette is 5 times more efficient than wt OTC in expressing a catalytically active protein, resulting in restoration of the physiological levels of urinary orotic acid and serum ammonia. Thus, this AAV-hOTC vector mediates efficient and safe correction of OTC deficiency in OTC-spf^{ash} mice. ImmTOR particles have been shown to inhibit the formation of antibodies to a highly immunogenic therapeutic enzyme, pegadricase, in a phase 2 clinical trial. We are now performing pre-clinical studies to assess the safety and the efficacy of ImmTOR to block the humoral immune response to AAV-hOTC and enable repeated dosing of gene therapy treatments.

26. Treatment of Metabolic Disorders Using Lipid Nanoparticle (LNP)-Encapsulated Messenger RNA Therapeutics (MRT)

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Background: Monogenetic metabolic disorders represent a class of rare diseases which, in general, lack effective therapies, but represent excellent targets for messenger RNA therapeutics (MRT). Urea cycle disorders (UCDs), including ornithine transcarbamylase (OTC) deficiency and argininosuccinate synthetase (ASS1) deficiency (citrullinemia), are examples of such disorders. Another group of inborn errors of metabolism, categorically denoted as organic acidemias (OA), are equally severe and include methylmalonic acidemia (MMA), which is most often caused by a deficiency of the enzyme, methylmalonyl-CoA mutase (MUT). Patients affected by UCDs and OAs are difficult to treat, and despite careful medical management, experience high morbidity and mortality. For this reason, many centers offer elective liver transplantation as an experimental surgical treatment. Because the liver is the most important site of nitrogen and organic acid metabolism, we have developed and tested a platform of hepatotropic lipid nanoparticles (LNPs) designed to incorporate synthetic messenger RNAs (mRNAs) that code for the missing enzymes in prototypical intermediary metabolic disorders such as OTC, ASS1 and MUT deficiencies. *Results*: Messenger RNAs encoding hOTC, hASS1, or hMUT enzymes were synthesized, incorporated in LNPs, and formulated for IV administration. LNP-delivered *hOTC* mRNA (MRT5201) was demonstrated to be active by measuring human OTC protein and an accompanying increase in OTC activity within the livers of treated *Otc*$^{spf\text{-}ash}$ mice. MRT5201-treated *Otc*$^{spf\text{-}ash}$ mice were also subjected to an NH_4Cl challenge. The duration of action following a single IV dose of MRT5201 was 3 weeks for protection of hyperammonemia, and 4 weeks for lowering of urinary orotic acid. In a mouse model of citrullinemia (*Ass1*$^{fold/fold}$), efficacy was demonstrated via repeated normalization of plasma ammonia

after multiple doses of LNPs encapsulating *hASS1* mRNA. Similarly, IV administration of LNP-encapsulated *MUT* mRNA in a murine model of MMA ($Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$) resulted in reduction of plasma methylmalonic acid levels. *Conclusions*: Our results demonstrate that a functional mRNA encapsulated in an LNP can be successfully delivered to the liver after IV administration in a variety of preclinical metabolic disease mouse models. The LNP-encapsulated mRNAs were translated to functional proteins and attenuated, prevented or normalized the characteristic metabolic abnormalities seen in these three disorders. Our results support further studies to investigate the potential of mRNA therapeutics as possible treatments for UCDs and OAs, conditions that, as a group, lack effective therapies.

27. Liver-Directed Lipid Nanoparticle mRNA Therapy Improves Survival and Reduces Serum Branched Chain Amino Acids in a Mouse Model of Maple Syrup Urine Disease

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Maple syrup urine disease (MSUD) is a rare metabolic disorder caused by mutations in one of the three genes encoding a multi-subunit, mitochondrial enzyme complex called branched-chain alpha-keto acid dehydrogenase (BCKDH). BCKDH deficiency is characterized by a buildup of branched chain amino acids (BCAAs) and their byproducts (alpha-keto acids) in peripheral circulation and in the brain. Excretion of keto acids gives the urine a distinctive sweet odor leading to the name of the disease. Currently, no cure exists for MSUD and treatment options are limited to carefully monitoring a restricted diet with the potential for liver transplantation, highlighting the unmet medical need to develop a novel therapeutic approach for this disease. The intermediate or hypomorphic MSUD mouse is deficient in the mouse E2 subunit of BCKDH but has low levels of expression of human E2, which is required to rescue the neonatal lethality seen in the classic mouse E2 knockout. Hypomorphic MSUD mice exhibit decreased survival beginning at weaning and display elevated BCAA levels reminiscent of MSUD patients. Here, we evaluated the use of liver-targeted lipid nanoparticles (LNPs) to deliver mRNA encoding either the E2 subunit of BCKDH or three of the BCKDH subunits (E1α/E1β/E2). We performed weekly intravenous administration of LNPs starting at the time of weaning in hypomorphic MSUD mice. Weekly dosing of either the E2 LNP or the combined E1α/E1β/E2 LNP significantly increased survival compared to the control groups (weekly administration of either green fluorescent protein LNP or phosphate buffered saline). Serum BCAA levels were reduced following administration of either E2 LNP or the combined E1α/E1β/E2 LNP relative to control hypomorphic MSUD mice, suggesting that the improved survival following LNP treatment is due to a reversal of the elevated peripheral BCAA levels observed in MSUD. Therefore, liver-directed LNP mRNA therapy may be an efficacious path forward for managing and treating MSUD patients.

28. Salmeterol with Liver Depot Gene Therapy Reversed Biochemical and Autophagic Abnormalities of Skeletal Muscle in Pompe Disease

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Gene therapy for Pompe disease with adeno-associated virus (AAV) vectors has advanced into early phase clinical trials. However, the paucity of cation-independent mannose-6-phosphate receptor (CI-MPR) in skeletal muscle, where it is needed to take up acid α-glucosidase (GAA), has impeded the efficacy of Pompe disease gene therapy. Long-acting selective β2 receptor agonists previously enhanced the CI-MPR expression in muscle, which increased the receptor-mediated uptake of GAA. In this study we have evaluated the selective β2 agonist salmeterol in GAA knockout (KO) mice in combination with an adeno-associated virus (AAV) vector expressing human GAA specifically in the liver. Previously salmeterol was evaluated with an AAV vector that expressed GAA ubiquitously with a chicken β-actin promoter and cytomegalovirus enhancer, and biochemical correction was improved by salmeterol administration only in the heart. That earlier study failed to demonstrate biochemical correction of skeletal muscle. Ubiquitous expression has provoked immune responses in GAA-KO mice, emphasizing the importance of immune tolerance induction during gene therapy in Pompe disease that can be achieved by liver-specific expression of GAA with an AAV vector. In the current study the glycogen content of the quadriceps was significantly decreased by administration of the liver-expressing AAV vector in combination with salmeterol, in comparison with the AAV vector alone ($p<0.01$). Importantly, glycogen content of the quadriceps was reduced to its lowest level by the combination of AAV vector and salmeterol administration. Male mice demonstrated lower glycogen content from combination treatment in the diaphragm, in comparison to gene therapy alone. Additionally, male mice had significantly decreased glycogen content in every muscle examined following combination therapy, in comparison with female mice. Thus, male gender consistently improved the response to treatment with salmeterol plus liver gene therapy. Rotarod testing revealed significant improvement following treatment with salmeterol, the AAV vector, or both treatments combined, in comparison with untreated mice. Salmeterol treatment improved wirehang performance, in comparison with untreated mice. Salmeterol treatment decreased abnormalities of autophagy in the quadriceps, as shown be lower LC3 and p62 either with or without concurrent gene therapy. Vector administration reduced the abnormal vacuolization and accumulation of nuclei in skeletal muscle, which correlated with improved autophagy in mice treated with both salmeterol and gene therapy. Given its benefits, salmeterol should be further developed as adjunctive therapy to improve the efficacy of liver depot gene therapy for Pompe disease.

29. Correction of Glycogen Storage Disease Type III with an AAV Vector Encoding a Bacterial Glycogen Debranching Enzyme

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Background: Deficiency of glycogen debranching enzyme (GDE) in glycogen storage disease III (GSD III) results in accumulation of abnormal glycogen with short outer branches (limit dextrin) in multiple tissues. Most patients have both muscle and liver involvements (type IIIa) while others have only liver involvement (type IIIb). There is no treatment for this disease. Adeno-associated virus (AAV) vector has emerged as the most effective tool for in vivo gene delivery. However, the small carrying capacity (<4.7 kb) limits its application to deliver a large therapeutic gene like human GDE. In this proof-of-concept study, we aimed to test the feasibility of treating GSD III with an AAV vector expressing a bacterial GDE (type I Pullulanase from *Bacillus subtilis*) in a mouse disease model (*Agl*-KO) and to evaluate whether the anticipated cellular immune response against the bacterial enzyme can be prevented by a tissue-restricted gene expression manner. **Methods:** Heterozygous Agl^{Tm1a} mice carrying a mutant *Agl* allele were purchased from the European Mouse Mutant Archive and crossed with the CMV-Cre mice (Jackson Laboratory) to generate the *Agl* knockout (*Agl*-KO) allele by deleting the exons 6-10. The homozygous *Agl*-KO mice exhibit widespread glycogen accumulation in the liver and all the muscle tissues. The codons in the sequence of the bacterial enzyme (Pullulanase derived from *Bacillus subtilis* strain 16, 2.2 kb) were optimized for the best expression in human cells. Then, the optimized sequence was cloned into an AAV vector containing either the universally active CMV enhancer/chicken β-actin promoter (AAV-CB-Pull) or a liver-specific promoter (AAV-LSP-Pull) and these vectors were packaged as AAV9. Ten-week-old *Agl*-KO mice were intravenously injected with the AAV9-CB-Pull or the AAV9-LSP-Pull vector at a dose of $5x10^{12}$ vg/kg and collected tissues after two weeks for analysis of biochemical and histological corrections. Age-matched untreated *Agl*-KO mice were used as controls. **Results:** Both AAV treatments dramatically reduced glycogen content in the liver by >90% compared to the untreated control (Table 1). Light microscopy of periodic acid-Schiff (PAS)-stained liver sections confirmed the reduction of glycogen accumulation and the restoration of normal hepatic morphology (not shown). Multiple $CD4^+$ and $CD8^+$ lymphocytic infiltrates were present in the AAV-CB-Pull treated liver but barely detectable in the AAV-LSP-Pull treated liver (Figure 1). These results suggest that the cellular immunity against Pullulanase expressed from the CB promoter was associated with waning Pullulanase levels in the liver. No significant increase of enzyme activities and a decrease of glycogen contents were observed in the heart and skeletal muscle (quadriceps) by either AAV treatment. **Summary:** Our data suggest that gene therapy with an AAV vector expressing bacterial Pullulanase is a possible treatment approach for GSD III and the Pullulanase-induced cellular immune response can be overcome by tissue-restricted gene expression. Future study will test the efficacy of this treatment in the muscle of *Agl*-KO mice using a muscle-specific promoter to drive Pullulanase expression.

Table 1. Biochemical correction of glycogen storage in *Agl*-KO mice by AAV treatment

		Liver	Heart	Quad
Pullanase activity (mU/mg protein)	*UT*	0.764±0.083	2.722±0.304	0.844±0.120
	CB	9.514±2.099	4.024±1.045	1.196±0.416
	LSP	28.305±3.039	2.382±0.228	1.048±0.240
Glycogen content (μmol glucose/g tissue)	*UT*	158.124±6.530	39.240±3.670	77.362±11.851
	CB	12.862±8.889	31.948±2.732	64.379±12.234
	LSP	9.181±3.880	33.670±6.040	83.972±8.322
AAV copy number per genome	*CB*	72.669±10.789	0.612±0.147	0.133±0.139
	LSP	46.593±8.274	0.951±0.264	0.276±0.043

Data represent as mean ± SD, n=5. Abbreviations: Quad, quadriceps; UT, untreated *Agl*-KO mice; CB, AAV9-CB-Pull treated *Agl*-KO mice; LSP, AAV9-LSP-Pull treated *Agl*-KO mice

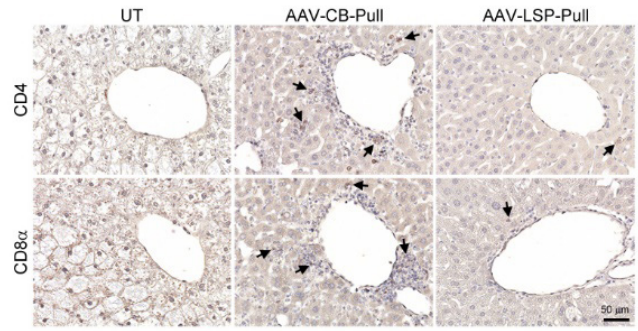

Figure 1. Immunohistochemical detection of CD8+ and CD4+ lymphocytes in the liver two weeks following AAV administration. Sections of the paraffin-embedded livers from untreated (UT), AAV9-CB-Pull treated, and AAV9-LSP-Pull treated *Agl*-KO mice were stained with an anti-CD4 or anti-CD8α monoclonal antibody (Abcam). Foci of lymphocytes are indicated (arrows).

30. Genome Editing with CRISPR/Cas9 in a GSD Ia Canine Model

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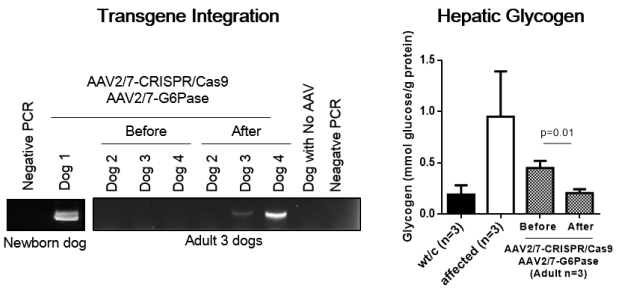
Glycogen storage disease type Ia (GSD Ia) is a rare inherited disease caused by mutations in the *G6PC* gene, which encodes glucose-6-phosphatase (G6Pase). Absence of G6Pase causes life-threatening hypoglycemia and long-term complications including renal failure, nephrolithiasis, hepatocellular adenomas (HCA), and a significant risk for hepatocellular carcinoma (HCC). The complications occur due to the accumulations of metabolic intermediates including glycogen and triglycerides in the liver, kidney, and small intestine. The canine GSD Ia model mimics the human disease more accurately than mouse models, given the longer lifespan and outbred genetics of dogs. AAV vectors have been developed for treatment of GSD Ia and shown effective at correcting hypoglycemia and greatly prolonging lifespan; however, these vectors have not prevented all long-term complications. AAV vector genomes remain almost exclusively in an episomal state in the cells, and therefore AAV derived transgene expression has diminished over time. To address this problem, we designed a CRISPR/Cas9 that cleaves the *G6PC* exon 1/intron 1 boundary and delivers a repair template to induce homologous recombination (HR) and to integrate a functional *G6PC* gene. We delivered two recombinant AAV7 vectors, the nuclease-containing AAV-CRISPR/Cas9 and the donor AAV-*G6PC*, to dogs with GSD Ia including a puppy on day one of life and to 3 adult dogs previously treated with gene replacement at the age of 3 years. The effect of genome editing by CRISPR/Cas9 was analyzed in

liver biopsies at 4 months after vector administration. Analysis of liver biopsies from the puppy and two out of 3 adult dogs revealed transgene integration in the *G6PC* locus. The puppy demonstrated higher vector genome copies (1.3 copies per cell) than two adult dogs (0.5 and 0.3 copies per cell). However, these two adult dogs had significantly increased vector genomes in the liver following genome editing, because vector genomes were undetectable at baseline. Furthermore, hepatic glycogen content was reduced after genome editing in two of 3 adult dogs. To demonstrate long-term efficacy of genome editing, we will perform repeat liver biopsies at 14 months following CRISPR/Cas9 administration. These data suggest that administration of AAV vectors early in life achieved a higher efficiency of genome editing by CRISPR/Cas9, in comparison with treatment of adult dogs. However, we confirmed that genome editing by CRISPR/Cas9 is possible in adult dogs. Further development of genome editing is warranted to provide a more stable treatment and to more effectively prevent long-term complications of GSD Ia.



Tools, Delivery and Neuro Capsids

31. A Safe and Reliable Technique for Central Nervous System Delivery of AAV Vectors via the Cisterna Magna

T. Taghian, M. G. Marosfoi, O. I. Cataltepe, AS Puri, D. Fernau, AR Batista, N. Aronin, R. M. King, M. Gounis, T. R. Flotte, D. McKenna-Yasek, P. W. L. Tai, M. Sena-Esteves, H. L. Gray-Edwards

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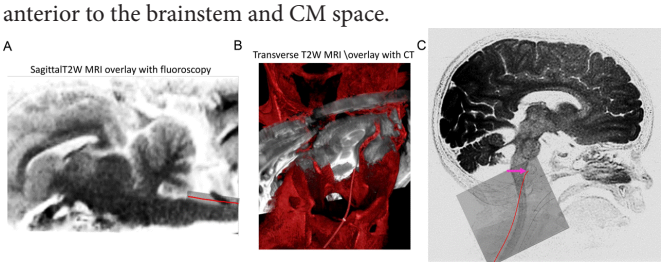
Tay-Sachs and Sandhoff disease are fatal neurodegenerative diseases that are characterized by accumulation of GM2 gangliosides in the central nervous system (CNS), resulting in severe neurodegeneration. Infantile forms are marked by a prolonged vegetative state with death occurring between 3 and 5 years of age, while juvenile onset patients survive into the second decade of life. Currently, there is no treatment for these diseases. GM2 accumulation is caused by a deficiency in Hexosaminidase A (HexA), due to mutations in genes encoding for α or β subunits that form the heterodimeric HexA enzyme. We have demonstrated efficacy of AAV gene therapy in mouse, cat and sheep models of GM2 gangliosidoses, however, efficacy is dependent on vector distribution in the brain and spinal cord. Here we report a minimally invasive technique for delivery of AAV vectors to the CNS. Cerebrospinal fluid (CSF) delivery of AAV vectors via the cisterna magna (CM) has shown efficacy in animal models of lysosomal storage diseases, but is contraindicated in patients due to increased risk of fatal mistargeting. To circumvent this limitation, we developed a safe CM delivery method in sheep and applied it to a child receiving AAV gene therapy. An intravascular microcatheter was inserted in the lumbar intrathecal space and threaded to the CM under fluoroscopic guidance. Pre-surgical MRI was merged with cone beam computed tomography to assist in catheter navigation of the spinal canal. VasoCT was acquired to confirm the final catheter position in the CM. By injecting 1ml of iodinated contrast through the microcatheter the pattern of contrast material distribution was recorded prior to vector injection. After removal of 7-10 mL CSF, 1E14 vg scAAV-CB-GFP-pI vector (in 15 mL) was infused at 1 mL/min. All sheep recovered well with no complications. Animals were sacrificed 3 weeks post injection (n=3) and no spinal cord damage was observed post-mortem. Histological evaluation of the tissue showed robust GFP expression throughout the spinal cord. Additionally, the cerebellum, hippocampus, thalamus, brain stem, striatum and frontal lobe were strongly transduced. After testing in eight sheep, this technique was then used to administer 1E14 vg of a 1:1 formulation of two monocistronic AAVrh8 vectors encoding the α and β subunits of HexA to a 30-month-old child with Tay-Sachs disease. A total of 14 mL of CSF was removed by passive flow and was followed by vector administration in a total of 12 mL at ~1 mL/min, 9 mL into the CM and 3 mL at the L2 level. Intracranial opening pressure was 42 mm H_2O, which was >2-fold above normal (~18 mm H_2O), and decreased to 21 mm H_2O after removal of CSF, and remained unchanged with AAV delivery. No adverse effects were noted from the infusion procedure, or otherwise. Therefore, this novel delivery technique is a safe, and highly effective method for increasing gene delivery to the brain via the CSF. *Authors contributed equally to this work. **Figure 1: A**. Overlay of MRI and fluoroscopy images in the sheep. Catheter location in the spinal canal in the CM space. **B.** CT overlay with MRI to show catheter location caudal to the cerebellum and dorsal to the brainstem. **C.** Overlay of fluoroscopy images and T2w MRI of a child that received AAV injection. The catheter (red) is anterior to the brainstem and CM space.



32. Highly Efficient Transduction of the Fovea Following 'Extrafoveal' Subretinal Administration of Novel AAV Vectors

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Purpose: Gene therapy for many retinal dystrophies requires targeting of transgene expression to foveal cone photoreceptors (PRs), the cells responsible for acute, daylight vision. In clinical trials for RPE65-LCA2, subretinal injection (SRI) of AAV under the fovea led to central retinal thinning and loss of visual acuity in some patients. To more safely target cones, intravitreal injection (IVI) has been explored. However, studies utilizing currently available capsids indicate that the barriers to IVI AAV (i.e. dilution/neutralization in vitreous, inner limiting membrane) may restrict transduction to 'sub-therapeutic' levels in a substantial proportion of patients. As such, alternative strategies that mediate efficient cone transduction without adversely affecting the retina remains an unmet need. We previously showed that several novel capsids display high transduction efficiencies that manifest, in part, from expansion of transduction outside of the limits of SRI blebs. These include an AAV2-based capsid containing 4 proteosomal avoidance (Y-F, T-V) mutations and substitutions to canonical HSPG binding residues- AAV2(4pMutΔHS), 2) the novel naturally occurring capsid, AAV44.9, and 3) a rationally designed version of the latter, AAV44.9(E531D). The purpose of this study was to evaluate the tropism of these novel capsids, and whether they promoted transduction beyond the area of detached retina (bleb) in a more clinically relevant, foveated species- cynomolgous macaque. **Methods:** AAV constructs containing CBA or hGRK1 promoters driving GFP were packaged into AAV44.9, AAV44.9(E531D), AAV2(4pMutΔHS), or AAV5. Seven macaques were SRI with vectors, each at 1e12 vg/ml. In 8 eyes, two 60μL blebs were made (subfoveal, and peripheral). In 6 eyes, three 30μL extrafoveal blebs were created. Ophthalmic examinations, optical coherence tomography (OCT), and confocal scanning laser ophthalmoscopy (cSLO) were performed at regular intervals. Care was taken to document the location and extent of blebs immediately post injection (p.i.) and then to follow transgene expression over time. At 6 weeks p.i., retinal sections were stained for cone arrestin. Rod/cone transduction was quantified within and outside the blebs. **Results:** Intentional subfoveal delivery of all vectors drove highly efficient GFP expression within foveal PRs. Loss of ellipsoid zone and foveal bulge on OCT were apparent within these eyes, with partial or complete recovery through study termination. Extrafoveal injection produced no OCT changes within the fovea of any eyes. GFP fluorescence extending beyond SRI bleb margins was apparent at 2 weeks p.i. with novel vectors, but not AAV5. Spread continued until study termination. GFP intensity mediated by all vectors increased through week 6. Extrafoveal injection of novel vectors led to transduction of up to 98% of foveal cones. **Conclusion:** AAV44.9, AAV44.9(E531D), AAV2(4pMutΔHS) exhibit increased PR/RPE transduction and enhanced lateral spread in SRI injected macaque relative to benchmark vector AAV5. Notably, they transduce foveal cones without the requirement of foveal detachment. These capsids will be useful for treating diseases where central retinal cones are the target, but detachment of the fovea is not preferred (i.e. Achromatopsia, Ushers syndrome). They may also be used to address IRDs that would benefit from an expansive treatment area (i.e. retinitis pigmentosa, choroideremia).

33. Cell Specific Transduction of a Vectorized Anti-Tau Antibody 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). However, for such an approach to be effective, delivery of antibody to specific cell types within affected or vulnerable brain regions may need to be achieved. We have previously demonstrated broad distribution and expression of a vectorized anti-tau antibody in the mouse brain using a novel blood brain barrier penetrant AAV capsid administered intravenously (IV) with a ubiquitous promoter. Here we describe studies characterizing different vectorized anti-tau antibody cassettes designed to optimize distribution and expression in specific cell types, using cell type specific promoters with a blood brain penetrant AAV capsid in mice. AAV vectors comprising a novel capsid and a transgene encoding an anti-tau monoclonal antibody were administered by IV bolus to wild type mice and one month later, gene transfer was assessed. 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 vectorized antibody in the mouse CNS was observed, including regions such as hippocampus, cortex, thalamus, brain stem and olfactory bulb. Furthermore, cell type specific expression of antibody was achieved using cell specific promoters. Importantly, brain levels of anti-tau antibody substantially higher than those measured after passive immunization could be achieved. Taken together, these results demonstrate improved vectorized antibody expression with specific AAV constructs and suggest that IV dosing of vectorized antibody using a blood brain barrier penetrant AAV capsid can deliver therapeutically relevant antibody levels in specific cell types in multiple brain regions.

34. mGAP: A Resource to Identify Nonhuman Primate Models of Human Genetic Diseases

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The Oregon National Primate Research Center manages more than 4,000 captive bred, Indian-origin rhesus macaque colony, current members of a pedigree spanning 9 generations. With over 1,000 macaques genomically sequenced, we have identified nearly 19 million sequence variants. Analysis of the variants identified 2,807 alleles that are identical to SNVs associated with human disease (ClinVar) and 24,237 SNVs predicted to be damaging to protein structure and likely to be pathogenic (PolyPhen, SnpEff). The variants implicate potential models of vision loss (Retinitis Pigementosa, Leber congenital amaurosis, Bardet-Biedl Syndrome), neurodegenerative disease (Neuronal Ceroid Lipofuscinosis, Leukoencephalopathy with ataxia or vanishing white matter), developmental disorders (Meckle-Gruber Syndrome, Caffey Syndrome), metabolic disorders (Glutaric Aciduria), respiratory disease (Cystic Fibrosis), dystonia (childhood onset with optic atrophy), among others. Rhesus macaques can be searched based on genotype, pinpointing subjects for physical examination or for targeted breeding to produce additional subjects for study. Complementing the genomic data is a database of life-long electronic health records (EHR), summarizing clinical, behavioral, imaging, histology and pathology data collected on each subject. Combining both genomic and phenotypic data sets, we are uncovering novel models of rare disease, including Bardet-Biedl Syndrome *(BBS7)* and Neuronal Ceroid Lipofuscinosis *(CLN7)*. These two models are currently being leveraged for biomarker development, gene-therapy and gene correction studies. The mGAP database (https://mgap.ohsu.edu/) provides an unprecedented resource to identify natural primate models of rare human diseases. The expansive genomic data sets, clinical data, combined with the onsite MRI and PET imaging facilities, veterinary clinicians, surgeons, and pathologists, and investigators with expertise in gene therapy, stem cell therapy and other state-of-the-art technologies, provide outstanding opportunity to leverage nonhuman primate models to develop precision medicine approaches for the treatment of debilitating rare human diseases. Additional macaque genomic and phenotypic data sets will be added to the mGAP database over the next two years. Disease model discovery will also continue using interdisciplinary approaches to validate new models, and as appropriate, model populations will be expanded through the targeted breeding of genetically characterized subjects.

35. Intraparenchymal Spinal Cord Delivery of AAV Gene Therapy Provides Robust SOD1 Knockdown in Large Mammal Spinal Cord for the Treatment of SOD1-ALS

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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. Delivery of AAV vectors to motor neurons along the spinal cord of large mammals is a key translational step for successful therapy. Here, we report the results from a series of in vitro and in vivo studies to select an AAV gene therapy targeting SOD1 with RNAi, and from studies in large mammals to optimize delivery paradigms to motor neurons throughout the rostral-caudal extent of the spinal cord. To select a RNAi sequence targeting human SOD1 (hSOD1), we identified 150+ sequences that were predicted to be highly selective for hSOD1. Synthetic siRNA duplexes were first screened in Hela cells. The most effective siRNAs were then compared using dose-response curves for SOD1 mRNA suppression in cultured cells. The most potent RNAi sequences were cloned into different pri-miRNA cassettes, then screened in human cell lines for SOD1 mRNA suppression. The best candidates were used to generate AAV vectors for in vivo studies in transgenic mice expressing human wild-type SOD1. We employed intrastriatal dosing as a surrogate route of administration to evaluate SOD1 mRNA suppression by RT-qPCR, and to assess precision and efficiency of miRNA processing with deep sequencing. The top pri-miRNA cassette/RNAi sequence was selected for further evaluation in pigs using intraparenchymal delivery of AAV vectors to the spinal cord. Significant SOD1 knockdown was observed 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. Suppression of SOD1 in motor neurons was also assessed by in situ hybridization. SOD1 protein reduction was analyzed by ELISA and LC-MS/MS assays. Notably, the identified lead substantially and safely suppressed SOD1 in motor neurons in cervical levels of the spinal cord, critical for respiratory function. Our findings support the use of AAV gene therapy targeting SOD1 with RNAi as a potential approach for the treatment of SOD1-ALS with an intraparenchymal spinal cord delivery paradigm.

36. Intrathecal and Intravenous Combination Gene Therapy in the Mouse Model of Infantile Neuronal Ceroid Lipofuscinosis Extends Lifespan and Improves Behavioral Outcomes in Moderately Affected Mice

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Infantile neuronal ceroid lipofuscinosis (INCL), also known as infantile Batten or CLN1 disease, is a severe neurodegenerative lysosomal storage disorder affecting the CNS and peripheral organs. The INCL mouse model (*CLN1*-KO) recapitulates the major features of the disease, with neurological deficits appearing at 4.5 months and a median survival of 8 months of age.

We explored dosing 1 month old "presymptomatic" mice and 6 month old "symptomatic" mice, with a single intrathecal (IT) injection of $7x10^{10}$ vg in the lumbar cistern of a self-complementary adeno-associated virus serotype 9 carrying the human CLN1 gene (scAAV9-hCLN1). Our results showed significantly increased survival when treating at 1 month, but no apparent benefits when treating at 6 months. We next explored the treatment at different ages (representing the different stages of the disease) and higher doses. We were able to establish that: 1) early treatment is better; and 2) a higher dose is better. Only modest survival effects were seen with the high IT dose (IT-HD, $7x10^{11}$ vg) administered at 6 months. In contrast, treatment with the IT-HD at 4.5 months (early symptomatic) showed significant survival benefits, delayed onset of symptoms, and improved behavioral performance. We next explored combining the IT dose with an intravenous (IV) dose, to increase the efficacy in these symptomatic mice. Administering scAAV9-hCLN1 IV in conjunction with IT delivery allows for dose escalation and potentially transduces regions of the brain not adequately treated by IT alone. A low dose IT along with a low, medium, or high IV dose did not provide greater therapeutic efficacy over the IT-HD alone in early symptomatic mice. However, treatment with an IT-HD and IV-HD combination provided significant benefits in this same age cohort (median lifespan of 17 months with function benefits). Our preliminary results in mice suggest the possibility of treating moderately affected patients. Ongoing studies are further evaluating the benefit of combined IV+IT dosing in presymptomatic animals. Data from a GLP toxicology study in rats assessing the safety of scAAV9-hCLN1 will also be presented.

Gene Therapy in Large Animal Models

37. Safety and Biodistribution Assessment of Scaav2.5-Eqil-1RA Gene Transfer to a Large Mammalian Joint

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Osteoarthritis (OA) is a chronic and incurable joint disease. Although highly debilitating, OA is not life-threatening, thus viable treatment options must be both safe and efficacious. Adeno-associated virus (AAV) offers many advantages as a gene delivery vehicle for OA treatment, particularly with regard to safety. As part of a 3-year efficacy study, we performed formal pre-clinical biodistribution studies in an equine system to establish a biosafety profile for self-complimentary (sc)AAV Interleukin-1 Receptor Antagonist (IL-1Ra) delivery in large mammalian joints. Due to similarities in size and tissue composition, the equine joint was used to model the behavior of delivery into a large human joint, such as the knee. To simulate human treatment conditions, we performed studies in the context of an OA osteochondral fragmentation (OCF) disease model. Biodistribution and expression profiles addressed two treatment phases: A) *Acute*- to detect pathologies associated with *vector administration* and systemic dispersion immediately following joint injection. For this, 6 animals were divided into two groups: i) treatment with scAAV.eqIL-1Ra at 1 $x10^{13}$ vg (*1x dose, clinical dose*), or ii) treatment with $1x10^{14}$ vg (*10x dose, "worst-case scenario"*) into the OCF joint. Two weeks post-injection, animals were euthanized for necropsy. B) *Long-term*- 12-month trial to determine efficacy and detect pathologies associated with *sustained local over-production of IL-1Ra and long-term vector persistence*. For this, the OCF joints of 10 animals received the 1x dose and 10 received saline control. At the conclusion, horses were euthanized for necropsy. For both phases, synovial fluid, blood and urine were collected throughout and analyzed for gene expression. Treated long-term animals from the efficacy trial had sustained stable eqIL-1Ra expression (~40ng/ml) for 12 months. There was no expression in contralateral joints, control animal joints or urine. Similar to previous results, we saw increases in eqIL-1Ra expression in blood in 5 untreated and 2 treated animals, which we attribute to endogenous production from causes unrelated to the transgene. Biodistribution studies demonstrated persistent vector genomes (vg) in injected cartilage and synovium for up to one year. Similar to long-term, Acute animals maintained transgene expression over the 2 weeks study. One 10x animal had increased expression in contralateral, uninjected synovial fluid. Interestingly, this animal was the only animal to have positive vg detection in contralateral cartilage and synovial tissues. Outside of the injected joint space, vg were only found in the spleen of the Acute 10x treatment group. Furthermore, only one Acute animal receiving the 10x dose had a significant increase in circulating antibodies (~525-fold increase in synovial fluid from the injected joint and ~230-fold increase in serum) to the vector capsid. No other animals had an increase in circulating anti-AAV2.5 antibodies. Overall the biosafety profiles generated were highly favorable and

demonstrate that at clinical doses, for both short and long-term stages of treatment, vector remained localized to the injection site. Further efficacy analysis is currently underway.

38. Gene Editing Restores Dystrophin Expression in a Canine Model of Duchenne Muscular Dystrophy

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Mutations in the gene encoding dystrophin, a protein that maintains muscle integrity and function, cause Duchenne muscular dystrophy (DMD). The deltaE50-MD dog model of DMD harbors a mutation corresponding to a mutational "hot spot" in the human *DMD*gene. We used adeno-associated viruses to deliver CRISPR gene editing components to four dogs and examined dystrophin protein expression 6 weeks after intramuscular delivery (n=2) or 8 weeks after systemic delivery (n=2). After systemic delivery in skeletal muscle, dystrophin was restored to levels ranging from 3 to 90% of normal, depending on muscle type. In cardiac muscle, dystrophin levels in the dog receiving the highest dose reached 92% of normal. The treated dogs also showed improved muscle histology. These large animal data support the idea that, with further development, gene editing approaches may prove clinically useful for the treatment of DMD.

39. SGT-001 Cardiac and Skeletal Muscle Microdystrophin Expression and Functional Efficacy in Preclinical Models of DMD

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Duchenne muscular dystrophy (DMD) is a severe muscle disorder caused by loss-of-function mutations in the DMD gene, which lead to the absence of the dystrophin protein. Without dystrophin, the structural link between the actin cytoskeleton and extracellular matrix is broken and critical signaling proteins are pathologically mislocalized. As a result, muscles are highly susceptible to contraction-induced damage and functional ischemia. Direct dystrophin replacement has thus far been unsuccessful due to the large size of the DMD gene. As a wide variety of mutations can be responsible for the disease, therapeutic strategies aimed at specific mutations only have the potential to benefit subsets of patients. SGT-001 is an adeno-associated virus (AAV) microdystrophin gene transfer candidate being evaluated by Solid Biosciences in a Phase I/II clinical trial, IGNITE DMD. The program is based on extensive research on the dystrophin protein to develop shorter yet functional microdystrophin variants, and to deliver genes to muscle. Significant preclinical work has been conducted to characterize SGT-001 in dystrophic mouse and dog models to evaluate microdystrophin expression and functional efficacy following systemic administration. Preclinical data show that a single dose of SGT-001 leads to robust long-term microdystrophin expression in cardiac and skeletal muscle. Importantly, SGT-001 microdystrophin successfully re-establishes the dystrophin glycoprotein complex and recruits nNOS to the sarcolemma, where it is functionally active. In dystrophic dog studies carried out for over two and a half years, data show that this response is sustained throughout the entirety of the study. Quantification of microdystrophin using immunofluorescence, Western blotting and mass spectrometry identified a dose-response increase in percent positive muscle and overall protein levels. In addition, in vivo and in vitro functional assessments demonstrated improved muscle function in a similar dose-dependent manner. Further studies are being conducted in dystrophic animals to assess critical components of microdystrophin function. These results will help improve our understanding of key aspects of DMD biology and advance SGT-001 as a potential therapeutic option for DMD patients, regardless of mutation.

40. In Vivo Correction of Dystrophin Expression in Old Dystrophic Dogs

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In vivo CRISPR/Cas9-mediated gene editing has emerged as a promising novel approach for correcting dystrophin expression following recombinant adeno-associated viral (rAAV) vector delivery into animal models of Duchenne muscular dystrophy (DMD). However, the most successful reports to date have focused on initiating treatment at very young ages where disease progression has not yet resulted in significant fibrosis and muscle wasting, ensuring optimal conditions for both AAV transduction and myonuclear gene editing. Furthermore, the use of young mice in particular facilitates delivery of very large doses of rAAV vectors, enabling near saturation of systemically targeted muscle groups for optimal experimental outcomes. These doses are not likely to be feasible in clinical trials and translation of results obtained from treatment of very young mice may significantly differ from those seen in older patients. Larger animal models of DMD, such as the CXMD canine model, are invaluable for the translation of experimental therapies into clinical application due to their closer resemblance to patients in terms of dosing, immunology and pathophysiology. Here we demonstrate successful muscle-specific correction of dystrophin expression in aged CXMD dogs following local rAAV6 vector delivery of CRISPR/Cas9 and offer a qualitative comparison to microdystrophin gene transfer. This proof-of-principle study demonstrates that our approach to restore an open reading frame via multi-exon deletion is applicable to large animal models but also suggests a link between treatment efficacy and state of muscle pathology at the time of intervention. Additional emphasis should be placed on optimizing gene-editing strategies with regards to subject age and state of disease progression during further development of CRISPR/Cas9-based approaches towards clinical translation into human DMD patients.

41. Utrophin Vector Protected by Central Tolerance as Potential Cure for Muscular Dystrophy

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The essential protein product of the Duchenne muscular dystrophy gene is dystrophin, a rod-like 427 kD protein that protects striated myocytes from contraction-induced injury by linking the cortical cytoskeleton to the extracellular matrix. Most patients with DMD have multi-exon frame-shifting deletions, while many with the milder allelic disease Becker MD have frame-preserving mutations that change the length of dystrophin's 150 nm rod domain. Utrophin, a dystrophin paralog, retains many of the structural and protein binding elements of dystrophin. Importantly, normal thymic expression in DMD patients should protect utrophin by central immunologic tolerance. Leveraging a deep analysis of dystrophin's molecular evolution with a focus on the stability of the rod domain, we designed a codon-optimized, synthetic transgene encoding a 25 nm miniaturized utrophin (μUtrophin), deliverable by AAV vectors. Here we show that μUtrophin is a highly functional, non-immunogenic substitute for dystrophin, preventing the most deleterious histological and physiological aspects of muscular dystrophy in small and large animal models. Following systemic administration of an AAV-μUtrophin to neonatal dystrophin-deficient mdx mice, all histological and biochemical markers of myonecrosis and regeneration are completely suppressed throughout growth to adult weight. In the dystrophin-deficient Golden Retriever model, μUtrophin non-toxically prevented myonecrosis even in the most powerful muscles. In a stringent test of immunogenicity, focal expression of μUtrophin in the deletional-*null* German Shorthaired Pointer model produced no evidence of cell-mediated immunity, in sharp contrast to the robust T cell response against similarly constructed μDystrophin. These findings support a model in which utrophin-derived therapies can be used to treat clinical dystrophin deficiency, with a favorable immunologic profile and preserved function in the face of extreme miniaturization.

42. Restoration of Dystrophin Expression by Genome Editing in the Canine X-linked Muscular Dystrophy (CXMD) with a Mutation in the N-Terminal Mutation Hotspot, a Dog Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal and devastating genetic disorder caused by a lack of dystrophin protein due to mutations in the *DMD* gene. DMD is mostly caused by out-of-frame mutations, leading to a premature stop codon. Genome editing holds the promise of treating DMD. Most studies currently aim to remove a part of the *DMD* gene by non-homologous end joining (NHEJ) and produce a truncated but functional dystrophin protein to shift the clinical prognosis towards its milder counterpart, Becker muscular dystrophy (BMD). Previous studies showed that genome editing restored dystrophin expression and improved the phenotype of DMD model mice. Recently, single-cut genome editing was shown to restore dystrophin expression in a DMD dog model deltaE50-MD. However, this method relies on the mutation of specific splice-acceptor sequences. Here, we sought to examine the effects of genome editing in the Canine X-linked Muscular Dystrophy (CXMD) with a mutation in the N-terminal mutation hotspot. They harbor a splice site mutation in intron 6, leading to a lack of exon 7 in mRNA. The removal of 3 exons, exons 6-8, using NHEJ would restore the reading frame. An alternative strategy is to remove exons 3-9. Although exons 6-8, 6-9, and 3-9 deletions are all associated with BMD, the exons 3-9 deletion is associated with a milder phenotype. We employed CRISPR/*Staphylococcus aureus* Cas9 (SaCas9) system to remove these exons in the *DMD* gene. We designed several guide RNAs (gRNAs) and selected the most effective ones by T7E1 assays. To evaluate the genome-editing efficiency in vitro, we transfected vectors expressing SaCas9 and a pair of selected gRNAs (intron 5/8, or intron 2/8) into primary CXMD dog muscle cells. We demonstrated that genome editing using these guide RNAs and SaCas9 deleted the targeted region in the genome and induced exons 3-9 or 6-9 skipping in dystrophin mRNA with nearly 100% efficiency. For in vivo testing, we injected vectors carrying SaCas9 with the ubiquitous cytomegalovirus (CMV) promoter and the guide RNAs into the tibialis anterior (TA) muscle of a dystrophic dog by utilizing recombinant AAV serotype 9 (rAAV9). Western blotting and immunohistochemistry revealed the recovery of dystrophin protein expression in the injected muscles. These results indicate that CRISPR/SaCas9 genome editing restored dystrophin expression in vivo. We are further investigating the efficacy of systemic treatment and functional recovery in the canine model.

Directed Evolution of AAV Vectors I

43. Development of Efficient AAV Vectors Using iTransduce, an Expression-Based AAV Selection System

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Generation of new AAV capsid variants with modified properties has demonstrated improved vector tropism for different tissues and in targeting cell types that are refractive to AAV. A common approach in AAV development is the introduction of randomized 7-mer peptides onto the surface of the viral capsid to modify its properties, followed by repeated rounds of selection in a target tissue to isolate capsid variants with improved ability to enter the target tissue/cell. Current AAV libraries do not directly select for transgene expression, the

ultimate feature of an AAV vector. We hypothesized that, by combining a randomized peptide library on the AAV9 capsid along with a transduction-sensitive reporter system, we could select efficient AAV capsid variants that mediate robust transduction in the context of a single-stranded genome. We developed a novel capsid selection system called iTransduce, which consists of an AAV9 peptide library with a single-stranded (ss) genome containing a Cre-expression cassette along with the AAV9 cap gene with peptide inserted at amino acid 588. Upon injection into Ai9 mice - which contain a floxed/Stop/tdTomato cassette in somatic cells - capsid variants which mediate Cre expression induce tdTomato fluorescence, allowing flow sorting of capsid DNA in this select population of cells. The capsid DNA containing the peptide insert is then amplified from the transduced cells, next generation sequencing (NGS) performed to detect the profile of peptides, and subsequent rounds of selection may be performed from repackaged library. We infused Ai9 mice via the tail vein with the iTransduce library and performed a selection for AAV capsids that could transduce the brain. On the second selection round we flow sorted tdTomato+ cells from the brain and NGS revealed two dominant peptides, AAV-iT^{X1} and AAV-iT^{X2}. These capsid clones were vectorized with a ssCBA-EGFP cassette and administered intravenously. Three weeks post injection, mice were euthanized, and brain harvested. Remarkably, sections of the brain revealed robust GFP expression throughout the entire brain (Fig. 1), without the need for anti-GFP immunostaining, which is usually required for systemically administered AAV vectors. GFP expression was observed in neurons, astrocytes, and endothelial cells. Following these results, we are currently performing head to head comparisons of the transduction efficiency of AAV-iT^{X1} and AAV-iT^{X2} with the parental AAV9 vector and AAV9-PHP.B (the standard for high transduction efficiency in C57BL/6 mice). To test the clinical potential of these novel vectors, we are also exploring the efficacy of iT^{X1} and iT^{X2} in other strains of mice, as well as larger animal species. Overall, the iTransduce system demonstrates the ability to produce efficient vectors with high transduction efficiency in the target tissue, that, given a selection marker, can be adapted to any cell type.

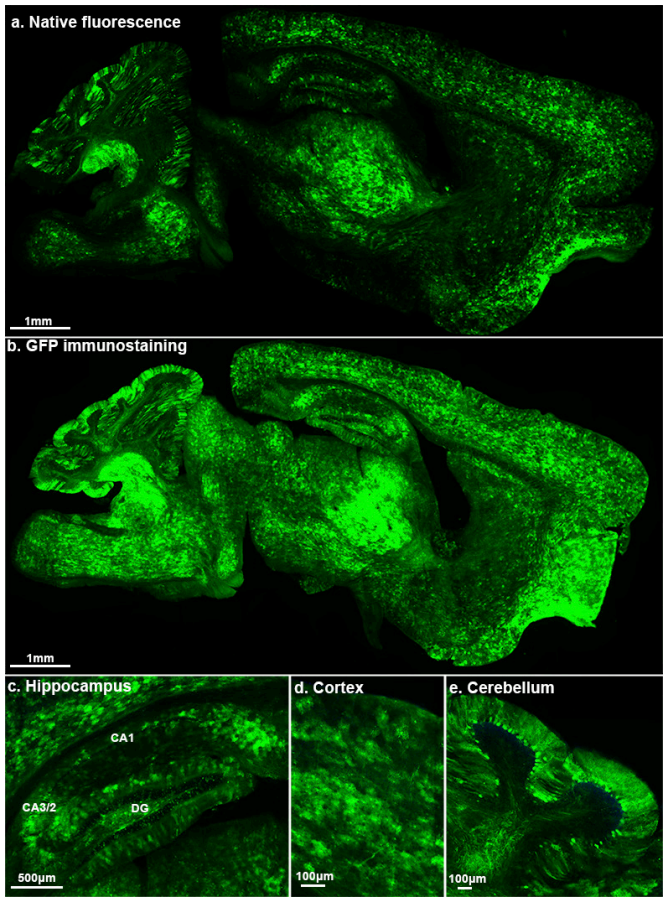

Figure 1: AAV-iT^{X1}-driven GFP expression in C57BL/6 mouse brain. a,b: whole brain sagittal sections showing native fluorescence (a) or fluorescent staining for GFP (b). c-e: Highlighted regions of brain. CA: cornu Ammonis. DG: dentate gyrus

44. Cell Type-Specific TRAnscription-Dependent Directed Evolution (TRADE) Identifies Novel AAV Capsids Capable of Enhanced Neuronal Transduction in Mice and Non-Human Primates

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Directed evolution of the AAV capsid is a powerful tool for identifying AAV variants with enhanced transduction; however, its application in large animals, including non-human primates, has remained a challenge. In order to overcome this limitation, we developed a next-generation directed evolution system that improves on currently available AAV capsid directed evolution approaches, including the recently described CREATE system. Here, we demonstrate proof-of-

concept and successful in vivo application of our directed evolution system, termed TRAnscription-dependent Directed Evolution (TRADE). TRADE employs a method of stringently screening AAV capsid libraries for variants that are capable of mediating mRNA expression in a specific cell type. Importantly, TRADE does not require the use of a recombinase to confer cell-type specificity. The TRADE system utilizes a bicistronic vector genome containing (1) an AAV2 p40-driven cap open reading frame, and (2) a cell type-specific promoter placed in the antisense direction that drives expression of the antisense cap sequence as a non-coding RNA containing an intron, as well as a GFP reporter. This vector configuration allows the recovery of capsid sequences from AAV variants that are capable of mediating cell type-specific mRNA expression by RT-PCR, and minimizes expression of immunogenic AAV capsids. In the proof-of-concept study, we sought to identify novel AAV capsids with enhanced neuronal transduction by utilizing a human synapsin I (hSynI) promoter in the TRADE system. We first confirmed neuron-specific antisense cap RNA expression by immunofluorescence microscopy of a TRADE vector-transduced mouse brain, using GFP as a surrogate for antisense cap transcripts. We also confirmed that RT-PCR can be used to selectively retrieve antisense transcripts by verifying splicing of the intron. Having established the key concept of TRADE, we next generated an AAV hSynI TRADE library consisting of an AAV9 liver-detargeted (AAV9-N272A) platform with an 8-amino acid peptide display inserted at position Q588 and flanked by glycine-serine linkers. We injected C57BL/6 mice and one rhesus macaque intravenously with this AAV library, harvested brain tissues 12 days post-injection, and recovered AAV capsid sequences from transduced hSynI-expressing brain neurons by RT-PCR. Following three rounds of selection in mice and one round of selection in a single rhesus macaque, we selected 5 mouse-derived and 21 macaque-derived AAV variants for subsequent validation studies in C57BL/6 and BALB/c mouse strains as well as rhesus macaques. We employed AAV DNA/RNA Barcode-Seq technology to compare our TRADE-derived mutants to AAV9 and AAV-PHP.B using barcodes expressed by the hSynI promoter. We found that one mutant, AAV-HN1, achieved similar brain neuronal transduction to AAV-PHP.B in C57BL/6 mice. Notably, AAV-HN1 also demonstrated substantially enhanced brain neuronal transduction in BALB/c mice and rhesus macaque whereas AAV-PHP.B showed no improvement over AAV9 in these animals. Preliminary immunofluorescence microscopic analysis using AAV-HN1-GFP supports the Barcode-Seq data. In conclusion, we have developed TRADE, a novel strategy for in vivo selection of AAV capsid mutants that selects at the level of vector-mediated cell type-specific transgene expression. Because the TRADE system does not require recombinase, it can be readily applied to a variety of animal species and cell types to rapidly advance the development of enhanced AAV vectors for research and clinical applications.

45. A Novel Adeno Associated Virus Capsid Variant selected on Human Islets Shows Robust Transduction in Many Cell Types In Vitro and In Vivo

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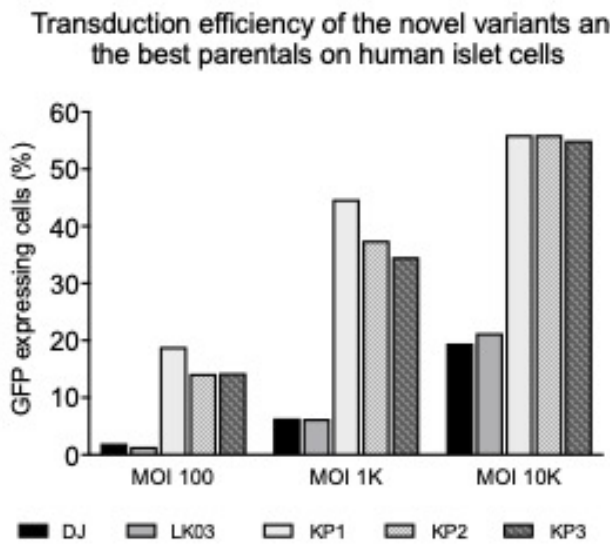
Gene therapy of islets may be a useful tool for the treatment of diabetes, a disease that is on the rise in the modern world. Adeno-associated virus (AAV) has been shown to be a very safe and effective vehicle for gene delivery into various human tissues, however gene transfer into islets has been hampered by a lack of serotypes that transduce those cells with high efficacy. In order to develop AAV capsids with improved tropism for human islets we infected human islets with two highly complex capsid shuffled barcoded AAV libraries. AAVs were replicated by super-infection with human adenovirus 5 and three rounds of consecutive selection on islets were performed. Enrichment of capsid variants during the selection process was tracked by high-throughput analysis of the barcodes. We identified three capsid variants that were capable of transducing human islet cells with 5- to 10- fold higher efficiency when compared to the previously identified best transducing capsids AAV-DJ and AAV-LK03. Two variants were tested for cell type specific transduction of intact islets at a high MOI. It was shown that the novel variants were capable of penetrating intact human islets and transduced beta-cells with significantly higher efficiency than the best previously known variant AAV-LK03. The top performing variant AAV-KP1 was tested for transduction of human embryonic stem cell derived beta-cells and found to exhibit considerably higher rates of gene delivery as compared to AAV-LK03. The AAV-KP1 capsid also exhibited strong mouse liver transduction when tested *in vivo* and transduced several human and non-human cell lines *in vitro* with higher efficiency than the extremely robust and versatile AAV-DJ capsid. In addition, AAV-KP1 was at least as efficient as AAV-LK03 (currently being used in Spark's AAV-FVIII clinical trial) at transducing both mouse and human hepatocytes in a humanized liver mouse model, thus providing a versatile vector with potential for human gene transfer application while maintaining transduction of mouse cells. If used in the clinic, AAV-KP1 unlike AAV-LK03 circumvents the need for using a surrogate capsid in preclinical animal testing. Finally, AAV-KP1 had a more favourable neutralization profile when compared to AAV-LK03 using pooled human immunoglobulins. We believe that our novel vector may prove to be a useful delivery vehicle for a variety of gene therapy applications.



46. Using Novel AAV Capsids to Maximize Gene Delivery Throughout the Rhesus Macaque Brain

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Studies investigating AAV-based gene delivery to treat and model neurological disorders have historically relied on MRI-guided stereotaxic injections directly into brain parenchyma, resulting in focal gene expression in the injected area with limited transport to remote brain regions. Focal AAV delivery has allowed for great advances in treating diseases stemming from pathology in circumscribed regions of the brain and eye and, excitingly, several of these therapeutic approaches that have made their way into clinical trials. Despite these advances, delivering genetic material efficiently to the central nervous system (CNS) still remains a hurdle in developing efficacious gene therapy strategies for CNS disorders characterized by widespread neuropathology throughout several brain regions where a focal, targeted gene delivery is far less likely to be efficacious. New AAV capsid mutants have been engineered by several groups by utilizing rational design or via molecular evolution technology in attempt to enhance transduction efficiency in brain tissue, including the AAV9 capsid mutant, AAV-PHP.B and the AAV2 capsid mutant, AAV2.retro. Here, we show that AAV-PHP.B-eGFP transduces neurons and astrocytes in widespread regions throughout the rhesus macaque brain and spinal cord when administered into either the internal carotid artery or the intra-thecal space of the cisterna magna. Interestingly, we found that cisterna magna delivery of AAV-PHP.B-eGFP results in significantly higher transduction, as measured by AAV vector genome copy per cell and eGFP immunofluorescence, in many cortical and subcortical brain regions compared to intra-carotid administration ($p<0.05$ for each brain region). In a separate cohort of animals, we show that stereotaxic infusions of AAV2.retro-eGFP into the caudate and putamen not only result in the transduction of neurons in these two brain regions, but also result in robust transduction of several striatal afferent brain regions including many frontal, motor and limbic cortical areas as well as subcortical brain regions including the thalamus, substantia nigra, amygdala and hippocampus. Together, these data show that recent advances in novel AAV capsid design allow for widespread delivery of transgenes throughout the rhesus macaque brain and highlight their valuable use in delivering promising gene therapies for a variety of neurological disorders that would benefit from a global, CNS-wide delivery strategy.

47. Development of Novel AAV Variants with High Retinal Transduction Efficiency

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Inherited retinal dystrophies (IRD) are blinding disorders caused by mutations in genes that are specifically expressed in photoreceptors or retinal pigment epithelium (RPE). Most IRDs are well characterized monogenetic diseases and are, thus, prime candidates for gene therapy. The most promising gene therapy approaches are based on recombinant adeno-associated virus (rAAV) vectors. To target photoreceptors or RPE, rAAV vectors require direct exposure to the surface of target cells, which can only be achieved by surgical detachment of the neuroretina from the RPE and injection of the rAAV vectors into a temporally formed cavity (subretinal bleb). This procedure can be deleterious to an already compromised retina. Moreover, only cells within the subretinal bled area are transduced. Using a directed evolution approach, we developed novel AAV vectors with high pan-retinal transduction efficiency when delivered to the intravitreal compartment via simple intraocular administration (intravitreal injection). In particular, we used AAV2 peptide display libraries carrying random 7-mer insertions flanked by 2-3 linker amino acid (aa) at position 587 of the VP1 protein. After in vivo application of approx. 8×10^{10} total vector genomes (vg) of the AAV library in wild type mice or mice expressing enhanced green fluorescence protein in cone photoreceptors. Subsequently, the retina was harvested, specific cone and rod cell populations were isolated using FACS or CD73 magnetic-associated cell sorting and DNA extracted for subsequent AAV sub-library construction and next generation sequencing. After three selection rounds we identified two novel AAV variants that enabled very efficient and fast-onset pan-retinal GFP-reporter gene expression in retinal cells including mouse rod and cone photoreceptors after a single intravitreal injection at a dose of 2×10^9 total vg. Both novel variants supported efficient transduction of photoreceptors after intravitreal injection in the dog and non-human primate model and of human photoreceptors after application to human retinal explant cultures. These modified AAV capsids hold promise as novel improved gene therapy vectors.

48. Targeted In Vivo Biopanning of AAV Capsid Libraries Using Cell Type-Specific RNA Expression

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Gene delivery to the adult central nervous system (CNS) remains a major challenge in gene therapy, and engineered AAV capsids with improved brain tropism represent an attractive solution. Attempts at improving the CNS tropism of AAV capsids upon systemic administration were met with limited success until the isolation of PHP.B (Deverman et al., 2016) and PHP.eB (Chan et al., 2017) capsids from an astrocyte-specific selection in CRE transgenic mice. This finding suggested that cell type-specific library selection could improve the outcome of directed evolution. However, the transgenic CRE system used by Deverman et al. is not tractable in other animal species. To address this problem, we have developed a broadly-applicable functional AAV capsid library screening platform for cell type-specific biopanning in non-transgenic animals. In the TRACER (Tropism Redirection of AAV by Cell type-specific Expression of RNA) platform system, the capsid gene is placed under the control of a cell type-specific promoter to drive capsid mRNA expression in the absence of helper virus co-infection. This RNA-driven screen increases the selective pressure in favor of capsid variants which transduce a specific cell type. Proof-of-concept experiments were conducted by placing the genes encoding an AAV9 peptide display library under the control of either the neuron-specific synapsin promoter (SYN) or the astrocyte-specific GFAP promoter. Following intravenous administration to C57BL/6 mice, RNA was recovered from brain tissue and used for further library evolution. Next-generation sequencing (NGS) showed sequence convergence between animals after only two rounds of selection. Interestingly, several variants highly similar to the PHP.eB capsid were recovered, suggesting that our method allowed a rapid selection of high-performance capsids. A subset of peptide sequences with high CNS enrichment was selected for capsid incorporation, and these were placed under control of either the SYN or GFAP promoter. Capsid pools were injected to three rodent species, followed by RNA enrichment analysis for characterization of transduction efficiency in neurons or astrocytes and cross-species performance. Top-ranking capsids were then individually tested, and several variants showed CNS transduction similar to or higher than the PHP.eB benchmark. These results suggest that the TRACER platform allows rapid *in vivo* evolution of AAV capsids in non-transgenic animals with a high degree of tropism improvement.

Clinical Gene Therapies for Blood Diseases

49. Gene Therapy for the Treatment of Adult and Pediatric Patients Affected by Transfusion Dependent BETA-Thalassemia

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Beta-thalassemia is a genetic disorder due to mutations in gene encoding the beta-globin chain causing a reduced or absent production of hemoglobin A leading to severe anemia and lifelong transfusion dependence. The only curative treatment is represented by allogeneic bone marrow transplantation (BMT), available for a minority of patients and associated with risk of complications and mortality. Gene therapy could represent an alternative to allogeneic BMT with these potential advantages: use of autologous cells, tailored conditioning with no need for immune suppression, no risk of GVHD or rejection. A phase I/II gene therapy clinical trial based on the autologous transplantation of G-CSF and plerixafor mobilized hematopoietic stem cells engineered by GLOBE lentiviral vector, expressing a transcriptionally regulated human beta-globin gene, was started in 2015. Transduced cells were infused by intraosseus injection following a myeloablative treosulfan and thiotepa conditioning. Nine patients with different genotype (β0/β0, β+/β+ and β0/β+) have been treated: 3 adults followed by 6 minors. A median cell dose of 19.5x106 CD34+ cells/kg containing a median 60% (range: 38-77%) of transduced clonogenic progenitors and a mean VCN/ genome of 0.93±0.27. At 1 year follow-up, primary endpoints of transfusion reduction and safety were achieved in 8 out of nine patients, with 4 patients transfusion independent. A robust and stable engraftment of genetically modified cells was showed in 7 patients at latest follow-up, with percentage of transduced BM progenitors up to 78%. Vector-transduced cells were detected in peripheral blood and BM purified lineages with a median VCN of 0.64 (range: 0.13-3.44). Polyclonal vector integrations profiles had the expected genomic distribution and no evidence of clonal dominance. At last follow-up the three adult patients had reduction of transfusion requirement, four pediatric patients are transfusion independent and two are still

receiving blood transfusions. Prolonged follow up analysis will provide additional information on the long-term safety and clinical efficacy of this treatment.

50. Gene Therapy for Sickle Cell Disease (SCD) Using RVT-1801 Lentivirus Vector and Arulite Reduced Intensity Conditioning Transplant Shows Promising Correction of the Disease Phenotype

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Myeloablative busulfan conditioning and transplant of autologous anti-sickling beta^{87}-globin gene modified hematopoietic stem cells (HSC) was shown to cure a child with SCD (NEJM 2017). The same approach was not successful in subsequent SCD adults treated similarly, until modifications to intensify busulfan dose, HSC dose and gene transfer were made (ASH, 2017, 2018). Other SCD trials (NCT03282656, NCT02247843) are also using ablative busulfan conditioning with mixed results. This conditioning comes with significant acute (cytopenia, mucositis) and chronic toxicities (infertility, secondary malignancy). We performed a Reduced Intensity Conditioning (RIC) Phase I/II Pilot Study on Gene Transfer In SCD Patients with a Modified γ-Globin Lenti-vector (RVT1801; NCT02186418) to determine its safety, feasibility and efficacy. RIC will have less toxicity, cost and implementable in many transplant centers, including resource-poor countries, where intense medical support required for myeloablative transplants is scarce, and where majority of SCD patients exist. Eligible adults with severe SCD underwent HSC collection via bone marrow (BM) harvests and/or plerixafor mobilized Peripheral Blood Stem Collection (PBSC). Patients received AruLite conditioning (IV melphalan) followed by infusion of RVT-1801-modified HSC. Patients were monitored for AEs, engraftment, vector copy number (VCN), modified fetal hemoglobin (HbF^{G16D}) expression and other laboratory and clinical parameters of SCD. As of Dec. 2018: P1 (35 yo) and P2 (25 yo), with HbS-β0 thalassemia received $1x10^6$ and $6.9x10^6$ CD34+ cells/kgbw, with VCN 0.22 and 0.46, respectively, 36 h following Arulite conditioning. Time to neutrophil recovery (> 500) was 7-9 days, and platelet recovery (> 50K) was 7-12 days. Both patients had severe disease and continued to have chronic pain requiring significant IV opiods for upto 6 mo post-transplant. 80% of the AEs were pain events; others were anticipated transient laboratory AEs associated with melphalan. Following gene-modified CD34+ cell infusion, both patients have shown a net rise in HbF^{G16D} starting from D30 post-transplant. Anti-sickling hemoglobin (HbF^{G16D}, HbF, HbA_2) levels were 32% in P1 and 22% in P2 at 15mo and 12mo, with a VCN of 0.2-0.4 in multiple lineages. Integration site analysis on both at all time points upto 6 mo shows a highly polyclonal pattern of integration. P1 had 20% HbF^{G16D} (2.1g/dl HbF^{G16D}, a rise in total Hb from 7-8.5 to 10.5-11g/dL) with stable VCN of 0.2-0.4 in multiple lineages in BM and blood at 15 mo. P2 has also shown a progressive rise in HbF^{G16D}, albeit at a lower trajectory. P1 and P2 had 48 and 20 acute sickle events, respectiely, in the 18 mo prior enrollment and chronic pain requiring chronic oral/IV opioids. Both have > 90% improvement in acute sickle episodes, and were weaned off chronic opioid use. Early results from 2 SCD patients treated with RVT1801-modified HSC using Arulite RIC showed good safety, feasibility, minimal post-transplant toxicity, rapid count recovery, and stable genetically modified cells in blood and BM. Both patients have sufficient anti-sickling globin expression to ameliorate anemia and remarkably reduce chronic pain and acute sickle events to date. These results with AruLite RIC, are promising and if replicated in future patients will provide a 'transportable' and feasible gene therapy for SCD.

51. Preliminary Conclusions Obtained in Fanconi Anemia Patients Treated by Lentiviral-Mediated Gene Therapy after 2 Years of Follow-Up

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In 2016 we started a gene therapy trial in patients with Fanconi anemia, subtype A (FA-A patients) using autologous $CD34^+$ cells. $CD34^+$ cells were mobilized to peripheral blood (PB) with G-CSF and plerixafor and transduced with the therapeutic PGK-FANCA.Wpre* lentiviral vector. Seven patients with ages between 3-6 years old have been infused either with cryopreserved or fresh $CD34^+$ cells with doses ranging from 50,000 to 400,0000 transduced $CD34^+$ cells/Kg. Patients FA-02002 and FA-02005 were treated in January and March 2016 and were infused with an estimated number of 250,000 and 230,000 transduced $CD34^+$

cells/Kg, respectively. Patients FA-02004 and FA-02006 were infused 6 and 12 months later with 170,000 and 410,000 transduced $CD34^+$ cells/Kg. More recently, three patients were infused with lower numbers of transduced $CD34^+$ cells. Although the proportion of corrected PB cells during the first 6 months post-infusion has been consistently low (generally less than 2%), progressive increases in gene marking have been observed thereafter in the first four treated patients through the most recent follow-up period (18 to 30 months post-infusion). Patient FA-02002 currently shows the highest level of gene marking, with percentages of marked cells in BM and PB above 50% at the most recent analyses (24 and 30 months post-infusion, respectively). Insertion site analyses did not reveal patterns consistent with insertion-site mediated clonal expansion, and confirmed the engraftment of multipotent HSCs. In each of the first four treated patients evident increases in the resistance of BM progenitor cells to mitomycin-C have been observed. Moreover, after *in vitro* challenge to diepoxybutane, significant decreases in the proportion of PB T-cells with chromosomal breaks have been observed in three of the four first treated patients since the first year post-infusion. Monitoring during a 2-3 year follow-up of our first gene therapy-treated non-conditioned FA-A patients shows the absence of severe adverse events and demonstrates progressive engraftment of phenotypically corrected HSCs. Data from this clinical trial strongly suggest that gene therapy will constitute a safe therapeutic approach for the treatment of the bone marrow failure characteristic of FA.

52. Genome-Wide Assessment of Lentiviral Integration Sites of Gene-Corrected Llympho-Hematopoietic Cells in FA-A Patients

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Fanconi anemia (FA) is a DNA repair syndrome resulting from mutations in any of the 22 FA genes that encode for proteins participating in the FA/BRCA pathway. Three years ago a gene therapy trial utilizing autologous cells gene-modified with lentiviral-vectors (LVs) started in Spain as a potential alternative treatment for bone marrow failure in FA. Based on risks of insertional oncogenesis observed in previous clinical trials with gamma-retroviral vectors, careful insertional site studies are mandatory in clinical trials involving vectors that integrate in the genome. Analyses of transduced cells in the first four non-conditioned FA patients had been infused with gene-modified autologous CD34+ cells have shown that LV-mediated hematopoietic gene therapy reproducibly confers a progressive increase of gene-modified cells in peripheral blood (PB) and bone marrow (BM). We have analyzed the LV-insertion sites in total and enriched PB and BM cell subsets from these patients, and investigated whether the repopulation advantage of these cells was due to integration site dependent dominant clones. Insertion site analyses of hematopoietic cells from these gene therapy treated FA-A patients were conducted with LAM-PCR. A total of 2,001 unique IS were obtained. This revealed a typical lentiviral integration pattern, showing that on average 72.6% of the LV-integrations occurred within genes (80.4% in genes ± 10kb) and that no preferential LV-integrations were found in close proximity to transcription start sites. No clones harboring genes associated with hematopoietic malignancies such as LMO2, CCND2 or MN1 were detected during the observation time points (up to 30 months post gene therapy in two patients). Importantly, progressive increases in the proportion of corrected cells were not associated with the continuous expansion of one or few clones of corrected cells but rather with an increase in the number of different clones. A high proportion of IS identified in total PB were also identified in different hematopoietic cell lineages, including myeloid and lymphoid lineages expressing the CD14, CD15, CD19 and CD3 lineage markers. Taken together, our data show that the progressive hematopoietic engraftment of non-conditioned FA patients is associated with an oligoclonal pattern of reconstitution, as expected given the limited numbers of gene-modified CD34+ cells (more than 1 log lower than other gene therapy clinical trials in non-malignant hematopoietic disorders). No common integration sites associated to known proto-oncogenes have been identified and no persistent clonal dominance was observed until the last observation time point (up to 30 months post gene therapy). Further ISA of these patients will allow us to confirm the safety of the LV-mediated gene therapy in FA, and will help to yield insights into clonal dynamics and repopulation kinetics of the blood forming system in this novel gene therapy trial.

53. A Diversity of Human Hematopoietic Differentiation Programs Identified through In Vivo Tracking of Hematopoiesis in Gene Therapy Patients

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Gene correction of human hematopoietic stem and progenitor cells (HSPCs) with integrating vectors marks each cell by the integration site (IS) position, providing an opportunity to track the progeny of each HSPC. Thus hematopoietic ontogeny in humans can be inferred by tracking the appearance of unique integration sites in fractionated blood cell populations. Here, we studied four patients corrected for Wiskott-Aldrich syndrome and two corrected for beta-hemoglobinopathies. We sorted peripheral blood samples for 5 cell types: myeloid (granulocytes and monocytes) and lymphoid subpopulations (T, B and NK cells), and analysed their IS profile (using our optimized pipeline, INSPIIRED). Clonal abundance was quantified using the sonicAbundance method which provided an estimate of the numbers of cells contributing to each IS cell clone, and therefore allow robust analysis of HSPC function in term of clonal lineage output. Using this approach, we have characterized up to tens of thousands IS per patients. However, the data generation process comprises several steps, which add uncertainty to determining the true population compositions. One challenge comes from sparse sampling, that we take into account by applying stringent abundance filtering (allowing 95% retrieval of IS between replicates). We also corrected IS data for residual contamination and unbalanced sampling, then quantified cell lineage output. Cluster analysis of long-term HSCs (focused on later time points) revealed the existence of several human HSC subsets with distinct lineage potential : myeloid-dominant, lymphoid-dominant, and balanced HSC subsets, that are detected up to 4-5 years in the two types of genetic diseases. A novel aspect of our study is the analysis of hematopoiesis in two distinct pathophysiologic contexts, which helps bypass possible biases introduced by the selective advantage of the gene-corrected cell populations. Our results thus highlight the heterogeneity of human HSCs and introduced a novel, rigorous approach for tackling the technical challenges associated with the use of IS data for human HSC lineage ouput tracking in gene therapy trials.

54. Restoration of Cellular and Humoral Immunity by Targeted Gene Correction of T Cells as a Treatment for X-Linked Lymphoproliferative Disease (XLP1)

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X-linked lymphoproliferative disease is a primary immunodeficiency arising from mutations in the SH2D1A gene encoding SAP, a key regulator of immune function expressed in T cells, natural killer, and NKT cells. Haematopoietic stem-cell transplantation (HSCT) is curative but positive outcomes post transplant can be limited in the mismatched donor setting. We have previously shown that gene correction using lentiviral vectors can restore immune function in mouse models of both haematopoietic stem-cell gene therapy and adoptive T cell transfer. However, the tightly controlled expression profile of SAP may be better replicated by using a targeted gene addition strategy that harnesses endogenous DNA regulatory elements surrounding the SH2D1a locus. We constructed SAP-specific TALE-effector nucleases (TALENs) capable of generating a site-specific DNA double-strand break (DSB) close to the translation start codon, allowing correction of most described SH2D1A mutations. Nucleofection of in-vitro-transcribed TALEN mRNA into stimulated primary T cells created indels at a rate of 64% and 71% measured by TIDE analysis and NGS respectively.To stimulate homology directed repair (HDR) of SH2D1a locus DSB, we produced a homology donor in AAV6 format designed to place SAP cDNA (and a co-expressed GFP fluorescent signal) under the control of the endogenous SAP promoter. When donor transduction was coupled with TALEN mRNA nucleofection, we demonstrated a HDR rate of 44% by stable GFP expression, and expression of SAP protein. To optimize the procedure, we tested different media compositions at the time of AAV transduction. In the most favorable condition we could reduce the AAV MOI 30 fold with no loss of editing efficiency. Ongoing phenotypic analysis will determine the suitability of this approach for clinical application. Next, we tested for restoration of humoral and cellular deficits in gene corrected XLP patient T cells. In a T follicular helper cell:B cell co-culture assay, edited patient CD4+ T cells expressed SAP protein, secreted IL-21 appropriately and could stimulate production of class switched immunoglobulins in B cells, all comparable to healthy controls.To assess cytotoxicity, we generated EBV-specific cytotoxic T lymphocytes (CTLs). When challenged against EBV+ targets, gene-corrected patient CTLs showed equal killing activity to healthy donors. We have established an in vivo tumour regression model in NSG mice, in which testing of the corrected patient CTLs is underway. This promising data indicates an autologous gene edited T cell product could fulfill an unmet clinical need for patients with XLP lacking a suitable donor for HSCT.

Oncolytic Viruses I

55. Intravenous Phase I Study of VSV-IFNb-NIS in Patients with Metastatic or Recurrent Stage III or IV Endometrial Cancer

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Background: VSV-IFNβ-NIS (Voyager-V1) is an oncolytic Vesicular Stomatitis Virus (VSV) ideally suited as a systemic therapy due to low VSV seroprevalence and selective replication in tumors. VSV-IFNβ-NIS

is engineered to encode interferon beta (IFNβ) for increased toxicity to cancer cells and amplification of cellular antitumor activity, and NIS (sodium iodide symporter) gene allows for noninvasive SPECT imaging of virus-infected cells. A Phase I first-in-human trial was initiated to evaluate VSV-IFNβ-NIS after IV administration to patients with metastatic or recurrent endometrial cancer (EC). **Methods**: The trial utilizes the standard 3+3 design (NCT03120624). Primary objective was to determine maximal tolerated dose of VSV-IFNβ-NIS. Secondary objectives include safety, tolerability, clinical response rate, progression free survival and overall survival. Exploratory correlative studies include pharmacokinetics (viremia, IFNβ levels), pharmacodynamics, shedding, tumor genetic signature and immune activation (immune cell phenotyping, immune cell infiltrates, anti-tumor cytotoxic T cells, and RNAseq). **Results**: To date, 7 EC subjects have received the virus, 3 at dose level 1 and 4 at dose level 2. Treatment related adverse events included grade 2 fever (N=3), grade 3 fever (N=1), and grade 2 chills (N=2), lymphocytopenia (grade 3 N=2; grade 4 N=2), grade 3 neutropenia (N=1), and grade 3 liver enzyme elevation (N=1). There was no appreciable viral shedding in body fluids: buccal swabs, mouth rinse or urine. Viremia was detected in all patients, peaking between 4h and 24h post infusion of virus, with variable decline thereafter, reflecting different extent of VSV replication. Importantly, IFNβ is a sensitive biomarker of virus infection. At dose level 1, 24h IFNβ levels were low (<50 pg/mL) in all 3 subjects. At dose level 2, peak 24h IFNβ levels in 2 subjects were >1000 pg/ml compared to 1 subject with IFNβ of 25 pg/mL. Low IFNβ level reflects poor susceptibility of the tumor to VSV infection, potentially due to a robust antiviral response. Subject 6 has the highest IFNβ peak and best response to treatment. Shrinkage of multiple tumors in the lungs and abdominal cavity was observed radiologically at day 29 and 90 (16% reduction, stable disease). She also has increased immune infiltrates of CD3+, CD8+, and PD1-expressing cells in tumor biopsies at 1 month after IV administration of virus. Her day 29 anti-VSV antibody titer was highest at 10,240 dilution. Importantly, antitumor CTL measured by ELISPOT assay showed increase in tumor reactive cytotoxic T cells at day 29 and 3 months after systemic VSV-IFNβ-NIS. **Conclusions**: Disease control with shrinkage of metastatic tumors was observed after one IV dose of VSV-IFNβ-NIS. There was increased CD3+ and CD8+ cells in tumor biopsies one month after systemic VSV, and induction of tumor antigen specific CTLs in peripheral blood. Data to support the combination of VSV-IFNβ-NIS with immune checkpoint inhibitors continues to be collected to guide subsequent clinical trial design.

56. Phase I Clinical Trial of Intravenous Administration of VSV-IFNb-NIS in Patients with Hematologic Malignancies

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Oncolytic Vesicular Stomatitis Virus (VSV, Indiana strain) encoding human interferon beta (IFNβ) and sodium iodide symporter (NIS) is an attractive agent for systemic therapy of disseminated cancers due to its low levels preexisting antiviral immunity and fast replication. To ensure tumor selective replication and spread, we designed VSV to encode IFNβ which also serves as a STING agonist to activate host immunity against the cancer. To non-invasively monitor viral infection of tumors using SPECT/CT imaging the NIS gene is inserted as a reporter gene into the genome. Promising preclinical efficacy data in mice with Multiple Myeloma (MM), Acute Myeloid Leukemia (AML), and T cell lymphoma (TCL) led us to a phase I trial for these indications. Here, we report a Phase I clinical trial of intravenous administration of VSV-IFNβ-NIS for relapsed or refractory MM, AML, and TCL. **Methods:** This is a classical 3+3 phase I trial, starting at $5x10^9$ $TCID_{50}$ (dose level 1) through $5x10^{11}$ $TCID_{50}$ (dose level 4), given as a single IV dose. There are two cohorts, cohort A consisted of patients with low tumor burden treated VSV-IFNβ-NIS monotherapy and cohort B is for patients with high tumor burden treated with VSV-IFNβ-NIS in combination with ruxolitinib. Ruxolitinib is a Jak1/2 inhibitor shown to increase viral replication in tumor cells and protect mice from supra-levels of IFNβ. The primary objective of this trial is to determine the maximum tolerated dose (MTD) of VSV-IFNβ-NIS alone and in combination with ruxolitinib; secondary objectives include estimating the safety profile and preliminary efficacy. Correlative objectives include monitoring the pharmacodynamics of viral replication through SPECT/CT imaging with NIS gene, viremia, virus shedding, and changes in the immune profile of peripheral blood leukocytes. Adverse events (AEs) are reported herein are based on CTCAE v4.3 with the exception of cytokine release syndrome (CRS) which is based on Lee (Blood 2014; 124(2):188-195) criteria. **Results:** To date, 12 patients have received IV VSV-IFNβ-NIS; 9 in cohort A and 3 in cohort B. In cohort A, 3 patients were treated at each dose level. Among the 8 evaluable patients in cohort A, there was one partial remission (TCL patient treated at dose level 2 - $1.7x10^{10}$ $TCID_{50}$) and one stable disease (TCL patient treated at dose level 2). In the cohort B, out of the 3 evaluable MM patients, 2 had stable disease. VSV-IFNβ-NIS had encouraging safety profile, virus was not shed, and no VSV related lesions appeared even in immune-compromised patients. No infectious virus was recovered in buccal swabs or urine and neutralizing anti-VSV antibodies were present by day 29. Extensive immune phenotyping and ELISPOT assays for shared antigens are ongoing. Multiple cytokines increased at 4h post infusion of virus, but most returned to baseline levels by 24h.Viremia was detectable in all patients at the end of infusion, and to varying levels at 30 mins, 1, 2, 4, 24, or 48 hours post virus infusion but no persistent viremia was observed. Infusion related reactions and adverse events were transient. Plasma IFNβ levels could serve as a valuable pharmacodynamics (PD) marker of viral replication, showing variable levels of viral replication and duration. Currently, Tc99m pertechnetate imaging for sites of viral replication has been below limits of detection by the SPECT modality for all patients. **Conclusion:** To date, no viral shedding has been detected in these patients with compromised immune systems. One partial response was recorded in a TCL patient at dose level 2. Dose escalation of is ongoing in both cohorts and updated results will be reported

57. A Dual Blockade of Death and Immune Checkpoints by SPG-275 Induces Complete Remissions in Treatment-Refractory 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. Irrespective of the treatments, efficient tumor-cell killing requires amplification of inflammatory signaling, which however is tightly regulated by various cell-intrinsic "checkpoint" mechanisms evolved by epithelial cells to prevent excessive tissue damage induced by virus and immune attack. Novel approaches to disabling theses conserved checkpoints may provide breakthrough and "tumor-agnostic" strategies to circumvent the innate treatment-resistance to unleash the full potential of C/T and immunotherapy in treatment-refractory tumors such as triple-negative breast cancer (TNBC) and pancreatic ductal adenocarcinoma (PDAC). **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 cytotoxic stress. Preclinical studies were used to validate a gene therapy strategy to disable the checkpoints identified from this process. **Results**: We uncovered that the cytotoxic and immunogenic death induced by C/T and immuno-stimulatory agents is constrained by repression of a toll-like receptor-2 (TLR-2)/TLR-3- and NF-kappa B-induced IRF-1 and interferon (IFN)-gamma anti-viral response program in various malignant tumor cells. Loss and gain of function studies implicated that co-repressor-2 (N-CoR2) translocates into cell nuclei in response to cytotoxic stress, wherein it serves as an epigenetic checkpoint of this death and inflammation program by mediating a histone deacetylase-dependent chromatin remodeling and repression of the expression of a specific panel of death and immune mediators. Blockade of the N-CoR2 death checkpoint using 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 a De-CoR2-armed vaccinia oncolytic virus (SPG-275) dramatically potentiated systemic C/T and immunotherapies, including anti-PD-1 and anti-CTLA-4 antibodies, and completely halted tumor growth or induced complete remissions in orthotopic or patient-derived xenograft models of TNBC and PDAC. **Conclusion**: Our findings suggest that malignant tumors can access intrinsically conserved anti-inflammatory mechanisms that enable them to escape from C/T and immunotherapy. The SPG-275 death checkpoint blockade oncoviral therapy constitutes the first-in-class strategy to override this cell-intrinsic defense program that may be applied to overcome resistance in treatment-refractory tumors and improve patient prognosis.

58. Oncolytic Virotherapy with Recombinant Measles Virus Induces Tumor Antigen-Specific T-Cell Responses

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Oncolytic virus therapy leads to immunogenic death of virus-infected tumor cells and this has been shown in preclinical models to enhance the T-lymphocyte response against tumor-associated antigens (TAAs), leading to killing of uninfected tumor cells as well. To investigate whether oncolytic virotherapy can increase immune responses to tumor antigens in human subjects, we studied T-cell responses against a panel of known myeloma TAAs in peripheral blood mononuclear cell samples obtained from 10 multiple myeloma patients before and after intravenous administration of an oncolytic measles virus encoding the sodium iodide symporter (MV-NIS). Despite their prior exposures to multiple immunosuppressive anti-myeloma treatment regimens, T-cell responses to some of the TAAs were detectable even before measles virotherapy. Measurable baseline T-cell responses against MAGE C1 and hTERT were present in 80% of patients and against NY-ESO-1 in 70% of patients. Furthermore, MV-NIS treatment significantly ($P<0.05$) increased T-cell responses against MAGE C1 and MAGE A3 (Figure 1). Interestingly, one patient who achieved complete remission after MV-NIS therapy had strong baseline T-cell responses against 8 of the 10 tested TAAs but these did not change markedly after virotherapy. Additionally, this patient had a higher mutational load compared to 664 MM patients and was predicted to express 68 neo antigens. However, functional validation of predicted tumor neoantigens did not result in a positive T cell recall antigenic response. TCRβ sequencing analysis of PBMCs also revealed minimal expansion of newly formed clones and comparatively higher expansion of pre-existing clones suggesting in this patient MVNIS therapy resulted in expansion of anti-tumor T cell clones that were pre-existing, leading to a durable disease control. Overall for the first time in human patients, our data demonstrate that oncolytic virotherapy can function as an antigen agnostic vaccine, increasing cytotoxic T-lymphocyte responses against TAAs in patients with multiple myeloma, providing a basis for continued exploration of this modality in combination with immune checkpoint blockade.

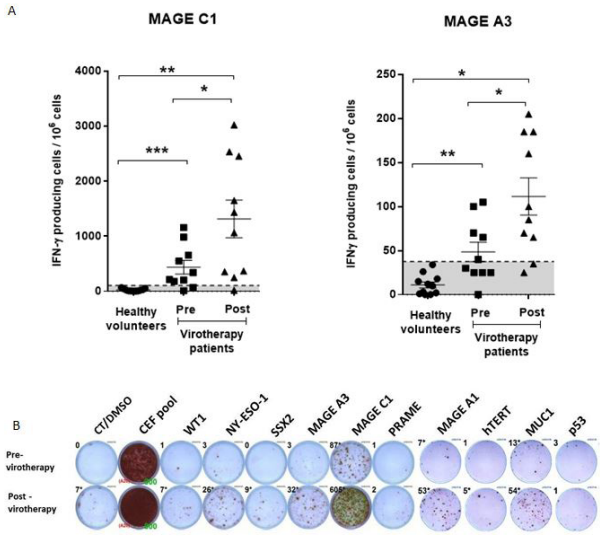

Figure 1. MV-NIS Virotherapy Induces Increased T-Cell Reactivity Against MAGE C1 and MAGE A3. A, Of the 10 tumor-associated antigens, T-cell responses against MAGE C1 and MAGE A3 were significantly increased in patient peripheral blood mononuclear cell samples after MV-NIS virotherapy. Grey-shaded area denotes the limit of negative response. B, Representative image showing increased IFN-γ responses against MAGE C1, MAGE A1, and MUC-1 at 6 weeks after MV-NIS virotherapy. CEF peptide pool was used as a positive control. Sole asterisk indicates $P<.05$, double asterisks, $P<.01$; triple asterisks, $P<.001$, compared with the corresponding groups. Error bars indicate mean with standard error of mean. CEF indicates CEF-positive control peptide pools; CT/DMSO, control/dimethyl sulfoxide; IFN, interferon.

59. Oncolytic Measles Virotherapy Increases the Potency of aPD1 Checkpoint Therapy to Establish Long Term Antitumor Immunity in Solid Tumors

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Introduction: Oncolytic measles virus (MV) Edmonston vaccine strains have shown significant antitumor efficacy against glioblastoma (GBM) and metastatic breast cancer in preclinical tumor models and are currently tested in clinical trials. Helicobacter pylori neutrophil-activating protein (NAP) is a Toll like receptor 2 (TLR-2) agonist and potent immunomodulator stimulating proinflammatory cytokines and cytotoxic Th1 immune responses. MV strains expressing secretory NAP (MV-NAP) induced local inflammatory cytokine release and significantly improved survival in mouse models of metastatic breast cancer and will be clinically tested in a soon to be activated clinical trial. We hypothesized that the immunostimulatory properties of MV-NAP will result in synergy when combined with anti-PD1 immune checkpoint blockade and, we investigated the antitumor efficacy of this combination in different solid tumor models. **Methods:** Novel measles virus (MV-s-NAP-UPAR) strains were engineered to encode secretory NAP transgene and retargeted to enter through the urokinase- type plasminogen activator receptor (UPAR). UPAR is a key regulator of plasminogen medicated extracellular proteolysis which controls the capacity of cells to migrate and allows viral entry in murine cells. Syngeneic 4T1 and E0771 breast cancer and GL261 glioblastoma murine models were used to evaluate immunotherapeutic anti-cancer 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-UPAR demonstrated significant oncolytic activity *in vitro* against all solid tumor cell lines tested. Intratumoral administration of MV-s-NAP-UPAR resulted in the development of a proinflammatory environment at the tumor site as shown by increased macrophage and infiltrating T cell recruitment. Repeat administration of combination therapy with intratumoral MV-s-NAP-UPAR and systemic aPD1 elicited a potent anti-tumor immune response: 80% of the mice bearing intracranial GL261 tumors achieved long term survival (>180 days) with combination therapy compared to 40% with single aPD1 immunotherapy (P= 0.021) mainly attributed to the massive influx of lymphoid cells in the brain. At 140 days following initial GL261 inoculation long term survivors were randomized to receive at the opposite side of the brain either B16-F10 melanoma or GL261 cells. All mice rechallenged with GL261 survived while all the B16-F10 challenged mice had to be euthanized due to tumor growth: these results further support the induction of tumor specific immunity. Combination treatment also had antitumor activity in the 4T1 and E0771 breast murine cancer models; experiments to assess the impact on survival rate are currently ongoing. **Conclusions:** Our findings suggest that combination of aPD1 immune checkpoint blockade and oncolytic MV-s-NAP virotherapy is an effective strategy to circumvent immune tolerance mechanisms in solid tumors and to heighten anti-tumor immune responses instigated by the virus.

60. Abstract Withdrawn

Gene Silencing Approaches

61. Combinatorial Gene Therapy for Spinocerebellar Ataxia Type 1

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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 treatment strategies for this disease. Previously, we showed that RNA interference (RNAi)-mediated silencing of *ataxin-1* mRNA provides therapeutic benefit in SCA1 mice models delivered prior to or after disease onset. Independently, we showed that overexpression of human ataxin-1-like equally improves disease readouts using pre-symptomatic delivery strategies. Here, we assess if combining ataxin-1-like overexpression with ataxin-1 knock down can expand the therapeutic dose that was identified for either therapy alone. To test this, we engineered dual expression vectors whereby the miRNA to knock-down ataxin-1 was expressed in the same vector as ataxin-1 like (AAV.miS1.Atxn1L). AAV.miS1.Atxn1L injection to transgenic

SCA1 mice was performed after rotarod deficit onset at varying doses, and impact on disease readouts assessed. Remarkably, our data show that the dual vector can arrest or improve motor deficits at doses orders of magnitude lower than AAV.miS1 alone. These studies show that we can take advantage of a combinatorial approach to reduce the load of RNAi required for therapeutic benefit in SCA1, and possibly other gain-of-function diseases for which RNAi gene therapy is being developed.

62. Evaluation of Programmable Zinc Finger Protein Transcription Factors for the Efficient Reduction of Tau in the Nonhuman Primate Brain

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Many neurodegenerative disorders are closely linked to the accumulation of toxic forms of the Microtubule Associated Protein Tau (MAPT). Collectively referred to as tauopathies, these diseases affect millions and include Progressive Supranuclear Palsy, Frontotemporal Dementia, and Alzheimer's Disease. Several anti-tau therapeutic modalities are in the early stages of clinical investigation, including passive and active immunotherapies, antisense oligonucleotides (ASOs), and small molecule inhibitors, each targeting different proposed intervention points in tau pathology. A large body of preclinical work suggests that lowering intracellular tau either genetically or at the level of the mRNA is well-tolerated and protective against tau and amyloid-beta induced toxicity. Intervening at this upstream stage provides the advantage of remaining agnostic of the ensemble of toxic tau species, which is known to be complex and varied across tauopathies. While repeat administration approaches (eg, ASOs) have shown promise for tau lowering in mice and nonhuman primates (NHP), the development of an AAV-delivered therapy targeting tau at the DNA level could offer important benefits. Among these are the potential for a one-time administration for the lifetime of the patient, and the ability to selectively target and lower tau in a specified cell population. We sought to develop such an approach using programmable zinc finger protein transcription factors (ZFP-TFs), which can be engineered to precisely target any gene and are compatible with AAV delivery technology. We have previously shown that ZFP-TFs can efficiently and specifically lower endogenous tau in the mouse brain, and that this effect is durable and well-tolerated out to at least 11 months. In this work, we fused the human KRAB repression domain to ZFPs that were designed to target the human and NHP tau transcriptional regulatory elements. ZFP-TFs were expressed under the control of a neuronal specific promoter and delivered to human iPS neurons using AAV vectors. We identified two ZFP-TFs for further study that reduced human tau by either ~50% or by >90% with no detectable off-target gene regulation following prolonged AAV ZFP-TF exposure (up to 19 days). Next, we administered the AAV ZFP-TFs to the cynomolgus monkey hippocampus using bilateral, stereotaxic real-time MRI-guided infusion. The procedure was well tolerated and there were no test article-related adverse clinical observations, clinical pathology findings or gross observations at necropsy. After 28 days, we examined tau and ZFP mRNA levels by qRT-PCR. In animals treated with the stronger tau repressor, we observed up to 90% tau lowering in the hippocampus and entorhinal cortex at the bulk transcript level. ZFP levels were strongly correlated with tau reduction both within single animals as well as across treatment groups. Consistent with our *in vitro* data, the ZFP-TF exhibiting ~50% repression in human iPS neurons also achieved up to ~50% repression of NHP tau *in vivo*. Importantly, both ZFP-TFs were equally well tolerated. Our results suggest that the degree of ZFP-mediated tau reduction *in vivo* can be predicted by our *in vitro* screening systems. The precision, efficiency and specificity of tau-targeted ZFP-TFs in preclinical models support their continued development to achieve sustained tau down-regulation for the treatment of human tauopathies.

63. Repression of mHTT Expression in Huntington's Disease Mouse Models by AAV-Mediated Expression of Zinc-Finger Protein-Repressor Transgene

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Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disease caused by a CAG-trinucleotide repeat expansion in exon 1 of the Huntingtin (*HTT*) gene. These CAG repeats lead to the expression and accumulation of mutant Huntingtin protein (mHTT). The degeneration process primarily affects the basal ganglia and cerebral cortex, and the disease is characterized by progressively worsening chorea, and cognitive and psychiatric dysfunction. It is hypothesized that the expanded polyQ region of mHTT acts as a protein-protein interacting domain, forming aggregates with transcription factors, proteasomes or other ubiquitin proteasome system components, resulting in the disruption of normal cellular functions. Since significant, long-term reduction of wildtype HTT protein (wtHTT) in the presence of mHTT poses unknown risk, we designed a drug molecule that can preferentially reduce the expression of mHTT while still allowing the expression of wtHTT. Our strategy was to identify an array of zinc finger proteins (ZFPs), which recognize and bind to trinucleotide DNA sequences, linked to a transcription repressor domain (RD) that would target the stretch of expanded CAG nucleotide repeats on the m*HTT* allele, repress gene expression and prevent the production of mHTT as a potential mechanism to treat HD. A collection of >1,600 ZFP arrays were designed and fused to a RD sequence to identify a ZFP-RD with such allele selectivity. The ability of these ZFP-RD transgene proteins to preferentially repress transcription of the m*HTT* over the wt*HTT* allele was assessed in HD patient fibroblasts and stem cell derived neurons. Quantification of wt*HTT* and m*HTT* transcripts by Taqman-based RT-qPCR assays were conducted to rank allele selectivity for the target mutant gene. In neurons transfected with mRNA encoding the lead ZFP-RD, approximately 90% reduction in m*HTT* mRNA levels was observed whereas levels

of wt*HTT* mRNA remained unchanged across all treatment groups. In addition, results from microarray and RT-qPCR-based analysis suggested that the lead ZFP-RD showed very limited off-target gene repression in the context of the human genome, suggesting that the lead ZFP-RD is selective for the m*HTT* gene. The lead ZFP-RD transgene was packaged into a recombinant Adeno-Associated Viral Vector (rAAV-ZFP-RD). Bilateral intrastriatal injection of rAAV-ZFP-RD was performed in the Q175 and R6/2 HD mouse models to test its effects on mHTT transcripts, soluble mHTT and mHTT aggregates levels. In Q175 mice, the lead rAAV-ZFP-RD reduced mHTT transcripts and decreased the amount of both soluble mHTT and mHTT aggregates in a dose-dependent manner. These effects were present for up to 33 weeks post-injection, in multiple brain regions, including striatum, forebrain and hindbrain cortex. Similarly, in R6/2 mice both mutant transcripts and protein were reduced. Additionally, motor deficits in R6/2 mice, as measured by rotorod and clasping, were improved by the injection of lead rAAV-ZFP-RD, in a dose-dependent manner. Based on these encouraging results, we will execute IND-enabling studies to evaluate this potential clinical candidate for the treatment of HD.

64. Astrocyte Transduction is Required for Rescue of Behavioral Phenotypes in the YAC128 Mouse Model with AAV-RNAi Mediated HTT Lowering Therapeutics

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Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disease caused by a CAG expansion translating into an elongated polyglutamine (polyQ) repeat near the amino-terminus of the huntingtin (HTT) protein. This results in production of a toxic mutant huntingtin (mHTT) protein that leads to neuronal dysfunction and subsequent neuronal death. Currently, no disease modifying treatments are available however numerous therapeutic strategies aimed at lowering HTT levels in the brain are under development. For all of these strategies, cell-type specific targeting is still an outstanding question. While it is clear that mHTT accumulation in medium spiny neurons play a role in their atrophy and ultimate death, there is mounting evidence that glial cells, and astrocytes in particular, play a key role in the disease process. Reducing levels of the disease causative mHTT protein in the brain has proven efficacious at ameliorating disease symptoms in a number of HD mouse models. To date these studies have not closely examined the contribution of mHTT reduction in neurons vs astrocytes and therefore a complete understanding of cell type specific targeting is outstanding. We sought to evaluate this question in the YAC128 mouse model of HD using AAV-mediated RNA interference (RNAi) to selectively lower HTT levels in neurons alone versus neurons and astrocytes. Previously we demonstrated that AAV-RNAi mediated HTT reduction using the AAV1 capsid (which transduces both neurons and astrocytes) is capable of ameliorating HD phenotypes in the YAC128 mouse model of HD. In the current preclinical study, a modified AAV2 capsid (AAV2-HBKO) with a similar distribution profile as AAV1 but strictly neuronal tropism showed comparable suppression of Htt mRNA and protein following injections into the striatum, however, it was not associated with any functional correction of HD phenotype in YAC128 mice. YAC128 mice show a significant rota rod deficit that was not ameliorated when mHTT levels were reduced only in neurons. As previously demonstrated, mice that received AAV1- miRNA HTT however showed significant improvement on behavioral deficits suggesting that AAV-mediated HTT reduction in neurons alone was not sufficient to support full therapeutic benefit in the YAC128 mouse. This work indicates that astrocyte dysfunction may play a critical role in HD pathogenesis and represent an important therapeutic target. Additionally, therapeutics that target only neurons may be inadequate at restoring normal function and ameliorating all disease symptoms.

65. The New Epigenome-Editing Approach for Targeting Dysregulated SNCA Expression: Novel Target Validation for Next-Generation Drug Discovery

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Elevated levels of *SNCA* are causative in the pathogenesis of Parkinson's Disease (PD) and other synucleinopathies, while, normal physiological levels of *SNCA* are crucial to maintain neuronal function. A so-far unmet need is the development of new therapeutic strategies targeting the regulatory mechanisms of *SNCA* expression to fine-tune *SNCA* levels, versus previous approaches that targeted directly the mRNA or the protein product resulting in robust reduction of *SNCA* levels associated with neurotoxicity. We developed a novel strategy targeting the transcription regulation of *SNCA*, based on targeted epigenome editing. Specifically, we established a system for targeted DNA-methylation at *SNCA*-intron 1 that comprises of an all-in-one lentiviral vector, for the delivery of CRISPR/dCas9 fused with the catalytic domain of DNA-methyltransferase3A (DNMT3A). To facilitate the drug discovery pipeline, we first applied the gRNA-dCas9-DMNT3A system into human induced pluripotent stem cells (hiPSC)-derived 'aged' dopaminergic neurons from a PD-patient with the *SNCA* triplication. The experiment resulted in fine-tuned downregulation of *SNCA*-mRNA and protein levels mediated by targeted DNA-methylation at intron 1. Furthermore, the reduction in *SNCA* levels by the gRNA-dCas9-DMNT3A system rescued disease-related cellular-phenotypes characteristics of the *SNCA*-triplication/hiPSC-derived dopaminergic neurons, *e.g.* mitochondrial ROS-production and cellular viability, and nuclear aging signatures (Kantor et al, 2018 Molecular Therapy). Furthremore, usign the methylation-based approach, we report here the correction of *Snca* overexpression in the rat model of PD. Collectively these proof-of-concept experiments provide a strong foundation for advancing the novel epigenome-editing- system further, towards PD therapeutic applications. **Key Word**: *SNCA* expression regulation, DNA methylation, CRISPR/Cas9 genome and epigenome editing, hiPSC-derived neurons, aging, epigenome editing, rat model of Parkinson's disease

66. Significant Reduction of Huntingtin Gene Expression in Cortex, Putamen and Caudate of Large Mammals with Combined Putamen and Thalamus Infusions of VY-HTT01, an AAV Gene Therapy Targeting Huntingtin for the Treatment 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. An expanded trinucleotide repeat in the huntingtin gene (HTT) results in toxic gain-of-function that causes neuronal dysfunction and death, especially in the striatum and cortex. 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 a HTT lowering therapeutic strategy. VY-HTT01 is a potent AAV gene therapy encoding a primary miRNA targeting human HTT mRNA selectively for knockdown. Here, we describe studies in the nonhuman primate on characterizing the pharmacology and tolerability of HTT knockdown with VY-HTT01 in the cortex, putamen and caudate. MRI-guided convection-enhanced delivery into the putamen and thalamus resulted in distribution of VY-HTT01 to the cortex, caudate, putamen and thalamus, and robust suppression of HTT in these regions. Distribution and HTT knockdown in primary motor and somatosensory cortical neurons was demonstrated in laser captured cortical neurons and supported by in situ hybridization (ISH) for vector genomes (VG) and HTT mRNA. Additional endpoints including quantitation of miRNA and HTT protein levels will be reported. Furthermore, good tolerability was supported by in-life observations, clinical pathology and histopathological analysis of the brain 5 weeks after dosing. Taken together, these results demonstrate the potential of VY-HTT01, an AAV gene therapy targeting HTT with RNAi, administered with combined infusions into the putamen and thalamus, for the treatment of Huntington's disease.

CNS Disorders

67. Liver-Directed Gene Therapy Clinical Trial for 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 excretion of glycosaminoglycans. Enzyme replacement therapy (ERT) is currently available for treatment of MPS VI. However, it has several limitations including the requirement of multiple and costly intravenous administrations, and limited efficacy on some disease manifestations. In previous preclinical studies in both small and large animal models of MPS VI, we showed that a single intravenous administration of an adeno-associated viral vector serotype 8 (AAV2/8) encoding *ARSB* (AAV2/8.TBG.*ARSB*) converts the liver into a factory organ for sustained production and secretion of ARSB at therapeutic levels. Based on these results, we have recently started a Phase I/II open label, dose escalation study based on a single intravenous administration of AAV2/8.TBG.*hARSB* in MPS VI subjects of 4 years of age or older. At the time this abstract was written, the first three subjects were enrolled to receive the starting dose (SD) of $6x10^{11}$ genome copies (GC)/kg of body weight of vector. Based on safety data, two additional subjects were enrolled to receive the high dose (HD) of $2x10^{12}$ GC/kg. To date, no severe adverse events related to the investigational drug were observed in all five treated subjects. A slight increase of alanine aminotransferase (ALT) was observed in two subjects (one of the SD and one of the HD cohort) that required treatment with oral prednisolone to blunt potential cell-mediated immune responses to transduced hepatocytes. Enrolled subjects have

withdrawn ERT at least one week prior to gene therapy and no evidence of worsening of disease manifestations was observed in the follow up period post-gene therapy of up to 12 months. Increased serum ARSB activity peaking between 20 and 30% of normal was detected in four of the five subjects and one subject with no detectable serum ARSB showed higher levels of anti-ARSB antibodies. Further clinical data will be collected in these five patients and additional subjects that will be enrolled to receive the HD of vector. Nevertheless, the data collected so far show that systemic administration of AAV8 is safe and can result in effective gene transfer as shown by sustained ARSB expression for at least one year.

68. A Clinical Glucocerebrosidase Lentiviral Vector Corrects the Pathology and Clinical Signs in a Mouse Model for Type 1 Gaucher Disease

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Type 1 Gaucher disease is an inherited lysosomal storage disorder with multisystemic effects, including hepatosplenomegaly, cytopenias and bone disease with varying degrees of severity. The disorder is caused by mutations in the glucosidase beta acid 1 (GBA1) gene resulting in insufficient activity of the enzyme glucosylceramidase (glucocerebrosidase), leading to a progressive accumulation of the lipid component glucosylceramide. Enzyme replacement therapy (ERT) is the standard treatment for Gaucher patients. Though effective in alleviating symptoms, ERT is costly, is not curative and restricts patients to lifelong infusions of the enzyme. Gene therapy represents a potential future therapy for type 1 Gaucher patients. Here, we use a clinical, GMP produced, single gene self-inactivating lentiviral vector (LV2) containing the glucocerebrosidase gene driven by the elongation factor 1α short (EFS) promoter to treat mice with type 1 Gaucher disease. Deletion of exons 9-11 of the GBA1 gene was accomplished by mating the mice with Mx-Cre mice and induction of Cre expression by polyIC. The mice develop increasing accumulation of glucosylceramide over time with splenomegaly and anemia (Enquist et al, PNAS, 2006). Lineage negative bone marrow cells were transduced with the LV2 vector and transplanted into irradiated Gaucher type 1 mice, 2-3 months after induction of the gene deletion (early intervention) and 5-8 months after induction of disease (late intervention, when considerable splenomegaly has developed). Mock transduction was used as a negative control. Five months after gene therapy, the mice were sacrificed and analyzed for enzyme expression, histopathology and glucosylceramide accumulation in bone marrow, spleen and liver. Histopathology showed prominent accumulation of Gaucher cells in bone marrow, spleen, liver and thymus of mock transduced mice whereas there was a prominent reduction of Gaucher cell infiltration in these tissues in vector treated mice. Mass spectrometry analysis of glucocerebroside in bone marrow, spleen and liver demonstrated highly significant reduction in glucocerebroside accumulation in vector treated animals ($p \leq 0.0001$ in the early intervention study and $p \leq 0.05$-0.001, depending on tissue, in the late intervention study). These data strongly support that gene therapy of Gaucher type 1 mice using this clinical lentiviral vector is very efficient and demonstrates robust efficacy in the mouse disease model. Furthermore, clearance of glucosylceramide resulted in reversal of splenomegaly, a restoration of blood parameters and reduced infiltration of Gaucher cells in hematopoietic tissues. Vector integration site analysis for the LV2 vector in the mouse disease model is in progress. Cumulatively, our preclinical findings support the initiation of a clinical trial for type 1 Gaucher disease using the clinical lentiviral vector described here to transduce hematopoietic stem cells from Gaucher type 1 patients followed by blood and marrow transplantation.

69. Gene Editing to Treat Both Tay-Sachs and Sandhoff Diseases

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The GM2-gangliosidoses are genetic disorders causing severe neurological diseases and premature death, thus developing effective treatment protocols is urgent. GM2-gangliosidoses result from deficiency of a lysosomal enzyme β-hexosaminidase (Hex) and subsequent accumulation of GM2 gangliosides. Genetic deficiency of HEXA, encoding the Hex α subunit, or HEXB, encoding the Hex β subunit, causes Tay-Sachs disease (TSD) and Sandhoff disease (SD), respectively. Previous studies have showed that a modified human Hex μ subunit (HEXM), incorporating sequence of both α and β subunits, can treat both TSD and SD by forming a homodimer to degrade GM2 gangliosides. To this end, we applied this HEXM subunit in our PS-813 gene editing system to treat neonatal SD mice. Four months after the intravenous administration of AAV vectors, plasma Hex A and Hex total activities reached up to 144 and 17 fold of wildtype levels in treated SD mice (n=10), respectively. Hex A and Hex total activities in the liver increased to 25 and 8 fold of wildtype levels, respectively. More importantly, Hex A and Hex total activities in the brain also increased significantly compared with untreated SD mice ($p<0.05$), which was further supported by immunohistochemistry of brain for Hex proteins. Further, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis showed that GM2 gangliosides in multiple tissues of treated mice were reduced to normal levels. Rotarod analysis showed that coordination and motor memory of treated mice were improved compared to untreated SD mice ($p<0.05$). Histological analysis of H&E stained tissues showed reduced cellular vacuolation in the brain and liver of treated SD mice. Results from this study will be directly applicable for developing a clinical protocol of *in vivo* genome editing

to treat TSD and SD patients. As a platform strategy, the proposed study is broadly significant in providing "proof of principle" evidence for treating other lysosomal diseases.

70. Sustained Long-Term Neurological Correction Following Haematopoietic Stem Cell Gene Therapy in Mucopolysaccharidosis IIIB Mice

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Currently there are no effective treatments to remedy the severe neurodegeneration experienced by Mucopolysaccharidosis type IIIB patients. These patients lack a functional copy of the NAGLU gene that codes for the lysosomal enzyme alpha-N-acetylglucosaminidase, an essential component of the heparan sulphate degradation pathway. Absence of functional NAGLU results in the accumulation of undegraded heparan sulphate (HS) in lysosomes causing cellular dysfunction which is particularly damaging to the brain and is accompanied by severe neuroinflammation, leading to cognitive decline. Lentiviral-driven (LV) haematopoietic stem cell gene therapy (HSCGT) is a promising therapy to enable delivery of NAGLU enzyme to the brain following the migration and engraftment of monocytes into the brain. We have previously reported on 6 month outcomes and here present long-term effectiveness of LV-NAGLU HSCGT in MPSIIIB mice compared to wildtype (WT) HSCGT at 10 months post treatment. We observed improved long-term expression of NAGLU in the brain of LV-NAGLU treated mice, with significant increases in enzyme levels detected over time with 26% wildtype NAGLU activity observed at 10 months post-transplant compared to 13% at 6 months post-transplant. HSCGT with WT donor cells only provided 4% WT enzyme levels at 10 months post-transplant, significantly lower than LV-NAGLU treatment. Nine-fold higher levels of HS were recorded in the brains of untreated MPSIIIB animals versus WT, with significant increases in HS sulphation levels. Effective NAGLU delivery to the brain by LV-NAGLU HSCGT leads to complete removal of excess HS, normalisation of HS sulphation patterning and reversal of neurodegeneration as confirmed by the sustained correction of astrocytosis, microglial activation and CNS lysosomal swelling in cortical, striatal and hippocampal regions. Furthermore, LV-NAGLU treatment provided sustained correction of HS storage in the periphery with normal HS levels and sulphation patterning observed in the liver, significantly better than WT HSCGT alone. Our data indicates that a minimum vector copy number of 1.07 in bone marrow cells had a 95% confidence of delivering sufficient enzyme activity in the brain for effective heparan sulphate clearance and reversal of neuropathology. This information will be critical in determining treatment condition requirements to obtain clinical efficacy and sustained benefit in MPSIIIB patients.

71. Infant Macaques Exhibit Safe and Sustained Expression of Human Iduronidase after Receiving an Intrathecal Cervical Injection of Adeno-Associated Virus 9

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Many neuropathic diseases cause early and irreversible neurologic deterioration, thus warranting therapeutic intervention during the first months of life. Mucopolysaccharidosis type I (MPS I) is a recessive lysosomal storage disorder resulting from deficiency of the lysosomal enzyme, α-l-iduronidase (IDUA). One of the most promising treatments for MPS I is the restoration of enzyme expression through gene therapy. Specifically, administering a neurotropic adeno-associated virus (AAV) that encodes IDUA into the cerebrospinal fluid via suboccipital injection into the cisterna magna is remarkably effective in large animals. Preclinical safety studies in adult nonhuman primates have supported a positive risk-benefit profile for this procedure while highlighting potential sub-clinical toxicity to the primary sensory neurons in the dorsal root ganglia. Here, we examined the long-term performance of a suboccipital AAV serotype 9 gene transfer of human IDUA into four infant (one-month-old) rhesus monkeys. Half of the animals (n = 2) were tolerized at birth via systemic administration of an AAV8 vector that expressed the same transgene from the liver. All animals exhibited a sustained expression of the transgene over nearly four years without demonstrating any acute or chronic toxicity. Transduced cells were mostly pyramidal neurons from the cortex and hippocampus, Purkinje cells from the cerebellum, lower motor neurons, and dorsal root ganglia neurons. Immunohistochemical analysis of the brain tissue revealed that both tolerized and non-tolerized animals maintained transgene expression. However, the presence of antibodies in the non-tolerized animals prevented detecting measurable levels of secreted enzyme in the cerebrospinal fluid. These results support the idea that it is safe and effective to treat newborn nonhuman primates with AAV9 gene therapy via suboccipital delivery.

72. Systemic mRNA Therapy as a Treatment for the Inherited Metabolic Liver Disorder Arginase Deficiency

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Urea cycle disorders (UCDs) are incurable genetic diseases that affect the body's ability to produce urea, typically leading to hyperammonemia. For arginase (A1) deficiency, a mutation in the ARG1 gene, the final step of the cycle, results in primarily hyperargininemia along with developmental delays, spastic diplegia, psychomotor function

loss, and uncommonly, death. There is currently no completely effective treatment available. While preclinical strategies to treat A1 deficiency have been demonstrated, significant disadvantages include risk for insertional mutagenesis and potential limited efficacy due to hepatocellular division with viral-based treatments and lack of availability of cellular transplant-based therapies. However, advances in mRNA codon optimization and synthesis and their encapsulation within biodegradable liver-targeting lipid nanoparticle (LNP) technologies have potentially enabled a new generation of safer, albeit temporary, treatments to restore liver metabolic function in patients with UCDs such as A1 deficiency. In this study, we applied such technologies to successfully treat a preclinical conditional A1-deficient murine model (A1-CKO). On Day (D)0, adult A1-CKO mice were administered intravenously (IV) $2x10^{11}$ genome copies of AAV-Cre recombinase to disrupt endogenous hepatic A1 expression. Beginning D14, mice were given IV either LNP-encapsulated control firefly Luciferase mRNA (LNP-Luc) or human codon-optimized A1 mRNA (LNP-A1). Group 1 was administered LNP-mRNA weekly and Group 2 every 72hrs. LNP-A1 Group 1 mice (n=10) demonstrated significant life extension compared to LNP-Luc mice (n=10) ($p<0.001$), but none exhibited long-term survival and stable weight; all mice died or were euthanized for humane endpoints by D62. In contrast, LNP-A1 Group 2 mice (n=10) exhibited significant life extension ($p<0.001$) and 100% survival with no signs of hyperammonemia or weight loss beyond D77 compared to LNP-Luc mice (n=10) that all perished by D22. Plasma ammonia levels showed significant increases in all Group 1 mice, peaking at death ($p=0.0007$) while LNP-A1 Group 2 mice demonstrated good control and no statistically significant increases throughout the study. LNP-A1 Group 2 mice demonstrated adequate urea cycle function by fully metabolizing an ammonium challenge after 1hr as assessed by plasma ammonia concentration and urea enrichment. Western blots of LNP-A1 Group 2 mice showed strong presence of exogenous A1 in the liver and IHC confirmed A1 localization in periportal and interportal hepatocytes similar to wild-type (WT) controls. A functional A1 assay of LNP-A1 Group 2 mice demonstrated significant recovery of functional liver A1 activity corresponding to 54% of WT liver levels ($p<0.001$). LNP-A1 Group 2 mice plasma amino acids demonstrated no statistically significant increases of arginine or glutamine throughout the treatment compared to LNP-Luc mice. No statistically significant increases of plasma ALT, a liver injury marker, compared to WT mice were detected. In this study, we demonstrated the ability to successfully treat a preclinical A1-deficient murine model by systemic administration of LNP-A1 leading to long-term survival. While not detected in our studies, future studies aim to minimize risk for long-term liver toxicity by further optimization of LNP-A1 dose and frequency. While viral- and cellular transplant-based strategies continue to be developed for clinical use, systemic mRNA administration by encapsulation in liver-targeting LNPs demonstrate a tangible, rapid, and potentially widely applicable modality to treat UCDs and other single enzyme liver deficiencies.

Development of Manufacturing Processes for Cell Based Therapies

73. Combining CD5-Targeted Chimeric Antigen Receptor Engineering and Genetic Editing of TGF-BR2 for the Treatment of T-Cell Hematologic Malignancies

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Patients with primary refractory or relapsed T cell lymphoma and leukemia (TCL) have very poor prognosis with few effective targeted therapies. This contrasts with patients who have B-cell acute lymphoblastic leukemia or large B cell lymphomas (DLBCL), for whom the striking clinical success of CD19-specific CAR-T-cell therapy has resulted in two FDA-licensed products. Development of equivalent ACTs that target neoplasms of T cell origin has been hindered by the shared expression of most targetable antigens on both malignant T cells and normal T lymphocytes, which would lead to fratricidal killing of the CAR-expressing effector cells during CAR-T-cell manufacturing and to potential severe T-cell immunodeficiency upon adoptive transfer. CD5 is an attractive target since it is expressed by all T cell malignancies, however it is also expressed on normal T cells; thus, engineering T cells to express a CAR against CD5 will be complicated by fratricide. NK cells have the advantage over T-cells that they do not express CD5 and will therefore not be targeted by CAR.CD5-mediated fratricide. Moreover, NK cells do not cause graft-versus-host disease (GVHD) and thus open opportunities to produce an off-the-shelf product for immediate clinical use. Our group has developed a novel strategy to genetically modify cord blood (CB)-derived NK cells to express a CAR, ectopically produce IL-15 to support NK cell proliferation and persistence in vivo, and express a suicide gene - inducible caspase 9 (iC9), to address any potential safety concerns. As proof of principle, we have initiated a first-in-human, phase I/II clinical trial of iC9/CAR19/IL15-NK cell therapy following lymphodepleting chemotherapy in patients with relapsed/refractory B-cell lymphoid malignancies with very promising results. We now propose to extend this approach to target T-cell malignancies by genetically modifying NK cells to express a CAR against CD5 and to ectopically produce IL-15. In a series of in vitro and in vivo experiments, we have confirmed the anti-T-ALL activity of iC9/CAR.5/IL-15 transduced CB-NK cells as evidenced by increased cytokine production (TNFa $p= <0.0001$, IFNg $p= 0.0019$), degranulation (CD107a $p= 0.0186$) and enhanced cytotoxicity against both T cell ALL cell lines (CCRF-CEM, MOLT3) and primary leukemic cells as shown by Cr^{51}-release assay ($p=0.001$ at 1:1 ratio, $p=0.008$ at 5:1 ratio, $p=0.01$ at 10:1 ratio, $p=0.02$ at 20:1 ratio). Moreover, In a xenogeneic NSG mouse model of CCRF-CEM T-ALL, iC9/CAR.5/IL-15 transduced NK cells exerted significantly better anti-tumor activity and improved survival

of mice compared to animals treated with non-transduced NK cells or IL-15 transduced NK cells (p=0.01). Finally, based on our findings that TCL express high levels of of the immunosuppressive cytokine TGF-β, we developed a protocol for combined iC9/CAR.5/IL-15 retroviral transduction and Cas9 ribonucleoprotein (Cas9 RNP)-mediated gene editing of *TGF-βR2* in order to protect CAR-NK cells from the highly immunosuppressive tumor microenvironment. *TGF-βR2* knockout resulted in significantly enhanced tumor killing by iC9/CAR.5/IL-15 transduced CB-NK cells compared to Cas9 control cells both in vitro and in vivo. These preliminary data indicate that *TGF-βR2* KO in iC9/CAR.5/IL-15-transduced CB-NK cells could further enhance their anti-tumor activity, making them more effective at circumventing the immunosuppressive tumor milieu

74. Single Day CAR Manufacturing Platform Using mRNA and Flow Electroporation Technology

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We have developed a rapid and potent cell therapy that utilizes mRNA in transient electro-transfection (ELT) to produce gene-modified cell therapy, termed CARMA™. The CARMA platform modifies peripheral blood mononuclear cells (PBMC) from an apheresis to generate a cryopreserved drug product in a single day manufacturing process using the cGMP-compliant, closed MaxCyte GT® Transfection System, dramatically reducing labor, facilities, and raw materials costs. We report here the implementation of CARMA platform to produce an anti-mesothelin scfv, modified PBMC cell therapy product, MCY-M11. MCY-M11 introduces a chimeric antigen receptor to all cells in the preparation, including active (NK- and T-cell) and bystander (B-cell and Monocyte) cells, which are processed and cryopreserved without the need for activation or expansion. The clinical production of MCY-M11 was manufactured by MaxCyte at a Contract Development Manufacturing Organization (CDMO). Six (6) Proof of Concept runs and seven (7) Engineering Runs were performed during tech transfer campaign, with analytical qualification and supply chain established. Cell yields of PBMC from apheresis processed using manual ficoll method fluctuated with donor variability, ranges from 40-60%. Cell yields from the pre-ELT to post-ELT samples averaged around 78% (37-92%). Four major subsets of mononuclear cells are generated and gene modified simultaneously: T-lymphocytes (CD4 and CD8), B-lymphocytes, Natural Killer Cells, and Monocytes. Expression of scfv is measured using biotinylated mesothelin, with an average CD3 subset expression of 73% (42-83%), and average NK expression of 59% (28-75%). Cytolytic potency is assessed via bioactivity in an in vitro cytolytic assay against mesothelin-expressing tumor cells. TNF-alpha cytokine release was demonstrated against ovarian cancer cell line OVACAR3. Manufacturing release specifications are preliminarily assigned, with multiple For Information Only (FIO) data points being accumulated during clinical production, before sufficient clinical data is available for evaluation to establish meaningful release specifications. Assays performed for individual production runs are monitored using a CARMA assay control material derived from one of the GMP non-clinical production runs. Animal studies using mesothelin-positive cell lines and non-clinical safety testing were performed. The product is currently being tested in a first in human clinical trial for advanced epithelial ovarian cancer and peritoneal mesothelioma (ClinicalTrials.gov Identifier: NCT03608618). The CARMA one-day manufacturing process using cGMP mRNA has the potential to significantly transform CAR therapy strategies by providing a meaningful reduction in wait time for patients receiving treatment.

75. Titratable and Reversible Regulation of Therapeutic Proteins in Cell and Gene Therapies Using FDA Approved Drugs and a Modular Protein Stabilization Platform

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Cell and gene therapies have shown remarkable clinical success and durable benefit in B-cell lymphomas as well as inherited diseases such as sickle cell anemia and hemophilia. Precise regulation of transgene expression can significantly improve safety and efficacy of cell and gene therapies and enable the use of such therapies in broader indications and patient populations. Ideally, transgene regulation should be rapid, reversible and titratable using exogenously administered drugs. Moreover, the direct regulation of protein half-life rather than through the encoding DNA or mRNA is a preferable regulatory strategy both for its broad applicability and for rapid kinetics. We used destabilizing domains (DDs) to achieve the desired performance metrics in the regulated control of therapeutic protein expression. DDs are small protein domains that are misfolded and inherently unstable in the cell but can be reversibly stabilized by the binding of a small molecule ligand. This conditional stability of DDs can be readily conferred to any protein of interest (POI) by fusing it to the DD. In the absence of ligand, the DD-POI fusion protein is rapidly degraded by the proteasome. However, addition of ligand stabilizes the fusion protein in a dose-dependent manner. We reasoned that DDs for use in cell and gene therapy should be (a) regulated by FDA-approved drugs for safety, translatability and to reduce development risk, (b) based on fully human proteins to reduce immunogenicity risk, and (c) small and modular to minimally impact the functionality of the therapeutic cargos. Specifically, we identified Dihydrofolate Reductase (DHFR), Phosphodiesterase 5 (PDE5), Estrogen receptor alpha (ERα), and Carbonic Anhydrase II (CAII) as substrates to engineer new DDs. We used a variety of strategies to identify and characterize multiple DD variants based on amino acid substitutions in these substrates with low basal expression and high dynamic range upon ligand addition. The DDs rapidly induced expression of DD-POI upon treatment with ligand, whereas expression reverted to basal levels within a few hours of ligand removal. These new DDs were used to regulate cytosolic proteins such as green fluorescence protein (GFP), transcriptional factors such as FOXP3, membrane proteins such as CD40 ligand and CD19 chimeric antigen receptor, and the secreted cytokines IL12 and IL15. After ligand addition, DD-regulated POIs showed functional responses indistinguishable from native POI. We could also regulate two different POIs in the same cell using distinct DD drug pairs, demonstrating the potential for multiplexed regulation. By incorporating various transcriptional and translational elements,

we were able to achieve drug-dependent regulation of DD-POIs over a wide range of expression levels. We found the performance of the DDs to be generally similar when expressed in a variety of cell types including a number of immortalized cell lines as well as primary human and mouse T-cells. We also assayed the effect of DDs on T-cell activation, cytotoxic activity, cytokine production and endoplasmic reticulum stress and did not observe any significant differences. We further tested DD regulation *in vivo* by adoptive transfer of primary human T cells transduced with lentivirus vector carrying DD-regulated IL12. After ligand administration, human IL12 was detected in the plasma of mice transplanted with IL12-DD modified human T cells. The level and duration of IL12 detected was proportional to the dose and timing of ligand administration. In summary, DDs can couple titratable and reversible transgene regulation with clinically approved drugs to enable safe and efficacious cell and gene therapies.

76. 3D Microculture Platform Enables Advanced, High-Throughput Screening for Differentiation of hPSC-Derived Cell Therapies

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Introduction: The promising outlook for hPSC-derived cell therapies motivates the development of manufacturing processes to meet the patient demand for such therapeutics. Toward this aim, 3D culture systems for hPSC differentiation are emerging because of their potential for higher expansion and yield of target cell types compared to 2D culture systems. Therefore, the ability to screen through a multifactorial parameter space of exogenous biochemical cues for 3D hPSC cultures would greatly accelerate the pace of discovery and development of efficient *in vitro* differentiation protocols for target cell types of interest. **Methods:** Here, we demonstrate the advanced capabilities of a 3D micro-culture platform to screen dosage, duration, dynamics, and combinations of 12 culture parameters, totaling more than 1000 unique 3D culture environments, to derive Olig2+Nkx2.2+ oligodendrocyte progenitor cells (OPCs) from hPSCs with 0.2% of the reagent volumes used in 96-well plates. Additionally, we leverage novel fluorescent hPSC reporter cell lines, mediated by Cas9 mediated knock-in, to monitor proliferation and differentiation *in situ* for over 80 days in the 3D micro-culture system. **Results:** We identified several early culture parameters that could be tuned to increase the Olig2 expression 10-fold. In addition, we observed different sensitivities to signaling pathways across time. Finally, a holistic analysis using statistical models uncovered and ranked parameters and combinations thereof according to their positive effect on OPC differentiation to prioritize them in future optimizations. To show the generalizability of the platform, we then applied it to simultaneously assay 90 unique differentiation protocols to derive TH+ midbrain dopaminergic neurons from hPSCs. **Conclusion:** Overall, we demonstrate a strong methodology for upstream microscale screening/optimization to inform downstream scale-up processes to improve 3D production strategies of hPSC-derived cell replacement therapies.

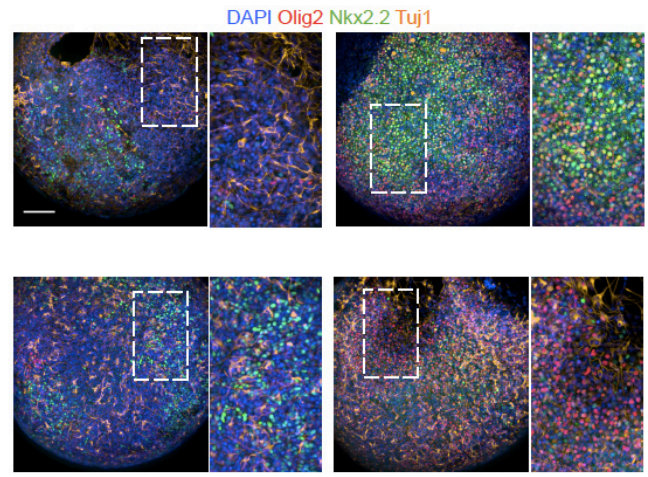

Figure 1. Four representative microcultures from the screen that depict wide variation in levels of Olig2, Nkx2.2, and Tuj1 expression; scale bar represents 100 microns.

77. 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. The most successful CARs used to date are those targeting CD19, which offer the prospect of complete remissions in patients with chemorefractory/relapsed B cell malignancies. The use of recombinant retroviral vectors for 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 variations in vector copy number. CAR T cells engineered in this manner are capable of tumor eradication, but these T cells are prone to tonic signaling and accelerated exhaustion which diminish their anti-tumor activity. We have developed a novel genetic engineering strategy to insert CAR genes into a precise genomic location in human peripheral blood T cells. Specifically, T cells are transfected with modified Cas9 mRNA and CRISPR guide RNA via electroporation, followed by the addition of a recombinant Adeno-Associated Virus (AAV6) that encodes the CAR sequence. Upon CRISPR/Cas9-mediated cleavage, cells repair this DNA double-stranded break using the AAV DNA, which contains DNA sequences flanking the CAR gene that are homologous to the target genomic DNA at the cleaved site. The repair process allows the insertion of the CAR gene upstream of the constant region of the expressed TCR alpha chain gene, which results in CAR expression controlled by the endogenous TCR promoter (TRAC) while at the same time disrupting TCR alpha chain expression leading to the abrogation of surface TCR expression. Directing a CD19-specific CAR under the transcriptional control of the human T cell receptor alpha constant (TRAC) promoter

not only results in uniform CAR expression in human peripheral blood T cells, but also delays T-cell differentiation and exhaustion, leading to enhanced T cell function and anti-tumor efficacy, with edited cells vastly outperforming retrovirally modified CAR T cells in a mouse model of acute lymphoblastic leukemia (Eyquem, Mansilla-Soto et al, Nature, 2017). The targeting of CARs to the TCR locus thus 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), leading to a better-defined T cell product and a more effective T cell. 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 (Feucht et al, Nat. Med. 2019). 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 a large-scale electroporator, we have demonstrated that we can knockout the TCR at large scale (100 E06 CD3+ T cells) with the same efficiency (>80%) as that obtained at small scale. We are evaluating different sources of AAV6 to deliver the CAR 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 in NSG mice bearing pre-established pre-B ALL NALM-6 tumors.

78. Reprogramming Human Immune Responses Using Ex Vivo MSC Bioreactor Therapy

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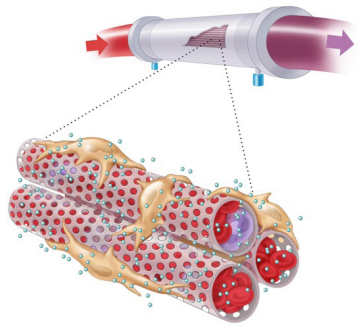
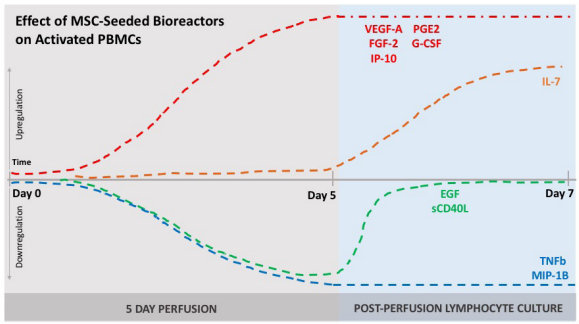
Mesenchymal stromal cells (MSCs) have long been shown to secrete potent immunomodulatory molecules and extracellular vesicles. However, delivery of these cells has yielded limited success in clinical applications thus far, likely due to ineffective dosing and reduced exposure times. To overcome these hurdles, Sentien Biotechnologies developed SBI-101, an allogeneic MSC-seeded bioreactor which may allow for increased potency and controlled delivery of MSC-produced factors. This product consists of a continuous flow bioreactor with MSCs immobilized on the extraluminal side of a semi-permeable hollow-fiber membrane. Patient's blood flows through the lumen of the hollow fibers and is conditioned by MSC-secreted factors, allowing for the blood cells and MSCs to sense their environment and react to it. The SBI-101 therapy is being tested in a Phase I/II clinical trial for treatment of acute kidney injury.



To study the effect of MSC-secreted factors delivered in this way, Sentien developed a scaled-down bioreactor system capable of probing the contact-independent communication of MSCs and inflamed human blood cells in vitro. We aimed to characterize the behavior of MSCs delivered via this system, then explore therapeutic efficacy through treatment of immune cell populations. MSCs were seeded into the extracapillary space of hollow-fiber bioreactors at doses ranging from 0 (acellular control) to 9 million cells per device. Bioreactors were then perfused with media up to 8 days, with metabolite and cytokine analyses performed on samples collected longitudinally. Data showed the consumption of nutrients and production of cellular byproducts in a dose-dependent manner, indicating the maintenance of metabolically active MSCs over the perfusion time-course. Furthermore, MSCs were found to secrete a range of factors, including immunomodulatory and angiogenic, when immobilized within this system. Upon introduction of inflammatory stimuli to the in vitro system, MSCs were observed to modulate their secretion of factors and extracellular vesicles in response. There was an increase in vesicle size as well as the production of immunomodulatory factors, including IL-6 and PGE2. Following characterization of the bioreactor, activated PBMCs were added to the perfusion medium to probe the effect of MSCs on inflamed blood cells. MSC treatment was found to regulate the inflammatory milieu through modulation of both cytokines and extracellular vesicles, including suppressed T cell proliferation as well as decreased cytotoxic and activated T cell populations. Conditioned medium and cells harvested from the perfusion circuits were further challenged via post-perfusion static assay. Treatment strategy was shown to influence lymphocyte and macrophage phenotype, proving a durable reprogramming effect. Thus, we report the ability of a scalable hollow-fiber bioreactor to effectively maintain MSC function and impart an immunomodulatory effect on inflamed immune cell populations, laying the framework for a new generation of ex vivo cellular therapeutics.



Pulmonary Gene Therapy

79. Assessment of a Second Generation, Oxidation Resistant Gene Therapy for Alpha 1-Antitrypsin Deficiency

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Alpha 1-antitrypsin (AAT) deficiency, an autosomal recessive hereditary disorder characterized by low serum levels of functionally active AAT, is associated with early development of panacinar emphysema. AAT, a 52 kDa serine antiprotease produced in the liver, functions to inhibit neutrophil elastase (NE), protecting the lung from NE destruction. Cigarette smoke and/or pollution-mediated oxidation of methionine (Met) residues located near the active site of AAT renders AAT incapable of inhibiting NE, accelerating the development of emphysema in AAT deficiency. Based on studies demonstrating that modification of Met residues at amino acid positions 351 and 358 to valine (Val) and leucine (Leu), respectively, results in an effective and oxidation-resistant modified AAT, we hypothesized that a one-time administration of a serotype 8 adeno-associated virus (AAV8) gene transfer vector coding for this second generation oxidation-resistant modified human AAT will maintain activity and inhibit NE even in the presence of an oxidative milieu. To evaluate this hypothesis, we administered $4x10^{10}$, 10^{11}, or $4x10^{11}$ genome copies (gc) of AAV8 coding for the expression of the oxidation resistant variant hAAT(A213/V351/L358; "8/AVL") via intravenous (IV) and intrapleural (IPL) routes to male and female 6-8 wk old C57Bl/6 mice (n=5/group). AAV8 coding for the wild-type AAT [hAAT(A213/M351/M358); "8(AMM)," $4x10^{11}$ gc], and AAV8Null ($4x10^{11}$ gc) were used as controls. Serum and lung epithelial lining fluid (ELF) hAAT levels and function were assessed at various time points. Human AAT levels (ELISA) showed high, dose-dependent levels of hAAT in 8/AVL administered to mice IV or IPL. Six month following the high dose IV administration of 8/AVL to male mice, serum AAT levels were 1826 ± 201 μg/ml and lung ELF AAT levels 29 ± 2.6 μg/mg protein; normalized to total protein, ELF to serum AAT level ratio (per mg protein) was 0.7 ± 0.1. Similarly, 6 months following IPL administration of 8/AVL to male mice, serum AAT levels were 1785 ± 455 μg/ml and lung ELF AAT levels 30.2 ± 4.7 μg/mg protein, ELF to serum ratio (per mg protein) 1.1 ± 0.2. A similar pattern was seen in female mice. While the serum and ELF levels with IV and IPL administration of 8/AVL was similar to that of the control 8/AMM ($p>0.09$, all comparisons), very different results were observed when the serum and ELF were assessed in the context of AAT anti-NE activity in the presence of oxidizing agents N-chlorosuccinimide (NCS) or H_2O_2. Importantly after 1, 3 or 6 months, serum and ELF from 8/AVL administered mice retained the ability to inhibit NE in the presence of high concentrations of oxidizing agents compared to controls, whereas serum and ELF from 8/AMM mice had 3-4-fold less NE inhibiting activity. Overall the data demonstrates that both intrapleural or intravenous administration of 8/AVL to C57Bl/6 mice provides high, persistent serum and ELF levels of human AAT equivalent to the wild-type (8/AMM) for at least 6 months. However, unlike the wild type 8/AMM which is rendered incapable of inhibition of NE in the presence of oxidant stress, expression of AAV8hAAT(A213/V351/L358) variant retains its anti-protease activity despite an excessive amount of oxidant stress. This 2nd generation gene therapy for AAT deficiency is ready to be tested in the clinic in AAT deficient individuals.

80. Lentivirus Gene Therapy for Autoimmune Pulmonary Alveolar Proteinosis

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Pulmonary alveolar proteinosis (PAP) is a lung disease characterised by the accumulation of surfactant, leading to respiratory failure and premature death. PAP is an ultra-rare disease with an estimated incidence of 0.36 cases per million. In 90 to 95% of cases, PAP is caused by the generation of anti-granulocyte-macrophage colony stimulating factor (GM-CSF) auto-antibodies (auto-immune PAP). The standard of care is whole lung lavage (WLL), which only leads to temporary remission of symptoms, needs to be performed repeatedly and has significant side-effects. More recently, administration of recombinant GM-CSF has shown encouraging results, but requires frequent administration and is expensive. We have developed a lentiviral vector, pseudotyped with the Sendai virus F and HN proteins (rSIV.F/HN) which transduces lung epithelium efficiently and persistently. We have now assessed whether rSIV.F/HN-mediated gene transfer can ameliorate PAP lung disease in GM-CSF knockout mice. Here we show that: (1) high titers of rSIV.F/HN-murine (m)GM-CSF (9.23e9 TTU/ml) can be produced using Good Manufacturing Practice (GMP) compatible serum-free production systems. (2) *In vitro* gene transfer leads to dose-related (MOI 0.1, 1, 10 and 100) mGM-CSF expression (median: 0; 290; 6,084; and 3,7322 pg/mg respectively; n=6). (3) mGM-CSF produced after lentivirus transduction is functional when assessed in a mouse myeloid factor dependent continuous-Paterson (FDC-P1)

cell proliferation assay. (4) Significant (mean 230.3 pg/ml ±26.8 (SEM), n=6) and stable expression (7 months) of mGM-CSF can be achieved in an *ex vivo* human air-liquid interphase model. (5) Transduction of mice by nasal sniffing (1e7 TU/mouse, n=10/group) generates significant (p<0.0001) levels of mGM-CSF in bronchoalveolar lavage fluid (BALF) (treated: median 3,321 (range: 331-7958) pg/ml; controls: 0.1 pg/ml. (6) mGM-CSF expression significantly ameliorates PAP-disease specific biomarkers in GM-CSF knockout mice two months after a single administration of rSIV.F/HN-mGM-CSF (1e7 TU/mouse, n=15-20/group), with significantly decreased BALF turbidity and surfactant protein D (SP-D) levels (p<0.001). These results provide proof-of-concept for gene therapy as a potential new treatment for PAP. Duration of gene expression as well as the efficacy-toxicity window is currently being characterised.

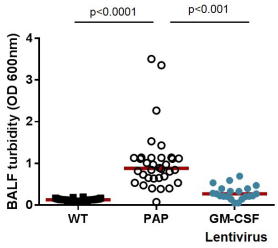

Figure 1. Turbidity (OD_{600nm}) of bronchoalveolar lavage fluid (BALF). Lung fluid turbidity is increased in the GM-CSF-KO mice (PAP, open circles) when compared to wild-type (squares) mice (n=28/group, p<0.0001). *In vivo* gene transfer significantly reduced BALF turbidity (1e7 TU/mouse, n=21, p<0.0001).

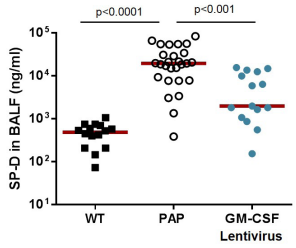

Figure 2. Surfactant protein D (SP-D) in bronchoalveolar lavage fluid. Surfactant component SP-D is increased in GM-CSF-KO mice (PAP, open circles) when compared to wild-type (squares) mice (n=20/group, p<0.0001). *In vivo* gene transfer significantly reduced SP-D levels (1e7T U/mouse, n=15, p<0.001).

81. Gene Correction of Cystic Fibrosis Mutations In Vitro and In Vivo Mediated by PNA Nanoparticles

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Cystic fibrosis (CF) is a devastating genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The location and nature of these mutations can dictate the severity of the disease, and while there are newly approved therapeutics for some of the most common mutations, such as the Class II mutation F508del, there are many mutations for which there are no currently available therapies. Class I premature stop codon mutations including W1282X and G542X are among the most severe and untreatable cases. Moreover, currently available therapies do not correct the underlying genetic defect. Genome editing therapeutics that are able to correct the disease-causing mutation(s) could provide a promising treatment option applicable to all CF patients. We have sought to correct CF-causing mutations using a non-nuclease-based peptide nucleic acid (PNA) gene editing technology. Triplex-forming PNAs are designed to bind genomic DNA and form PNA/DNA/PNA triplexes, which stimulate endogenous DNA repair via nucleotide excision repair and homology-dependent repair pathways. When PNAs are co-delivered with a mutation-correcting donor DNA in a polymeric nanoparticle (NP) formulation, site-specific gene correction can occur. We have previously demonstrated that biodegradable polymeric NPs loaded with PNA and donor DNA designed to edit the F508del mutation resulted in significant gene correction in the lung and nasal epithelium accompanied by disease amelioration after intranasal administration *in vivo* in mice homozygous for the F508del mutation (1). Motivated to correct the many other organs affected by CF, we subsequently administered PNA NPs *in vivo* intravenously (IV) to treat the disease systemically. In these new studies we have also explored novel polymeric formulations (2) that are able to load PNA and DNA editing agents with high efficiency and be readily taken up by cell types of interest. Following intravenous administration, our NP formulations exhibit favorable biodistribution; within hours after injection, NPs accumulate within cells in organs of the respiratory and gastrointestinal (GI) tracts. Systemic IV administration of PNA NPs designed to correct the F508del mutation resulted in a gain of CFTR function into the wild-type range in both the nasal and rectal epithelia as measured by nasal potential difference and rectal potential difference. Ussing chamber analyses on nasal and GI epithelia from treated mice also revealed increases in short-circuit current consistent with increased CFTR-mediated chloride transport. Together, these data suggest that systemic delivery of PNA NPs designed to correct CF-causing mutations is a viable treatment option to ameliorate the disease phenotype in multiple affected organs. To our knowledge, this is the first report of systemic correction of a CFTR mutation in adult animals. Encouraged by our success treating the F508del mutation, we have also designed PNA-based editing reagents for the Class I stop codon mutations W1282X and G542X which are currently being studied. Following a course of PNA NP treatment, *in vitro* air-liquid interface (ALI) cultures of human bronchial epithelial cells homozygous for the W1282X CF mutation exhibited increased short-circuit current in Ussing chamber analyses. These promising results suggest that our PNA NP approach can be used in the treatment of many CF-causing mutations.

References (1) McNeer N, et al. *Nat Commun*. 2015. (2) Kauffman A and Piotrowski-Daspit A, et al. *Biomacromolecules*. 2018.

82. Delivery of AAV-CFTR to Bronchial Epithelial Cells from Cystic Fibrosis Patients Augments Functional Recovery of Chloride Conductance

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Cystic fibrosis (CF) is a life-threatening, autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a channel that conducts chloride and bicarbonate ions across epithelial cell membranes. Impaired CFTR function leads to inflammation of the airways and progressive bronchiectasis. Because of the single-gene etiology of CF and the various CFTR mutations in the patient population, gene therapy potentially provides a universal cure for CF. The standard of care for CF currently attempts to modulate the activity of defective

CFTR using modulators, for example, Lumacaftor / VX-809 (a channel corrector), Ivacaftor / VX-770 (a channel potentiator) or Orkambi (a combination of the drugs). While these approaches are promising, they are limited by their specificity for only subsets of the known CFTR mutations. In our current studies, we have generated a novel AAV vector featuring a capsid that is highly efficient at transducing human airway epithelium in the apical membrane. Specifically, we have used AV.TL65-SP183-CFTRΔR to deliver an R-domain-partially-deleted CFTR mini gene and AV.TL65Luciferase-mCherry, a dual reporter vector, to express luciferase and fluorescent mCherry protein. We have also made use of small molecule augmenters (proteasome inhibitors) to significantly enhance recombinant AAV transduction by stimulating endosomal processing and nuclear trafficking of the viral transgene. We have shown that combining AV.TL65Luciferase-mCherry with doxorubicin or idarubicin provides non-toxic enhancement of Luciferase expression by more than 600-fold of air-liquid interface (ALI) human bronchial epithelial (HBE) cultures from 5 separate CF (homozygous dF508/dF508 CFTR) and non-CF donors compared to AV.TL65Luciferase-mCherry without proteasome inhibitor. We have also shown that AV.TL65-SP183-CFTRΔR, when paired with doxorubicin or idarubicin, yields a mean correction of forskolin-stimulated, CFTR-mediated chloride transport in ALI HBE cultures from 6 separate CF donors that is a least 104% that of 6 separate non-CF donors. Furthermore, we have shown this complementation of forskolin-stimulated current is up to four times greater than the standard of care treatment drugs, Lumacaftor and Ivacaftor, in ALI HBE cultures from two separate HBE CF cell donor lines. In summary, we have developed a novel method to augment CFTR expression using an AAV viral vector to correct chloride channel defects in HBE cells from CF patients. These studies lay the ground work for testing the effectiveness of AV.TL65-SP183-CFTRΔR *in vivo* using the CF ferret model and for clinical studies in CF patients.

83. Systemic in Utero Gene Editing as a Treatment for Cystic Fibrosis

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Recent advances in non-invasive genetic testing allow for prenatal disease detection using cell free fetal DNA obtained from a single maternal blood draw as early as seven weeks of gestation. Despite the ability to obtain an early diagnosis, many neonates and children with cystic fibrosis (CF) continue to suffer substantial morbidity and mortality, as currently available therapies do not correct the underlying gene defect. There is a growing consensus that treating CF patients early is crucial in preventing or delaying irreversible organ damage. Even at birth, there are signs of multi-organ disease, evidenced by the occurrence of pancreatic insufficiency, reduced birth weight, meconium ileus, tracheomalacia, biliary cirrhosis, and congenital bilateral absence of the vas deferens. We hypothesize that early intervention through in utero gene editing could correct disease causing *CFTR* mutations during the initial stages of pathogenesis, which could potentially allow for normal organ development, disease improvement, and possibly cure. We previously showed that site-specific gene editing to correct the F508del mutation can be achieved efficiently and safely in adult animals via intranasal administration of polymeric, biodegradable nanoparticles (NPs) loaded with peptide nucleic acids (PNAs) and single-stranded donor DNA. Further, we established that NPs can be safely administered to developing fetal mice, using either an intravenous (IV) or intra-amniotic (IA) delivery approach. The aim of this study was to demonstrate that NPs loaded with PNA/DNA can correct the F508del mutation in utero, resulting in sustained postnatal CFTR function. We first used a GFP reporter mouse model to assess the safety and activity of PNA/DNA NPs to edit lung tissue after IA or IV in utero administration. We found that both NP delivery routes resulted in gene correction in the lung, with more significant editing achieved after IV NP treatment. Next, we determined if in utero treatment with PNA/DNA NPs targeting the *CFTR* locus can be used to correct the F508del mutation and lead to sustained postnatal functional CFTR activity in multiple disease-affected tissues in a mouse model of CF. We found that in utero PNA/DNA NP delivery to F508del fetal mice resulted in significant mutation correction and functional CFTR activity after both IA and IV NP treatment. Systemic fetal treatment resulted in sustained chloride flux, at a level similar to that of wild-type mice, in both nasal and gut tissue. Additionally, analysis of bronchoalveolar lavage fluid indicates a decreased inflammatory response in the lung of mice that received fetal NP treatment, compared to untreated mice with CF. PNA/DNA NPs can be safely administered to fetal mice, and can effectively correct the F508del mutation, resulting in sustained postnatal functional disease improvement. Our findings represent the first systemic in utero correction of a *CFTR* mutation and additionally establish the feasibility of a fetal gene editing approach that could be used in the treatment of numerous CF disease-causing mutations.

84. Development of Genetic Tool for Testing Cftr Gene Targeting in Pigs

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Background: Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Mutant CFTR channel is defective in ion transportation of epithelial cells. CF patients suffer from impaired lung function due to the inflammation and infection in the airway. Traditional medical therapies treat only symptoms but not the cause of the disease. Despite current CFTR-directed drugs, such as Ivacaftor and a combination of Ivacaftor and lumacaftor, are making a major impact on CF patients, novel therapeutic strategies are needed for targeting mutations that are not effectively treated by any drugs. We

have been developing CRISPR/Cas9-based gene targeting strategies for permanently correcting CF lung disease. We used helper-dependent adenoviral (HD-Ad) vectors to integrate functional human *CFTR* gene (*hCFTR*) into a genomic safe harbor of pig cells. The objective of this project is to test our *CFTR* gene targeting strategy in cultured CFTR deficient pig cells *in vitro*. **Methods:** To test our *CFTR* gene targeting strategy, a CFTR deficient pig cell line was generated. The HD-Ad-Cas9-hCFTR vector was constructed to deliver CRISPR/Cas9 and *hCFTR* donor template into $CFTR^{-/-}$ cell line. The *hCFTR* cassette (8.7 kb) was flanked by two 3 kb homology arms. Pig *GGTA1* locus was chosen as a safe harbor to receive transgene integration via homology directed repair (HDR). CRISPR/Cas9 cleavage efficiency and transgene integration frequency were quantified by digital PCR (ddPCR). The function of transduced *hCFTR* was assessed by forskolin-induced CFTR channel opening through membrane potential sensitive dye-based assay (FLIPR). **Results/conclusions:** The pig $CFTR^{-/-}$ cell line was successfully generated via CRISPR/Cas9 gene editing. The pig endogenous *CFTR* transcripts were not detectable in the $CFTR^{-/-}$ cell line by qRT-PCR analysis. In HD-Ad-Cas9-hCFTR transduced cells, the targeted integration of *hCFTR* transgene at *GGTA1* locus was confirmed by junction PCR and DNA sequencing. We also detected *hCFTR* RNA and protein expressions from transduced cells. The cleavage efficiency was ~56% in cells transduced with 50 MOI of vector and was ~50% and ~45% with 25 and 10 MOI of vectors, respectively. The integration efficiency of *hCFTR* transgene was ~10% at 50 MOI, ~4.2% at 25 MOI, and ~3.1% at 10 MOI. In FLIPR assay, we detected that the transduced and integrated *hCFTR* was functionally expressed at cellular passages of 10 and 20. Increased hCFTR channel-mediated conductance was detected after forskolin stimulation. Meanwhile, the inhibition of hCFTR channel conduction was detected by adding CFTR inhibitor (CFTRinh-172). Our study shows that the *hCFTR* cassette could be precisely integrated into pig *GGTA1* locus through CRISPR/Cas9 induced HDR by HD-Ad vector delivery *in vitro*. Transduced *hCFTR* was functionally expressed in infected cells. These results show the potential to further design *CFTR* gene targeting therapies for CF patients and test them in CF pig models.

Gene Editing for Musculo-Skeletal and Skin Diseases

85. DUX4 mRNA Silencing with CRISPR-Cas13 Gene Therapy as a Prospective Treatment for Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral Muscular Dystrophy (FSHD) is an autosomal dominant disorder associated with progressive muscle wasting and weakness, typically in the face, shoulder girdle, and arms. However, this pattern of muscle involvement is not universal, and some patients may develop weakness in other muscles of the body, possibly leading to wheelchair dependence and caregiver assistance. There are currently no treatments that alter the course of FSHD and therapy development is an unmet need in the field. The pathogenic mechanisms underlying FSHD have only become clear during the last decade. FSHD arises from contraction of D4Z4 repeats leads to de-repression of the myotoxic *DUX4* gene in muscle. Therefore, the most straightforward method for treating FSHD is to turn the *DUX4* gene 'off' or mutate it so it is non-functional. One way is genomic knockdown of *DUX4* using the CRISPR-Cas9 approach that is promising but also has challenges, partly because hundreds of identical or nearly identical *DUX4* copies are embedded throughout the human genome within DNA repeats (called D4Z4), and this similarity in DNA sequence makes it challenging to make a precise CRISPR-Cas9 system that would disrupt only the *DUX4* copy associated with FSHD. In contrast to the traditional CRISPR-Cas9, an alternative CRISPR-Cas13 system does not cleave DNA, but can be specifically directed to a RNA transcript of interest using a sequence-specific guide RNA (gRNA). We hypothesize that Cas13/CRISPR-mediated *DUX4* mRNA silencing would offer an effective therapeutic strategy for FSHD, without altering host cell DNA. In this study, we developed a new *DUX4* silencing strategy designed to target *DUX4* mRNA using the CRISPR-Cas13b. To do this, we designed several Cas13b-gRNAs targeting different parts of *DUX4* mRNA and demonstrated their ability to suppress *DUX4* and prevent cell death *in vitro*. We detected >90% reduction of DUX4 protein in treated cells, and improved their viability. *DUX4* mRNA levels were markedly decreased in treated FSHD myotubes, as determined by RNAscope *in situ* hybridization. This reduction was accompanied by complete or near complete reduction in levels of DUX4-responsive biomarkers in treated patient myoblasts/myotubes. In our first *in vivo* studies in tibialis anterior muscles of TIC-DUX4 mouse model, our lead constructs led to near to 100% reduction in DUX4 expression. As of this writing, additional *in vivo* studies are underway, including testing the capabilities of our AAV6-delivered CRISPR/Cas13 system to suppress *DUX4* long-term in vivo, and improve histopathological, functional, and molecular outcomes. This study provides proof of concept for silencing *DUX4* expression using the RNA targeting CRISPR-Cas13b system and has implications for future FSHD therapy.

86. Long-Term Evaluation of AAV-CRISPR Genome Editing for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a debilitating muscle wasting disease that leads to loss of ambulation in the teen years and cardiac and respiratory failure in the 30s. DMD is a monogenic disorder and a candidate for therapeutic genome editing with several recent reports of adeno associated virus delivery of CRISPR/Cas9 (AAV-CRISPR) in preclinical models of DMD. However, long-term persistence of gene correction and safety of AAV-CRISPR is still being addressed. Here,

AAV-CRISPR was administered intravenously to adult and neonatal *mdx* mice harboring a stop codon in exon 23 of *Dmd*. The smaller Cas9 derived from *Staphylococcus aureus* (SaCas9) was used to accommodate AAV packaging limits. Two gRNAs were targeted to intronic regions flanking exon 23 to excise the region and restore dystrophin protein expression. Genome editing outcomes at the on-target site were characterized after 8 weeks or one year with an unbiased, unidirectional sequencing method. The levels of exon 23 excision were sustained or improved over one year at the DNA and mRNA level in mice treated systemically as neonates. In contrast, genome editing levels decreased in mice treated by intramuscular injection as adults. In systemically treated neonates, dystrophin protein levels were maintained for one year as measured by western blot and immunofluorescence histology. Creatine kinase levels were also improved in these systemically treated mice, indicating improved muscle membrane stability. Previous reports have indicated humoral and cellular responses to AAV or Cas9. We confirmed the presence of antibodies against the AAV8 or AAV9 capsid and SaCas9 in all mice treated as adults regardless of the injection route. However, all mice treated as neonates had no detectable humoral immune response to either the vector or Cas9. Similarly, we detected SaCas9-reactive T cells by ELISPOT in all mice treated as adults, but no response was observed in mice treated as neonates. Using unbiased unidirectional sequencing of genomic DNA, we found multiple types of on-target modifications in AAV8-treated neonates including the intended deletion (14%), indels at a single gRNA target site (33%), inversions (6%), and AAV genome integrations into the predicted gRNA cut sites (48%). Analysis of multiple capsids (AAV8 and AAV9), tissues, and injection routes showed the same diversity of on-target genome modifications with overall levels depending on the tissue. Transcript variants were detected using the same method including the intended removal of exon 23, multi-exon skipping, putative circular RNA formation, and fusions with AAV-derived sequences. Most of the vector integration at the target site resembled canonical integration within the inverted terminal repeats (62%) with the remaining integrations occurring throughout the vector genome. We also used this approach to map AAV vector integrations genome-wide, including sites previously identified as AAV integration sites and at sites with sequence similarity to the gRNA target sites used in this study. This study further establishes genome editing as a therapeutically relevant approach for inherited muscle disease with edited cells and dystrophin protein restoration persisting for one year *in vivo*. The treatment was well tolerated with no apparent toxicity despite an immune response and unintended genome modifications. This work highlights the potential opportunity to enhance genome editing outcomes by modulating immune responses and increasing the efficiency and accuracy of intended on-target genome modifications. It also underscores the necessity to carefully monitor these issues throughout preclinical development of genome editing therapies.

87. CRISPR/Cas9-Base Editing Mediated Correction for Recessive Dystrophic Epidermolysis Bullosa

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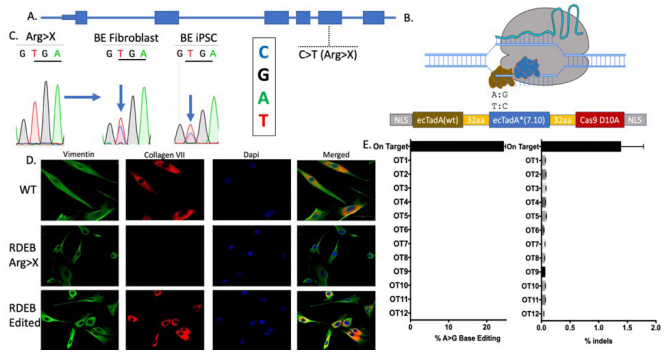
Genome editing represents a promising strategy to correct *COL7A1* gene mutations that cause recessive dystrophic epidermolysis bullosa (RDEB). Previously, we used programmable nucleases that create double-stranded DNA breaks (DSBs) to repair *COL7A1* mutations through homology-directed repair (HDR) with an exogenous repair template. Delivery of this template can be cytotoxic and DSBs induce undesired insertions and deletions (indels) that compete with desired HDR. To overcome these limitations, we used base editors (BE), a CRISPR/Cas9-based system that uses naturally occurring or laboratory-evolved deaminating enzymes to directly convert A>G, C>T, T>C, or G>A. BE does not lead to significant DSBs, obviates the need for a repair template, and typically offers higher editing efficiencies for point mutations than HDR. We used an optimized A>G base editor (ABEmax) for two RDEB causative nonsense mutations in the *COL7A1* gene (Fig 1b). We delivered ABEmax mRNA with minimal toxicity into primary fibroblasts or induced pluripotent stem cells from two patients with RDEB, and observed mutation correction rates of up to 50% in along with concomitant restoration of COL7A1 protein production (Fig 1c.d). Indel occurrence was minimal, with an observed frequency of ~2%, consistent with previous studies. Twenty predicted off-target loci were analyzed by high-throughput sequencing. Treatment with the one reagent showed no off-target effects, while the second candidate showed A>G editing at one exonic off-target site at a frequency of ~6% with <0.5% indels (Fig. 1e). Base-edited human fibroblasts were injected into an immune-deficient mouse model of RDEB, and human COL7A1-expressing cells were observed *in vivo*. 3D bioprinting was used to deposit base-edited fibroblasts in a biopolymer complex that allowed for significant fibroblast expansion in support of a scalable, ex vivo approach for skin graft generation. These findings suggest that an optimized base editing approach may provide an efficient and precise genome editing method for individualized autologous cell therapy for RDEB.



88. Excise-and-Replace Strategy Using AAV-Mediated Delivery of CRISPR/Cas9 and HITI Donor Restores Full-Length Dystrophin Expression in Human Cardiomyocyte Model of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked progressive muscle wasting disorder caused by mutations in the large *DMD* gene that lead to early termination of the dystrophin protein, making it an attractive candidate target for therapeutic gene editing. Due to the low capacity of post-mitotic muscle cells for homology-directed repair (HDR), most therapeutic strategies employ reframing or exon skipping mediated by non-homologous end joining (NHEJ) to bypass mutations that disrupt the *DMD* open reading frame and produce a truncated but partially functional dystrophin protein, thereby achieving the clinically milder phenotype of Becker muscular dystrophy (BMD). While these methods possess considerable potential for improving voluntary muscle function, cardiac abnormalities remain common in BMD and often manifest as cardiomyopathy that becomes lethal. To treat cardiac muscle degeneration associated with DMD and BMD, we developed a novel gene correction approach that harnesses AAV-mediated co-delivery of CRISPR/Cas9 and a homology-independent targeted integration (HITI) donor to restore full-length dystrophin expression in human cardiomyocytes (CMs). In this proof-of-concept study, we first applied HITI to tag the cardiac troponin T locus in human embryonic stem cell (hESC)-derived CMs with a GFP reporter and confirmed targeted knock-in by PCR genotyping analysis and immunocytochemistry. HITI enabled in-frame GFP expression in approximately 20% of treated hESC-CMs, as quantified by flow cytometry. We then devised a novel dual-cut HITI strategy to repair the dystrophin gene in CMs derived from DMD human induced pluripotent stem cells (hiPSCs) that harbor a deletion of *DMD* exon 51. Guide RNA pairs were designed to excise and replace exon 50 or 52 with a donor construct containing conjugated exons 50-51 or 51-52, respectively, that drives unidirectional transgene insertion to yield a complete dystrophin transcript. Site-specific donor integration was validated by end-point PCR, and correctly spliced dystrophin transcripts containing the missing exon were detected by RT-PCR analysis. In ongoing work, we are quantifying gene correction efficiency via immunocytochemistry and Western blot and further optimizing gene targeting frequency. Our broadly applicable excise-and-replace strategy can potentially be utilized to correct various exon deletions and small mutations in the *DMD* gene, which together constitute nearly 90% of known genotypes, for functional rescue of both skeletal and cardiac muscle in DMD.

89. DNA Base Editing to Modulate mRNA Splicing as a Therapeutic Strategy for Duchenne Muscular Dystrophy

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The removal of introns and inclusion of selected exons during mRNA splicing is critical to normal gene function and is often misregulated in genetic disorders. Technologies that modulate mRNA processing and exon selection, such as exon skipping approaches, may be used to study and treat these diseases. Exon skipping aims to restore the correct reading frame or induce alternative splicing by blocking the recognition of splicing sequences by the spliceosome, leading to removal of specific exons along with the adjacent introns. For example, Duchenne muscular dystrophy (DMD) is typically caused by deletions of one or more exons from the dystrophin gene, leading to disruption of the reading frame. Expression of dystrophin protein can be restored by correcting the reading frame by inducing the exclusion of one or more additional exons. Studies have shown that by targeting Cas9 to the splice acceptor of exons, the indels produced during DNA repair can disrupt the splice site and induce exclusion of the exon. In contrast to the semi-random indels generated by the conventional CRISPR-Cas9 system, base editing technologies have been developed for the precise modification of a single base pair without inducing double-stranded DNA breaks. Base editors can change a C directly to a T, or a G to A on the reverse strand, and they have been targeted to both splice donors "GT" and acceptors "AG" of a variety of exons to modulate mRNA splicing. We first designed gRNAs to target the base editor systems with both *S. pyogenes*- and *S. aureus* Cas9 proteins to human dystrophin exons within the hotspot for deletions in the *DMD* gene between exons 45 and 55. We assayed splice acceptor G>A editing at four dystrophin exons by plasmid transfection of human HEK293T cells followed by deep sequencing of the target sites. While some exons showed poor editing efficiency, we observed that 7-8% of alleles were edited at exon 45, which is the dystrophin exon whose removal could treat the second largest group of DMD patients (~8%). In order to test the effect of splice site disruption on exon skipping, we generated a human iPSC line harboring a deletion of dystrophin exon 44. This pluripotent cell line models an inherited DMD mutation with a disrupted reading frame of the *DMD* gene that is correctable by removal of exon 45. Myogenic differentiation of this Δ44 iPSC line by lentiviral transduction of MyoD cDNA confirms that the mutation ablates dystrophin protein expression. We delivered the *S. pyogenes* dCas9-based AncBE4max and a gRNA cassette to these cells by lentiviral transduction. Deep sequencing showed that 22% of splice acceptors were disrupted after 12 days. MyoD overexpression in this edited Δ44 iPSC line followed by RT-PCR confirmed that splice acceptor base editing results in skipping of exon 45, which restores the dystrophin reading frame. Future studies will evaluate the functional impact this single base pair change can make on the DMD phenotype.

90. Allele-Specific Gene Correction Using CRISPR-Cas9 in Compound Heterozygote SGCAmutations

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Muscular dystrophies comprise a group of more than 40 progressive debilitating diseases of whom most are inherited in an autosomal-recessive pattern. Compound heterozygous patients with different mutations on either allele outnumber those with homozygous mutations, but only few gene editing strategies for compound heterozygote mutations have been reported. Using limb girdle muscular dystrophy 2D (LGMD2D) caused by *SGCA*mutations as a model we developed a tool box to test gene editing strategies in compound heterozygous mutations. We isolated primary patient myoblasts, generated muscle stem cell derived iPS cells, established a protocol for redifferentiation of iPS cells into muscle cells, and created three mouse models in which the murine *Sgca*sequence (exon 1-8) was exchanged for the human wildtype or mutated *SGCA*sequences. In these systems, our measures can uniformly been tested. We designed mutation and allele specific gRNAs for *SGCA*c.157G>A and *SGCA*c.748-2A>G. We find that allele specific gRNAs cut with high efficiency and that in iPS cells mutation correction can occur without external template. We name this "internal-template HDR". Transfer in our other experimental models is currently explored and compared with efficacy after adding external templates. In conclusion, gene editing of compound heterozygous mutations is possible and good instruments are just being developed.

Cardiovascular Gene Therapy

91. AAV9.LAMP2B Reverses Metabolic and Physiologic Multiorgan Dysfunction in a Murine Model of Danon Disease

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Danon disease (DD) is a rare, X-linked autophagic vacuolar myopathy caused by loss of function mutations in the gene encoding Lysosomal-Associated Membrane Protein type 2 (LAMP-2), a lysosomal transmembrane protein essential for autophagy. Penetrance of disease-causing mutations in DD is high and the cardiac phenotype is generally severe. Patients develop significant cardiac arrhythmia and hypertrophy first manifesting in early adolescence. In addition to heart failure, patients also suffer from liver dysfunction, skeletal myopathies, as well as retinal and cognitive impairments. No specific therapies for the treatment of DD exist and most patients either die from heart failure or require cardiac transplant by age 30. LAMP-2 has three spliced isoforms (LAMP-2A, B, and C); LAMP-2B is the predominant isoform expressed in cardiac muscle and is considered to be critical for autophagosome-lysosome fusion in cardiac myocytes. Given that cardiac disease is severe in DD patients and that mutations resulting in isolated LAMP-2B deficiency result in the disorder, this isoform has been identified as critical in the pathogenesis of the disease. We evaluated the efficacy of human LAMP-2B gene transfer with adeno-associated virus 9 (AAV9) in a *LAMP-2* KO mouse, a previously described model of Danon disease. PBS or AAV9 encoding human LAMP-2B (AAV9.LAMP-2B) at doses of $1x10^{13}$, $5x10^{13}$, $1x10^{14}$ and $2x10^{14}$ vg/kg, were intravenously injected into 2 and 6-month-old *LAMP-2* KO male mice and evaluated at 24 weeks and 12 weeks post-treatment respectively, to assess efficacy in adolescent and adult phenotypes. Untreated age-matched WT mice were used as controls in both cohorts. *LAMP-2* KO mice that received LAMP-2B gene transfer showed dose-dependent restoration of human LAMP-2B transcript and protein levels in heart, liver and skeletal muscle in both cohorts. Impairment in autophagic flux, shown by an increase in LC3-II levels and by the accumulation of autophagic structures in *LAMP-2* KO mice, was abrogated by LAMP-2B gene transfer in all three tissues in both cohorts. Cardiac contraction and relaxation as evaluated by dP/dt max and dP/dt min was significantly improved in a dose-dependent manner in the AAV9.LAMP-2B treated *LAMP-2* KO mice compared to PBS injected controls. The increase in transaminase levels (alanine aminotransferase and alkaline phosphatase) observed in PBS-injected *LAMP-2* KO mice compared to WT control mice was significantly reduced following LAMP-2B gene transfer, indicating improvement in liver function. Survival was significantly higher in mice that received $\geq 1x10^{14}$ vg/kg dose of the vector. In summary, LAMP-2B gene transfer improves the principle metabolic and physiologic sequelae as well as survival in a mouse model of Danon disease. AAV9 has been previously evaluated safely in human studies; our findings have direct implications for the development of this gene therapy for patients with Danon disease.

92. Ancestral AAV Delivery Catalyzes Rapid and Robust Myocardial Gene Expression within 72 Hours- A New Frontier for Early Stage Cardiac Disease

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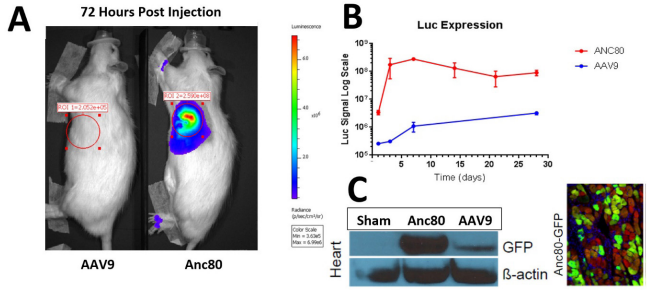
Introduction: Early stage heart diseases following major coronary events such as myocardial infarction demand upregulation of pro-survival genes as soon as possible to salvage myocardium prior to myocyte death. Adeno-associated virus (AAV), especially serotype 9, is the de facto selection for the heart. Despite its reliability, AAV9 however has a delayed time course of expression that does not address very early disease, stem cell repair, or matrix alteration biology that occur within 1 week following the cardiac event. Ancestral AAV designed via

evolutionary selection most recently has promised superior kinetics relative to standard AAV9. Here, we evaluate ancestral AAV80 (AnC80) versus AAV9 in an adult rat model. **Methods:** Six 500gr rats were divided between AAV9 and AnC80 vector groups, n=3 ea. Rats were subjected to a thoracotomy to expose the heart, then 1e11 of vector expressing luciferase was delivered directly into the myocardium with 3 intramuscular injections at 100 uL each. IVIS imaging was performed at 1, 3, 7, and 28 days following gene transfer. A separate parallel arm with the same design was performed with EGFP marker to quantify protein, vector genome copies and microscopy. **Results:** AnC80 vector resulted in rapid, robust cardiac gene expression with luciferase detection as early as 24 hours, reaching high levels at 72 hours (Fig1A), with none in AAV9 $p<0.05$. AAV9 did reach significant levels just after 1 week, and also significantly lagged AnC80 for the remaining course to 4 weeks (Fig1B), all $p<0.05$. At 4 weeks in the parallel EGFP groups, AnC80 mediated transfer resulted in much higher total EGFP protein as compared with AAV9 (Fig1C). Cardiac specificity was confirmed with 20x mag. confocal analysis with > 70% efficiency in both groups, however AnC80 transduced more whole myocardial cross section area 60+/-10% versus AAV9 36+/-12% at 4x mag., indicating a greater degree of myocardial trafficking post injection. QPCR results for both groups in injected areas demonstrated >1-100 AAV GC/cell. **Conclusion:** AnC80 virus offers incredible transduction time performance (<3 days) in adult myocardium compared to standard AAV. These vectors have the potential to address the critical need of rapid expression onset for various early stage survival and repair mechanisms.



93. First in Human Phase I/Phase II Safety and Preliminary Efficacy Study Using Low Frequency Ultrasound in Addition to Adipose Derived Stem Cells in Patients with Moderate to Severe Lower Extremity Peripheral Arterial Disease

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Peripheral arterial disease (PAD) afflicts 8 to 10 million patients in US alone, and 200,000 of them require amputation each year. Perivascular delivery of autologous adipose derived stem cells (aADSCs) has been shown to improve micro-circulation in ischemic tissues; however, one of the main problems in stem cell therapy is the lack of stem cell (SC) homing in the targeted area. Research at NIH (Tebebi PA, Sci Rep, 2017) has shown an increase of 5-6 fold SC in ischemic areas by using low frequency ultrasound (LoFU) after SC injection in rodents (1). The aim of this pilot study was to assess safety and preliminary efficacy of ADSCs in combination to LoFU in patients with severe PAD that exhausted all available clinical non-amputation treatments. **Methods:** This is the first in-human, single center, open label, prospective, randomized study in patients with non-revascularizable moderate or severe lower extremity PAD with a Rutherford class 3-5. Patients were randomized 1:1 to one of two groups: ADSCs only and ADSCs + LoFU. ADSCs were isolated from lipo-aspirates of abdominal subcutaneous fat. Both groups received ADSCs, injected intra muscularly along the target vessel path and intra-adventitially proximal to the lesion. In the LoFU group, LoFU was delivered at 1 MHz frequency in pulse mode (50 LoFU pulses per site) to induce mild focal- inflammation to the targeted ischemic area prior to ADSC injection. Main endpoints were for safety and for preliminary efficacy such as change from baseline of Rutherford classification, six minute walk test (6MWT), ankle brachial index (ABI), transcutaneous partial oxygenation (TcPO2) by angiosome and quality of life (EQ- 5D 5L and VascuQol) at 6wks, 3 and 6 months. **Results:** A total of 13 patients were screened and 10 patients enrolled. For safety endpoints, both groups had 0 procedural complications or investigational product (IP) related adverse events. For efficacy endpoints: ABI and TcPO2 60% (ADSC n=3) vs 80% (LoFU + ADSC, n=4) of patients were stabilized at 3 months. For ABI, LoFU group shows more patients (4/5) improved at 3 month compared to SC only (3/5). For TcPO2, LoFU group shows more improvement at 3 month in dTcPO2 (4/5) and sTcPO2 (3/5), compared to 3/5 and 2/5 in SC only group, respectively. ABI Rutherford, 6MWT and QoL had less decline/deterioration in both groups. ADSC viability was 91.9% ($246 \pm 38 \times 10^6$ cells) and 92.6% ($228.2 \pm 47.4 \times 10^6$ cells) for ADSC and ADSC + LoFU respectively ($p=0.4$). **Discussion:** Despite the fact that the patients enrolled in this study had no other treatment option but amputation, stem cell treatment particularly when used after ultrasound, is safe and shows promise stabilizing vascular hemodynamics of the affected area. Due to the heterogeneous disease process and small sample size, the results do not show statistical difference between groups for preliminary efficacy endpoints. However, the LoFU study group showed slight trending of improvement in Rutherford class, ABI and diastolic TcPO2. This study utilized a single injection of the IP, repeated doses may also enhance the benefit of LoFU. **Conclusion:** Pre-treatment of LoFU over the affected areas prior to ADSCs treatment of patients with moderate to severe non-revascularizable PAD is safe and shows promising beneficial effects in improving the function of compromised lower extremity vascular systems. Further studies are needed to expand on efficacy of ultrasound coupled with stem cell therapy. Study originated by Arbitrage Medical.

94. MiR-125a-5p Regulates Cardiomyocyte Function and Prevents Pressure-Overload Induced Cardiac Dysfunction

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Introduction: Cardiomyocyte hypertrophy enables the heart to maintain function during increased cardiac load. Although beneficial on the short-term, persistence of increased load results in detrimental cardiac remodelling and eventually heart failure (HF). Recent findings associate cardiomyocyte hypertrophy with increased cell cycle activity. The classical paradigm proposes that cardiomyocytes proliferate during fetal life, whereas adult cardiomyocytes are forced to withdraw permanently from the cell cycle. Recent studies overturned this traditional belief and show that pathologically stressed cardiomyocytes re-enter the cell cycle, allowing hypertrophic cell growth. Although cardiomyocyte hypertrophy and cell cycle activity have been shown to be associated, the underlying mechanism and regulating factors have not been identified. Therefore, we first aimed to identify miRNAs able to regulate cardiomyocyte hypertrophy. Second, we investigated the mechanism underlying the hypertrophy-regulating function of miR-125a and modulated its activity *in vivo* to prevent the development of HF. **Methods:** High-throughput screening for miRNAs with a hypertrophy-regulating function was performed by overexpressing 194 HF-associated miRNAs in primary cardiomyocytes. Cardiomyocyte size was determined through immunofluorescence staining and automated high content imaging. *In vitro* miRNA modulation was achieved through transfection of miRNA mimics or inhibitors. Overexpression of miR-125a in the heart of mice subjected to sham surgery or transverse aortic constriction (TAC) was performed using AAV9-mediated gene transfer. **Results:** Using high-throughput screening, we identified miR-125a as one of the strongest inducers of cardiomyocyte growth. Deciphering its role revealed that miR-125a increases contraction frequency and amplitude, and increases G1-to-S-phase transition of neonatal cardiomyocytes. Strikingly, miR-125a overexpression induced hypertrophy accompanied with a decreased expression of fetal genes, while miRNA inhibition induced hypertrophy with an up-regulated expression of fetal genes. Silencing of miR-125a also increased the progression into the G1-phase. In line, AAV9-mediated overexpression of miR-125a in the adult murine heart increased the left ventricular ejection fraction of healthy mice and prevented TAC-mediated cardiac dysfunction, left ventricular dilatation, and induction of fetal genes. **Conclusions:** High-throughput screening identified miR-125a as a potent inducer of hypertrophy. In cultured neonatal cardiomyocytes, miR-125a controls hypertrophic growth, fetal gene expression, cell cycle activity, and contraction. In the adult murine heart, miRNA overexpression increases contractility and protects against pressure overload induced cardiac remodelling. Whether miR-125a inhibition induces pathological remodelling and/or diminishes physiological hypertrophy in the adult heart will be subject of further investigations.

95. Preclinical Evaluation of an AAV-Based Gene Therapy Approach for the Treatment of Friedreich Ataxia Cardiomyopathy

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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 AAV-based gene therapy approach using a conditional mouse model of the disease that reproduces the cardiac phenotype (FXN MCK mouse model). AAV containing a cassette for the expression of a HA-tagged human frataxin was administered intravenously before or after symptom outset, at varying doses. Longitudinal echocardiography was performed to assess cardiac function. Molecular analyses, including HA immunohistochemistry, succinate dehydrogenase (SDH) staining, vector/genome copy number determination, transgene mRNA and protein expression, were performed on isolated heart samples. Together, our data shows that restoring frataxin expression in approximately 50% of cardiomyocytes broadly corrects the molecular and functional phenotypes of FXN MCK mice and significantly extends survival, consistent with recently published studies.

96. Optimizing AAV Gene Therapy Strategies for Barth Syndrome

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Barth syndrome (BTHS) is a rare but debilitating and potentially fatal disease caused by loss-of-function mutations in Tafazzin (*TAZ*), an X-linked gene that is critical for proper mitochondrial function. Patients with BTHS present with symptoms including dilated cardiomyopathy, skeletal myopathy, neutropenia, and early growth retardation. There is no targeted therapy for BTHS, although a gene therapy proof-of-concept study was reported recently in a mouse model with partial *TAZ* depletion. Here we characterize a novel *Taz* knockout mouse model of BTHS and use it to optimize parameters for TAZ gene therapy. Mice globally deficient in *Taz* displayed partial embryonic and neonatal lethality. The few surviving $Taz^{-/Y}$ mice exhibited growth retardation, cardiac dysfunction, decreased motor activity, and shortened life span, all of which closely resemble BTHS patients. *Taz* ablation specifically in cardiac muscle resulted in progressive loss of cardiac contractility and severe defects in mitochondrial respiration and energy production.

As a result of gene therapy, neonatal lethality and cardiac dysfunction were largely rescued with self-complementary AAV expressing full length human *TAZ* (scAAV-fl*TAZ*). Standard single-stranded AAV, or AAV expressing other human *TAZ* isoforms, were not as effective as scAAV-fl*TAZ*. Remarkably, treatment of adult *Taz*$^{-/Y}$ mice with established cardiac dysfunction not only halted the progression of heart failure, but also successfully restored cardiac function to normal. These studies indicate that AAV-based TAZ gene replacement is a promising therapeutic approach for treating BTHS patients.

Directed Evolution of AAV Vectors II

97. Machine-Guided Design of AAV Capsid Proteins with Experimentally Augmented Evolutionary Data

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Adeno-associated Virus (AAV) capsids have shown great clinical potential as gene therapy vectors. However in their natural form they present several drawbacks that need to be addressed before they can be applied more broadly, especially the high prevalence of pre-existing immunity among patients due to prior infection from related serotypes. Currently, experimental methods for optimizing AAV capsids for efficient production, low immunogenicity, and tissue tropism are laborious and costly. The process of generating synthetic variants stands to benefit enormously if computational models can predict viral protein functionality before the need for experimental validation, especially when attempting to generate capsid sequences that greatly differ from naturally occuring AAV serotypes. Recently, we reported an unsupervised machine learning approach, known as a Variational Auto-Encoding (VAE), as a general method to predict the effects of mutations from evolutionary data. We tested this technique on several protein families with rich multiple sequence alignments (MSA) and reported their results. This is particularly of interest because MSA data is widely available, and can inexpensively provide a guiding paradigm to increase the chance of producing functional variants. In this work, we focused our efforts on generating variants of the AAV2 capsid that are able to successfully package their genomes (we refer to this as viable) using both evolutionary history and experimental data. We selected a 28-amino acid window near the heparin binding site, for which we have collected data previously. The MSA data includes 560 sequences from AAV2-related strains. Our experimental data consisted of 80,000 variants, generated either at random or based on a single-site mutation model. We investigated two partitions of data, and two models: our models included an independent-site model, as well as a higher-capacity VAE model; our datasets were composed of (i) Evolutionary data (ii) Evolutionary data augmented by 19,000 viable mutants 1-21 mutations from wild-type (WT) AAV2. Using this hybrid approach of augmenting evolutionary data with experimentally assayed proteins, we generated 7,500 capsid variants, and experimentally validated them for packaging. About half of the mutants generated by the independent-sites model trained on dataset (ii) are viable, however they closely resemble the original experimental data. In contrast, only 1% of the mutants generated by a independent-sites model trained on dataset (i) are viable. The best performing mutants generated by the VAE are more diverse, with 12% and 16% of the mutants trained on dataset (i) and (ii), respectively, being viable. With the same technique we also find a viable variant that is only similar to wildtype in 3 positions (out of the 28 possible positions). Our results suggest that VAEs can be trained on evolutionary data to produce novel engineered AAV capsids at a distances far from naturally occurring capsids, with higher efficiency than a simple independent-sites model. Furthermore we demonstrate that when evolutionary data is sparse, it can be augmented with information from mutational scans to improve performance. We propose that this approach can be extended to optimize other viral proteins, as well as other traits of interest for AAV delivery.

98. Overcoming Transgene-Related Immunological Confounders in High-Throughput In Vivo AAV Capsid Screens Using Barcoded Non-Coding RNAs

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Recombinant adeno-associated viruses (rAAVs) have become favorable gene delivery vehicles for expressing therapeutic transgenes. Capsid engineering efforts to produce novel AAVs that have improved transduction efficiencies, unique tissue specificities, and reduced host immunities, are a direct response to the high demand for treatment needs that preexisting rAAVs cannot currently fulfill. New AAV capsids ultimately require extensive characterization in relevant *in vivo* models. Many capsid screening methodologies are based on the detection of an index inserted into the 3'-UTR of a reporter gene, coupled with high-throughput sequencing approaches. Unfortunately, adaptive immunity to these foreign transgenes in large animal models may mask the performance of candidate capsids, and can drastically limit the persistence of tissue transduction and ability to accurately probe vector durability in those models. This constraint represents a key challenge for studying cross-species translatability of engineered capsids. To address the current limitations in screening for capsid performance *in vivo* in large animal models, we have developed a vector and transduction tracking system that employs the indexing of a non-coding RNA, specifically a barcoded Tough Decoy (*bcTuD*), that express highly stable RNA transcripts that can be used as readouts for transduction efficiency. The pseudo-hairpin structure of the *bcTuD*

contains a variable region that is amenable to barcode insertion and can be detected by target amplicon sequencing. The described approach, which we have named AAV-*bcTuD* screening, offers a new alternative for *in vivo* assessment of rAAV that can accurately quantify vector genomes and transcript abundances in tissues, as exampled by our demonstration in liver and brain infections. We demonstrate that vector genome and transcript detection in tissues with this method is accurate and consistent for upwards of four logs of vector concentration difference in a mixed vector injection, showing that this method is robust, sensitive, and applicable for multiplexed screening of capsid performance *in vivo*. Our novel design of AAV-*bcTuD* screening provides a new alternative for AAV *in vivo* screening that can quantify relative amounts of vector genomes and transcripts in animal tissues. Ultimately, this approach will allow better accuracy for assessing tropism and biodistribution profiles for new capsid designs, particularly in large animal models where transgene immunity becomes a major factor that can influence interpretation of transduction data. #Co-corresponding authors

99. Multiplexed-CREATE Selection Yields AAV Vectors Targeting Different Cell Types of the Central Nervous System Following Systemic Delivery

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Recombinant adeno-associated viral (rAAV) capsids are widely accepted as safe gene delivery vehicles in research laboratories and in gene therapy clinical trials and there is potential to further improve their usage by evolving the surface of the capsids to enhance their affinity to specific cell-types or tissues after intravenous (IV) delivery. To this end, we built upon our previous Cre recombination-based AAV targeted evolution (CREATE) method (Deverman et al, Nat. Biotech., 2016; Chan et al., Nat. Neurosci., 2017) to develop Multiplexed-CREATE (M-CREATE). M-CREATE facilitates both positive and negative selections and minimizes the propagation of biases from successive rounds of selection via synthetic library generation. In addition to increasing the confidence in the selections, M-CREATE enables a detailed characterization of the selection process, improving our understanding of selection progression and outcome. We applied M-CREATE to evolve AAV capsids to efficiently target different cell types of the central nervous system (CNS) after systemic administration, by performing parallel *in vivo* selection in different Cre transgenic mouse lines, such as Tek-Cre (for endothelial cells), GFAP-Cre (for astrocytes), SNAP25-Cre and Syn-Cre (for neurons). The outcome of these selections yielded several AAV9 variants targeting CNS cell types including one with biased tropism towards endothelial cells and one with greater specificity toward neurons brain-wide. We also show that M-CREATE minimized the selection bias carried through the rounds of selection, and detailed characterization of the recovered capsid libraries has identified a family of variants that carries recurring amino acid combinations within the diversified region of the highly enriched variants. In addition to our efforts on capsid engineering, we also demonstrate the integration of regulatory elements to expand the use of engineered capsids to target specific cell types. For instance, the Ple261 (de Leeuw et al., Mol. Brain, 2016) MiniPromoter can restrict gene expression to endothelial cells. In summary, the new M-CREATE methodology is highly versatile, yielding several novel viral capsids for use in neuroscience and gene therapy related applications.

100. Engineering A Humanized AAV8 Capsid through Iterative Structure-Guided Evolution

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Recombinant Adeno-Associated Viruses (AAV) are facing an acceleration in their development as mainstream vectors for human gene therapy. Innovative approaches that enable "humanization" of AAV vectors will enable desirable properties such as human tissue tropism and immune evasion, as well as the possibility of cross-species compatibility. Several studies to date have demonstrated that AAV capsids can display transduction and immune profiles that differ markedly between pre-clinical animal models and clinical trials. To address this challenge, we utilized iterative structure-guided evolution to re-engineer the AAV8 capsid, a rhesus macaque isolate, into a clinical trial ready, humanized AAV vector. Our approach was guided by cryo-electron microscopy (cryoEM) analysis of AAV8-antibody complexes and high throughput sequencing analysis of new AAV8 variants that were evolved by iteratively engineering antigenic footprints on the capsid surface. Our lead synthetic variant, dubbed AAV hum.8, provides vector yields similar to AAV8 and displays markedly enhanced transduction of human hepatocytes compared to parental AAV8 both *in vitro* (~40 fold) and in a chimeric human liver xenograft mouse model (FAH-/-) *in vivo*. Further, the synthetic AAV hum.8 variant displays a similar transduction profile within the liver in naive mice, displaying potential for streamlined mouse-to-human translation. Importantly, AAV hum.8 efficiently evades neutralizing antibodies from multiple species, including mouse monoclonal antibodies, pooled human IVIG as well as canine, primate and human antisera that neutralize conventional AAV8 vectors. Our approach draws parallels to monoclonal antibody-based therapeutics, wherein naturally occurring AAV strains can be humanized for clinical gene therapy modalities.

101. Engineered AAVs for Enhanced Transduction of Submucosal Cells within the Lung Following Intravenous Delivery

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Lung diseases are one of the leading causes of morbidity and mortality worldwide, highlighting the need for vectors capable of efficiently transducing cells within the lung. Recent developments in gene delivery have demonstrated that the use of gene therapies to treat disease is a realistic and achievable goal. Adeno-associated viruses (AAVs) have quickly become the vector of choice in both laboratory and clinical settings due to their strong safety record and stable expression *in vivo*, especially when compared to other viruses. However, the naturally occurring AAVs tend to have severely limited tropisms in lung tissues, as expression following intranasal delivery is generally inefficient and restricted to the airway epithelium. One solution to this obstacle is to utilize a systemically injected AAV in conjunction with inhalant based delivery to cover a broad population of cells throughout the lung. Unfortunately, systemic administration of naturally occurring serotypes fails to traverse through the endothelium, severely limiting vector coverage within the lung. To address this need and provide a delivery mechanism targeting submucosal cells within the lung, we utilized a modified version of CREATE, a Cre recombination-dependent viral engineering platform we reported on previously, to evolve a novel variant that elicits robust and widespread transduction throughout the mouse lung following intravenous delivery. Quantification shows that systemically injected AAV9.DT-L transduces submucosal cells at ~15 fold greater efficiency than AAV9, and ~30 fold greater than AAV5. Similarly, transduction of type II pneumocytes is ~30 fold greater then AAV9, and ~60 fold greater than AAV5, and comprises ~60% of the cells transduced. AAV9.DT-L provides novel, non-invasive access to widespread populations within the lung, enabling access to cell types implicated in lung diseases like pulmonary fibrosis.

102. Engineered AAVS for CNS Transduction and Peripheral Organ De-Targeting across Species after Systemic Delivery

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In recent years, we have witnessed the development and clinical use of gene therapies at an accelerated pace. The option to introduce, silence, or edit genes greatly increases the therapeutic avenues for a variety of diseases, with adeno-associated viruses (AAVs) being the vehicle of choice due to their low immunogenicity, stable expression, and strong clinical safety record. While the route of delivery has traditionally been localized, systemic administration via the blood is an option in cases where direct injection is impractical or widespread cell populations are affected. However, the naturally occurring AAV serotypes have evolved to broadly infect tissue, an undesirable characteristic for targeting therapeutics to distinct cells. To ensure strong enough transduction for adequate coverage while minimizing off-target effects, AAV specificity needs to be tuned according to application. Toward this purpose, protein engineering allows for the manipulation and screening of AAV capsids for selectivity toward defined cell-types or tissues. Utilizing a modified version of CREATE, an AAV evolution strategy that employs a Cre-dependent capsid recovery selection in transgenic mice, we engineer diversity in the capsid surface and perform parallel positive and negative *in vivo* selections for viral sequences recovered from tissues of interest and explicitly not recovered from others. Through this method, we've identified a panel of novel engineered capsids capable of high levels of production, extremely efficient transduction of the central nervous system (CNS) after intravenous delivery, and negligible presence throughout the rest of the body. Notably, AAV9.DT-N exhibits the highest transduction of neurons in the brain of any engineered variant to date, with less than 1% of cells in the liver transduced, a decrease of over 100-fold from AAV9. Most importantly, these capsids' ability to efficiently cross the blood-brain barrier and transduce CNS neurons is not limited to the rodents they were selected in, having also been validated in non-human primates.

New Advances in Chemical and Physical Gene Delivery

103. Heat Shrinking DNA Nanoparticles for In Vivo Gene Delivery to the Liver

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Control over DNA nanoparticle size is essential to achieve targeted gene delivery to the liver. DNA nanoparticles must possess a diameter of 100 nm or less to cross fenestrated liver endothelial cells and gain access to hepatocytes, whereas larger particles remained trapped in the circulation. We have previously reported the development of a highly optimized polyacridine PEG-peptide that forms stable 170 nm DNA nanoparticles that remain transfection competent for up to four hours when i.v. dosed in mice[1-2]. Despite their circulatory stability, these 170 nm DNA nanoparticles are too large to cross fenestrated endothelial cells. Here we report a simple and efficient method to "heat shrink" DNA nanoparticles to as low as 70 nm in diameter. Heat shrinking is accomplished by heating plasmid DNA at 100°C for 10 min to achieve partial denaturation, followed by the addition of a polyacridine PEG-peptide prior to cooling. The size of heat shrunken DNA nanoparticles is controlled by the heating time, temperature, buffer and the amount of polyacridine PEG-peptide added. The addition of a polyacridine PEG-peptide results in the conversion of supercoiled DNA to open circular plasmid DNA. The heat shrinking mechanism involves relieving plasmid strain by forming single stranded regions with increased flexibility that fold into smaller particles. Biodistribution analysis of 70 nm heat shrunken DNA nanoparticles resulted in a rapid and transient uptake by the liver whereas 170 nm DNA nanoparticle avoid uptake by the liver. Heat shrunken DNA nanoparticle are transfection competent when directly hydrodynamically dosed and when allowed

to circulate in mice for hours. The results demonstrate a completely novel approach to decrease the DNA nanoparticle size for delivery to the liver. 1.Kizzire, K.; Khargharia, S.; Rice, K. G., High-affinity PEGylated polyacridine peptide polyplexes mediate potent in vivo gene expression. *Gene Ther* **2013,** *20*, 407-16. 2.Khargharia, S.; Kizzire, K.; Ericson, M. D.; Baumhover, N. J.; Rice, K. G., PEG length and chemical linkage controls polyacridine peptide DNA polyplex pharmacokinetics, biodistribution, metabolic stability and in vivo gene expression. *J Control Release* **2013,** *170*, 325-33.

104. Engineered Amphiphilic Peptides Promote Delivery of Protein and CRISPR Cargo to Airway Epithelia In Vitro and In Vivo

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Efficacious delivery of therapeutic cargo to differentiated airway cells of the lung has potential to correct or treat many genetic and acquired diseases, including cystic fibrosis, chronic obstructive pulmonary disease, asthma and number of infectious diseases. However, efficient and safe delivery is extremely challenging due to formidable entry barrier imposed by the specialized and differentiated cells of the lung. Here, we report a novel and efficient peptide-based method to deliver protein and CRISPR/Cas nuclease cargoes and provide evidence of its effectiveness in well-differentiated primary human airway epithelial cells *in vitro* and in mouse airway epithelial cells *in vivo*. We engineered several soluble amphiphilic peptide transduction reagents that when combined with a protein of interest enabled their rapid and efficient delivery without compromising the activity of the cargo. The peptides are 25 to 41 amino acids long and contain both positively-charged hydrophilic and hydrophobic sections, in some cases separated by a glycine-rich linker. These amphiphilic peptides are non-covalently mixed with the payload to facilitate delivery of protein-based cargoes without a requirement for multiple incubation steps or covalent conjugation. GFP protein, when co-incubated with the peptide reagent, gained rapid and widespread entry into cultured human well-differentiated ciliated and non-ciliated epithelial cells, human tracheal tissue explants, and the large and small airway epithelial cells of mice *in vivo*. Delivery *in vivo* was rapid and restricted to lung as revealed by bioluminescent imaging of mice given luciferase protein intranasally. When peptide reagents were non-covalently combined and delivered with CRISPR ribonucleases (SpCas9 or AsCas12a) and their respective guide RNAs (ribonucleoproteins-RNPs), we saw rapid entry of CRISPR cargo and resultant editing at different gene loci (*CFTR* and *HPRT*) in primary well-differentiated human airway epithelia. To demonstrate *in vivo* CRISPR/Cas gene editing, we utilized two-color fluorescent Cre-reporter ROSA$^{mT/mG}$ mice. When CRISPR/Cas9 or CRISPR/Cas12a RNPs targeting the LoxP sites were delivered with the peptide reagent to mice intranasally, we observed efficient editing in large and small airway regions as demonstrated by the color switch from red (tdTomato) to green (GFP) fluorescent expression. We observed no evidence of short-term toxicity following peptide-mediated RNP delivery to mouse airways based on bronchoalveolar lavage fluid cell counts and differentials, and histological analysis of cytokine mRNA expression at 1 and 7 days post-delivery. Studies are ongoing to investigate the mechanism of the rapid entry of protein cargo facilitated by amphiphilic peptides in primary airway epithelia. This includes entry at 37°C vs 4°C and following exposure to various inhibitors of endocytosis. This amphiphilic peptide platform provides a flexible, chemical-, virus-, and DNA-free strategy for the rapid delivery of protein and CRISPR cargoes to hard-to-transduce airway epithelial cells and other cell types *in vitro* and *in vivo*.

105. Structure-Function Relationships of Branched Ester-Amine Quadpolymers for Non-Viral Retinal Gene Therapy

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Introduction: Safe and effective delivery of genes to the retina has the potential to effectively cure many heritable and acquired retinal diseases and has long been the focus of much research using adeno-associated virus. To-date, non-viral delivery of nucleic acids to post-mitotic cells of the retina has lagged behind advances in adeno associated virus for delivery. Here, we present the development of branched ester-amine quadpolymers for delivery of DNA to post-mitotic retinal cells that achieves safe, effective and persistent expression *in vitro* and *in vivo.*
Methods: Branched ester amine quadpolymers composed of four constituent monomers were synthesized via Michael addition reactions (Fig. 1A) to yield biodegradable, pH sensitive, cationic polymers with varying degrees of branching as well as highly defined primary, secondary and tertiary cationicity. Using post-mitotic retinal pigment epithelial cell monolayers with high content analysis screening and confocal microscopy, the delivery efficacy and structure-function relationships of these polymers was assessed *in vitro*. A diverse set of nanoparticles were then assessed for transfection activity *in vivo* following injection into rat retinas assessed by histology and anti-GFP ELISA.

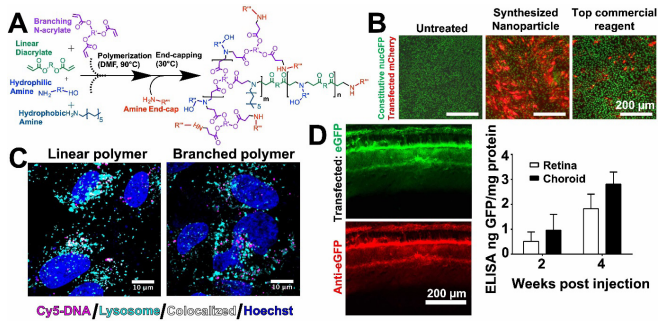

Fig. 1. A) Synthesis scheme of branched ester amine quadpolymers. B) High content analysis screening techniques for effective nanoparticle formulations in human embryonic stem cell derived RPE monolayers. Cells with constitutive nuclear-GFP expression were transfected with nanoparticles to express mCherry. C) Confocal microscopy of RPE cells having internalized nanoparticles carrying fluorescently labeled plasmid DNA. Lysosome colocalization and nuclear uptake were quantified. D) Rat retina histology images and quantification of conformation specific ELISA demonstrate long term expression in rat retina following injection of nanoparticles carrying plasmid DNA coding for eGFP. **Results:** Polymer design and testing *in vitro* was iterated using high throughput synthesis and high content imaging techniques, yielding >2x higher transfection than the top commercial transfection reagents tested (Fig. 1B). Local optimums for specific primary, secondary and tertiary amine density as well as hydrophobicity were identified. Moderately branched amphiphilic polymers were demonstrated to have improved stability driven by a combination of increased cationicity and branching structure. Branching further improved lysosomal avoidance, serum stability assessed by confocal laser scanning microscopy (Fig. 1C) and polymer efficiency on a per-mass basis. In contrast, lipophilic polymers were not improved by branching but did demonstrate improved stability, especially when combined with stabilizing and helper lipids. Assessment of materials *in vivo* following supra-choroidal injection in rats identified top nanoparticle formulations for translational development (Fig. 1D). **Conclusions:** Synthetic, biodegradable, branched ester amine quadpolymers were demonstrated to effectively delivery plasmid DNA to post-mitotic cells both *in vitro* and *in vivo*. Expression *in vivo* was demonstrated to persist for at least 1-month post injection.

106. Transcutaneous Ultrasound Mediated Gene Delivery in Canine Liver

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Ultrasound 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, which causes a deficiency of functional Factor VIII and inhibits proper execution of the clotting cascade. Previous studies in our lab showed successful transfection of swine liver using luciferase reporter plasmids and a transcutaneous UMGD procedure. In this study, we used the established transcutaneous UMGD procedure to transfer human factor VIII plasmid in a canine model. A high-expressing liver-specific human factor VIII plasmid was injected in combination with RN18 MBs into the canine liver lobes through a fluoroscopy-guided balloon catheter inserted into the hepatic vein via jugular vein access. Simultaneously, transcutaneous therapeutic ultrasound was applied to induce cavitation in the liver using an eight-minute pulsed US treatment (4.8 MPa, 1.05 MHz, 50 Hz PRF). Blood was collected prior to gene delivery and then at days one and three followed by weekly time points for four weeks. A Western blot was performed to examine the presence of human factor VIII in the treated animals. ELISA was also carried out to evaluate the levels of human factor VIII expression and transaminase (AST/ALT) assays were performed to assess potential liver damage at various time points following treatment. The Western blot confirmed the presence of human factor VIII protein in the plasma of treated dogs. ELISA data showed notable expression of human factor VIII at approximately 9% of normal plasma levels at day three, followed by lower expression levels at day seven. We are investigating the formation of species-specific inhibitor antibodies at later time points. The AST/ALT data showed minor elevation above normal levels at day one, but these levels had completely normalized by day three. All the other parameters from CBC and whole blood chemistry analysis showed little or no changes from the normal levels over the entire experimental period. Transcutaneous UMGD of human factor VIII plasmids achieved successful transfection of liver tissue and produced therapeutic levels of human factor VIII expression in a canine model. Taken together, these results show significant promise for a minimally invasive UMGD as a clinically feasible therapy for Hemophilia.

107. The Balancing Act between Required Stability and Sufficient Cargo Release: A Systematic Investigation of the Impact of Stabilizing Units within pDNA Lipo-Polyplexes

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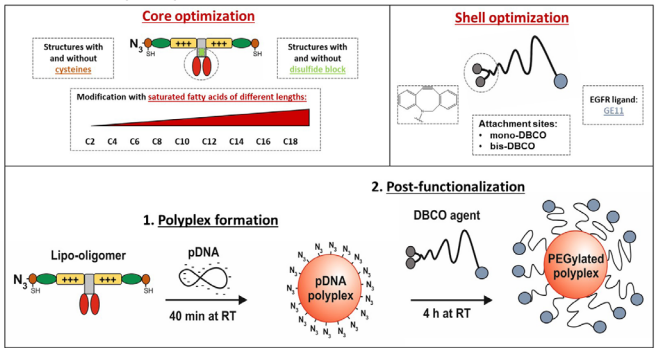
Appropriate carrier systems are required for effective pDNA delivery, comprising extracellular protection, efficient cellular uptake and intracellular release of pDNA. One option is the use of lipo-polyplexes formed by electrostatic interactions between anionic pDNA and cationic lipo-oligomers. Solid-phase assisted synthesis presents a feasible way to create such lipo-oligomers in a sequence-defined manner. Thereby, functional moieties and their position within the oligomer can be altered to derive relations between oligomer structure and polyplex properties. In the current work, the influence of different structure variations within the lipo-oligomers on stability, transfection efficiency and toxicity of the resulting polyplexes was investigated. For this purpose, a library of lipo-oligomers was synthesized, differing in the chain length of the incorporated saturated fatty acids. In addition, analogous structures were synthesized without cysteines and with or

without a bioreducible disulfide building block between the diacyl domain and the cationic backbone. The findings were as follows: Shorter fatty acids as well as the lack of cysteines show a destabilizing effect. Less stability seems to be beneficial for high transfection efficiency *in vitro*. However, certain stability is required, so that there exists a maximum in transfection efficiency for an acyl chain length of C6 - C10. Structures with cysteines exhibit the best transfection results, followed by the disulfide block containing ones. Those, lacking both, show almost no transfection. Longer fatty acids (C14 - C18) cause toxicity. By incorporation of the redox-sensitive unit, toxicity can be reduced. As the lipo-oligomers all contain an azide function, the pre-formed core-polyplexes could be modified with a shell via orthogonal click-chemistry in a next step. Therefore, two different ligands for EGFR targeting have been evaluated, bearing one or two DBCO units as attachment sites. Post-functionalization with the bis-DBCO agent shows a notable decrease in zeta-potential. Furthermore, functionalization with the bivalent agent leads to better transfection efficiency compared to the mono-DBCO agent. Best performer of the whole study is the lipo-oligomer that contains cysteines and decanoic acid (C10) and that is post-functionalized with the bis-DBCO agent for EGFR targeting in Huh7 wt cells.



108. Characterization of the DNA Nanoparticle Interactome and Potential Targets that Enhance Gene Delivery

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Gene therapy has the potential to treat many pulmonary diseases but gene delivery to the lung has poor efficacy due to rapid degradation and low cellular uptake. DNA nanoparticles (DNPs) are a non-viral vector capable of transfecting non-dividing cystic fibrosis (CF) airway epithelial cells with minimal toxicity in animals and CF patients. Nevertheless, the lung is difficult to transfect as it has many extracellular barriers, including blood, mucus, and inflammatory cells. The DNP compacts DNA with a cationic polymer consisting of 1 L-cysteine and 30 L-lysines conjugated to a 10 kDa PEG. Despite PEGylation, which aids in circumventing many extracellular barriers, DNP transfection of cultured primary airway epithelial cells (AECs), which lack barriers such as inflammatory cells, is less efficient than viral vectors. This suggests that intracellular barriers are important. A significant intracellular barrier are the interactions between the vector and cellular molecules that inhibit the vector from reaching its target compartment (e.g. the nucleus for DNA and the ribosome for RNA). Previous study of DNP biology identified nucleolin as a DNP interactor that is crucial to cellular uptake and nuclear trafficking. The glucocorticoid receptor (GCR) interacts with nucleolin and our group found that GCR activation with cortisone enhanced vector efficacy 3-4 fold. This evidence highlights the importance of cellular interactions and supports our hypothesis that defining the DNP interactome would identify targets that modulate gene transfer. We used tandem mass spectrometry to identify the DNP interactome examining immunoprecipitations of DNPs during transfection of AECs. The biology of the 463 interactome proteins was analyzed using gene ontology (GO) term enrichment. We found that interactors have a broad range of molecular functions, with significant interactors belonging to proteins that bind nucleic acids. Significant cellular compartments of the interactors localize DNPs to major organelles, with the ribosome, nucleus, and cytoskeleton being the most significant. Over half of the interactors can be found in the nucleus, demonstrating it is where the majority of DNPs localize. Furthermore, we analyzed the biological processes attributed to interactors and found that translational, cytoskeletal, transcriptional, and transport machinery are highly enriched. The GO process viral transcription was also significantly implicated, consistent with the presence of SV40 enhancer elements in our vector. The data suggest that the DNP interfaces not only with transcriptional machinery but also with other processes, such as translation. Since nucleolin is essential for transfection, we hypothesized that interactors which affect nucleolin would modulate gene transfer. Therefore, we determined which proteins in the DNP interactome also interact with nucleolin. We found 79 DNP interactors, including non-erythrocytic spectrin alpha 1 (SPTAN1), adenomatous polyposis coli (APC), and heat shock protein 90 (HSP90), that interface with nucleolin. AECs treated with shRNAs that knockdown either SPTAN1 or APC exhibited a 5- or 37-fold increase, respectively, in levels of transfection by DNPs compacting a luciferase expressing plasmid compared to scrambled shRNA treated cells. This increase in gene transfer was significantly greater than that achieved by modulating GCR with cortisone. Conversely, inhibition of HSP90 by 17AAG decreased gene transfer by 24% in HeLa cell studies. These results supported our hypothesis and identified 2 protein targets whose disruption enhanced gene transfer. Therefore, manipulation of other DNP interactors is a logical approach to modulating gene delivery.

109. New Clinically Compatible Nanoplexes for Molecular-Genetic Imaging of Human Cancers

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We have previously shown that systemic delivery of a non-viral imaging vector comprised of a tumor-specific promoter and a reporter enables efficient visualization of metastatic lesions of human cancer in mouse

models.[1,2] The vectors and non-viral gene delivery carrier (linear polyethyleneimine, *l*PEI) used in these studies contain features not amenable to human use. To develop a clinically compatible molecular-genetic imaging system, here we created and tested new vectors featured with a CpG-minimized backbone, a kanamycin resistant gene or RNA-OUT, and scaffold/matrix attachment regions. We also developed a scalable flash nanocomplexation (FNC) manufacturing method[3] for production of lyophilized, shelf-stable pDNA/*l*PEI nanoparticles using a cGMP grade *in vivo*-jetPEI® with an excellent reproducibility under quality assurance and quality control standards. These well-defined pDNA/*l*PEI nanoparticles delivered systemically were well tolerated in mice. The *Peg-3* promoter (PEG-Prom) was chosen for its tumor-specific activity in a broad-spectrum of human cancers. PEG-Prom contains 42 CpG sequences. Mutagenesis studies on each CpG sequence revealed that the majority of them are essential for maintaining promoter activity; therefore, the original CpG-containing PEG-Prom was used for the study. By reducing CpG sites in other parts of the plasmid construct, one vector pCpGlow-PEG-Prom-fLuc (cfPEG) given systemically in pDNA/*l*PEI nanoparticle form revealed the metastatic lesions in the lung of LL2 and B16/F10 murine tumor models with enhanced sensitivity compared with pPEG-fLuc (pPEG) (**Fig 1A**). The pDNA/*l*PEI nanoparticles prepared with another clinically compatible vector, pCpGlow-PEG-HSV1tk (ctPEG) successfully depicted metastatic lesions in multiple tumor models via SPECT/CT imaging following systemic administration. These nanoparticle formulated with ctPEG induced significantly lower levels of acute inflammatory cytokines, IFN-γ, TNF-α, and IL-12 when injected into immunocompetent mice, compared with those induced by the CpG-rich research grade plasmid, pPEG (**Fig 1B**). The new clinically compatible vectors and nanoparticle formulation exhibited promising characteristics for both efficacy and safety to serve as a plasmid backbone for clinical translation of the molecular-genetic imaging system for human cancers. **Reference:** 1. Bhang, H. E. *et al.* Tumor-specific imaging through progression elevated gene-3 promoter-driven gene expression. *Nat. Med*, *2011; 17(1), 123-129.* 2. Minn, I. *et al.* Molecular-genetic imaging of cancer. *Adv, Can. Res. 2014; 124:131-69.* 3. J. L. Santos et al., Continuous production of discrete plasmid DNA-polycation nanoparticles using flash nanocomplexation. *Small, 2016; 12(45), 6214-6222.* **Acknowledgements:** This study is partially supported by a research contract from Cancer Targeting Systems, Inc. (CTS) and an NIH grant R01EB018358. The authors thank Chris Ullman and Christine Carrington of CTS for helpful discussions.

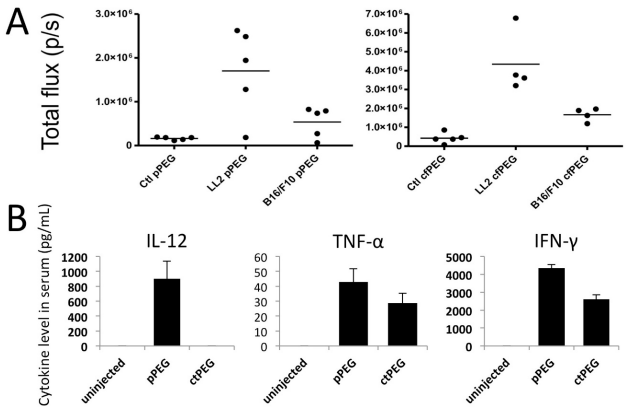

Figure 1. New clinically compatible nanoplex exhibited enhanced efficacy and safety. (A) Summary of data from BLI images of non-tumor-bearing NSG mice (Ctl) or NSG mice carrying lung metastatic lesions of LL/2 and B16/F10 cells. Left graph: CpG-rich research grade plasmid. Right graph: CpG-low clinical plasmid. Note: vertical scales differ. Each dot represents the calculated total flux from region of interest of a single mouse. (B) Inductions of acute inflammatory cytokines were measured 48 h post injection of the indicated plasmids into immunocompetent mice.

Rational Engineering of AAV Vectors I

110. An RNAseq Directed Screening Method to Identify Genes Modulating Titer in rAAV Producing Cell Lines

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Background: As rAAV gene therapy trials progress from Phase I/II to Phase III clinical trials and commercialization, it is essential to develop and establish manufacturing processes that consistently produce large amounts of vector in a cost-effective manner. At Ultragenyx Gene Therapy, the HeLaS3 producer cell line system has shown successful scalability up to 2000L with high yields. To build on this progress, we are devising approaches to engineer a producer cell line for even greater yields. An impediment to engineering improved producer cell lines is the lack of understanding of which genes are essential for, or detrimental to, high titer production. RNAseq offers a powerful tool for understanding global gene expression from a range of vector production. Armed with knowledge of differential gene expression, one can devise gene augmentation and or gene knockdown strategies to improve vector yields. **Methods:** RNAseq was performed on multiple producer cell lines prior to and during rAAV production to determine global gene expression under these conditions. Differential analysis revealed a small gene set with similar magnitude fold changes conserved across two distinct producer cell lines. RNAseq results were then confirmed through RT-qPCR of two genes with the largest fold changes. Additionally, an siRNA knockdown method was established to analyze the effects of individual genes on productivity. Finally, the small gene set was put through an siRNA screen to evaluate the effect of knockdown on rAAV titer compared to a missense control. **Results and Conclusions:** A subset of 11 genes was identified as potentially important for rAAV productivity based on RNA sequencing and differential analysis. siRNA knockdown of those genes resulted in the

identification of 4 genes that, when individually silenced, improved titer 2-4 fold. These results support further utilizing an RNAseq-guided siRNA knockdown method to identify targets for the purpose of engineering permanent modifications. Ultimately this work could lead to the development of improved methods for AAV gene therapy vector production and of superior cell substrates for high producing cell lines.

111. AAV Gene Transfer with Tandem Promoter Design Prevents Anti-Transgene Immunity and Provides Persistent Efficacy in Neonate Disease Mouse Models

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The ability of achieving therapeutic levels of transgene expression in multiple affected tissues concomitant with transgene immune tolerance represents an important goal for the field of *in vivo* gene therapy with Adeno-associated virus (AAV) vectors. Hepatocyte-restricted, AAV-mediated gene transfer is being used to provide sustained, tolerogenic transgene expression in gene therapy for the treatment of a variety of diseases caused by protein deficiencies. However, given the episomal status of the AAV genome, this approach cannot be applied to pediatric disorders where hepatocyte proliferation may result in significant loss of therapeutic efficacy over time. In addition, many multi-systemic diseases require widespread expression of the therapeutic transgene that when provided with ubiquitous or tissue-specific non-hepatic promoters often results in anti-transgene immunity. Here we have developed tandem promoter monocistronic expression cassettes that, packaged in a single AAV, provide combined hepatic and extra-hepatic, tissue-specific transgene expression and prevent anti-transgene immunity. We validated our approach in infantile Pompe disease, a prototype disease caused by the lack the ubiquitous enzyme acid-alpha-glucosidase (GAA), presenting multi-systemic manifestations and detrimental anti-GAA immunity. We showed that the use of efficient tandem promoters prevents immune responses to GAA following systemic AAV gene transfer in immunocompetent $Gaa^{-/-}$ mice. Then, we demonstrated that neonatal gene therapy with either AAV8 or AAV9 in $Gaa^{-/-}$ mice rescued the disease phenotype whole-body and autophagy block in skeletal muscle only when using a tandem liver-muscle promoter (LiMP) but not the single muscle- and hepatocyte-specific promoters. The tandem configuration of the tissue-specific regulatory elements within LiMP provided synergistic effects on transgene transcription and high, persistent transgene expression in muscle tissues. In conclusion, the tandem promoter design overcomes important limitations of AAV-mediated gene transfer and can be beneficial when treating pediatric conditions requiring persistent multi-systemic transgene expression and prevention of anti-transgene immunity.

112. Novel AAV1 Mutants for Robust Transduction of the Central Nervous System Following Intravenous Injection

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AAV9 has garnered significant attention as a gene therapy vector for central nervous system (CNS) disorders due to its superb ability to cross the blood-brain barrier (BBB) and globally transduce cells in the CNS following systemic administration. Indeed, we have recently used AAV Barcode-Seq technology to demonstrate that AAV9 vectors express more transgene RNA transcripts per DNA vector genome than many other AAV vectors, including AAV1, following intrathecal (IT) injection into the cisterna magna of non-human primates (rhesus and cynomolgus macaques). However, the same study found that IT-injected AAV1 vectors deliver vector genome DNA more efficiently than AAV9 vectors. This indicates that, by a mechanism that has yet to be elucidated, AAV1 is superior to AAV9 with respect to delivery of vector genome DNA into the brain, while vector genome DNA delivered by AAV9 becomes more transcriptionally active than vector genome DNA delivered by AAV1. Here, we show that three novel AAV1-derived mutants whose capsid amino acids are 96-97% identical to those of AAV1 exhibit AAV9-like robust neuronal transduction following intravenous (IV) injection in mice. These three mutants, AAV1.9mt30, AAV1.9mt76 and AAV1.9mt100, carry 21 to 26 amino acids from the AAV9 capsid at defined positions. We predicted that novel AAV capsids possessing the advantages of both AAV1 and AAV9 would provide a novel platform for further capsid evolution and the development of next-generation AAV capsids for CNS gene delivery. In order to create such novel capsids, we identified seven potential regions in the AAV9 capsid that confer robust CNS transduction following IV injection and distinctively slowed blood clearance, and created 127 AAV1-derived mutants with AAV9 grafts. The 127 mutants represent all possible combinations of the AAV9-derived regions grafted into the AAV1 capsid. In vivo biological phenotypes (blood clearance rates and tropism following IV injection) of all 127 mutants, as well as the parental AAV1 and AAV9 serotypes, were then determined by AAV DNA/RNA Barcode-Seq technology. The RNA Barcode-Seq data analysis revealed that AAV1.9mt30, mt76, and mt100 transduced the brain much better than the parental wild-type AAV1 and at levels comparable to that of AAV9. These three mutants were selected for a downstream validation study and vectorized with a double-stranded AAV-hSynI-GFP vector genome. We then injected mice with each vector intravenously at a dose of 1×10^{12} vector genomes (vg), and harvested the brain tissues six weeks post-injection. Immunofluorescence microscopic analysis of the brain tissues showed strong GFP expression across the brain (cortex, hippocampus, caudate putamen, thalamus, hypothalamus and amygdala). All three mutants showed comparable levels of expression to

the AAV9 control, while AAV1 vector injection resulted in a very low level of brain transduction. We also quantified neuronal transduction by counting the number of GFP positive cells within HuC/D-stained neuronal cells, which conformed to the AAV Barcode-Seq data. In summary, we identified novel AAV1-derived mutants that may display strengths of both AAV1 and AAV9 with respect to CNS transduction. These AAV mutants are a promising platform for the development of more robust AAV capsids.

113. Design Principles for AAV Mediated Circular RNA Expression in the Brain

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Circular RNAs (circRNAs) are covalently closed RNA molecules that are more stable than their linear counterparts. These molecules are highly abundant in the mammalian brain, synthesized during neuronal differentiation and dynamically expressed during development. Recently, our lab has shown that recombinant adeno-associated viral (AAV) vectors can be utilized to express translatable circular RNAs generated by backsplicing. Here, we describe design principles for highly efficient circularization of RNA transcribed from AAV vector genomes. Specifically, we have engineered novel circRNA yielding constructs by manipulating different intronic elements flanking a split GFP exon and different internal ribosomal entry sites (IRES) driving translation. In addition to comparing natural and synthetic intronic elements, we have mapped the expression profile of a panel of AAV-circRNA vectors at the regional and cellular levels in the murine brain. We characterize the circular RNA products through a battery of biochemical and cellular assays demonstrating the versatility of this new platform. These results contribute to our understanding of circRNA biogenesis in the brain and reveal the potential for translational control through different IRES elements in the CNS as well. This multi-component AAV-circRNA platform has the potential to enable robust control over tissue-specific and durable expression of therapeutic transgenes and noncoding RNAs.

114. Cryo-EM, Biophysical and In Vivo Tropism Analyses of a Novel Adeno-Associated Virus Capsid Isolated from Human Tissue

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Recombinant adeno-associated viruses (rAAVs) have recently gained a lot of attention within the human gene therapy field, as safe and reliable gene delivery vehicles. AAV2 is currently most commonly used in preclinical and clinical studies. However, AAV2-based drug Luxturna is the only FDA approved virus-based biotherapeutic, so it is critical to improve the pharmaceutical properties of AAV. AAV2 is known to be a "poor producer" for vector production and "underperforms" in many tissue- and cell-types. We therefore aimed to isolate virus variants with improved properties, using a library of AAV2 variants isolated from clinical samples. A variant named AAVv66 emerged as the most abundant pro-viral capsid variant in a clinical pancreatic neoplasm sample. The AAVv66 capsid harbors 13 residues that differ from AAV2. The variant exhibits favorable tropism in the CNS following subcranial injections. Furthermore, AAVv66 demonstrates better packaging efficiencies than prototypical AAV2. Using differential scanning fluorimetry (DSF), we found that the melting temperature of AAVv66 is ~6°C higher than AAV2 across a pH range spanning pH4 - pH7. Furthermore, DSF analysis shows that at pH4, AAVv66 expunges its vector DNA at higher temperatures than AAV2. We also demonstrate that AAVv66 confers superior CNS transduction. Cryo-EM structure at 2.9Å resolution reveals structural differences between AAV2 and AAVv66 at the 3-fold protrusions and at the interface of the 5-fold axis of symmetry, suggesting that critical residues at these positions confer improved stability and function for vector transduction. Our data collectively suggest that AAVv66 is superior to AAV2 in capsid stability and retaining its genome at low pH environments, likely contributing to virus stability within late-endosomes and lysosome compartments. Our work demonstrates that characterizing natural AAV capsid isolates continues to reveal novel aspects of AAV biology and may pave the way to improved gene therapy vectors. *Co-first authors; #Co-corresponding authors.

115. Development of AAV Vectors with the Potential to Dampen the Host Humoral Immune Response

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Although AAV vectors have taken center stage in the potential gene therapy of a wide variety of human diseases, the host immune response to these vectors remains a major challenge. As a consequence, repeat dosing with AAV vectors is not possible. We previously reported that a cellular protein, which we designated as the double-stranded D-sequence-binding protein (dsD-BP), interacts with the D-sequence in the AAV inverted terminal repeats (ITRs) and plays a crucial role in AAV DNA replication and encapsidation (*J. Virol.*, 70: 1668-1677, 1996). We subsequently provided evidence that the dsD-BP also binds to the X-box sequence of the major histocompatibility complex type II (MHC-II) promoter, and suggested that the dsD-BP might be a

putative RFX transcription factor (*Mol. Ther*., 1: S190, 2000). Indeed, RFX1 and RFX3 transcription factors were recently shown to bind to the AAV D-sequence (*Sci. Rep*., 8: 210, 2018). In the present studies, we document that MHC-II promoter-driven expression of a firefly luciferase reporter gene was strongly inhibited both by X-box and the D-sequence synthetic oligonucleotides, whereas no inhibition was observed with non-specific synthetic oligonucleotides. Transgene expression from a CMV promoter was not inhibited by either the X-box or the D-sequence oligonucleotides. Furthermore, treatment with interferon-gamma, known to lead to activation of the MHC-II promoter, resulted in enhanced transgene expression, which was also significantly inhibited both by the X-box and the AAV D-sequence oligonucleotides in human cells *in vitro*. We have now generated AAV vectors expressing the EGFP reporter gene under the control the MHC-II promoter, which should facilitate further *in vivo* studies designed to evaluate the extent of transduction of antigen-presenting cells, such as dendritic cells, B-cells, and macrophages, by AAV vectors. We have also generated AAV vectors containing the X-box, or the entire MHC-II promoter sequences. Studies are currently underway to evaluate the efficacy of these vectors in a murine model *in vivo*. Taken together, these studies suggest that the D-sequence-mediated down-regulation of expression of the MHC-II genes, which may be further augmented by the inclusion of the X-box or the MHC-II promoter sequences, might be exploited to dampen the host humoral immune response in order to make it feasible to achieve repeat dosing with recombinant AAV serotype vectors.

116. The Potential Role of Surface-Exposed Tyrosine Residues on AAV Capsids in the Diminution of Vector Neutralization by Human Antibodies

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One of the major challenges in the use of AAV vectors for gene therapy of human diseases is the presence of pre-existing antibodies that neutralize these vectors. We have described the development of next generation of AAV vectors in which specific surface-exposed tyrosine (Y) residues were replaced with phenylalanine (F) residues. The transduction efficiencies of 3 of these Y-F mutants (Y444F, Y500F, Y730F) was significantly higher than their WT counterpart (*Proc. Natl. Acad. Sci, USA*, 105: 7827-7832, 2008). When the 3 most efficient mutations were combined into one capsid, the resulting triple-mutant (Y444F+Y500F+ Y730F) vector was ~30-fold more efficient than its WT counterpart (*Mol. Ther*., 18: 2048-2056, 2010). Furthermore, this triple-mutant AAV2 vector was shown to minimizes *in vivo* targeting of transduced human hepatocytes by capsid-specific $CD8^+$ T cells (*Blood*, 121: 2224-2233, 2013). In the present studies, we wished to evaluate whether the Y444F+Y500F+Y730F triple-mutant (TM) AAV2 vector was also capable of escaping neutralization by pooled human intravenous immunoglobulins (IVIG). To this end, both WT and TM scAAV2-EGFP vectors were pre-incubated with PBS, or with 10-fold serial dilutions of pooled human IVIG (Privigen), and used to transduce HeLa cells in triplicates under identical conditions. Transgene expression was determined by flow cytometry 72 hrs post-transduction. The results indicated that both WT and TM AAV2 vectors were completely neutralized at a conc. of 1 mg/ml of IVIG. This was perhaps not unexpected given that ~90% of humans are sero-positive for anti-AAV2 antibodies, and thus, the pooled IVIG would be expected to have significant levels of anti-AAV2 antibodies. We next extended these studies to include the WT AAV6 and a Y705+731F+T492V+K531R quadruple mutant (QM) AAV6 vectors. Again, both WT and QM AAV6 vectors were pre-incubated with PBS, or with 10-fold serial dilutions of pooled human IVIG, and used to transduce human K562 cells in triplicates under identical conditions. Transgene expression was determined by flow cytometry 72 hrs post-transduction as described above. The results indicated that whereas 100% of the WT AAV6 vectors were neutralized at a conc. of 1 mg/ml of IVIG, only ~25% of the QM AAV6 vectors were neutralized at the same IVIG conc. These results are remarkably similar to those reported in a recent publication (*Mol. Ther. Meth. Clin. Dev*., 9: 323-329, 2018) in which ~80% of a F129L+Y445F+ Y731F triple-mutant AAV6 vector was shown to be resistant to neutralization by 1 mg/ml of IVIG, whereas only ~3% of the WT AAV6 vector was resistant under identical conditions, albeit in HeLa cells. These observations have led to the hypothesis that specific surface-exposed Y residues may be a critical part of antigenic epitopes on AAV capsids. Additional studies are currently underway with WT and various permutations and combinations of specific surface-exposed amino acids on other clinically-relevant AAV serotype vectors, such as AAV3, AAV8, and AAV9, to further corroborate this hypothesis, which would be instrumental in the development of novel AAV serotype vectors capable of evading neutralization by pre-existing human antibodies.

Cancer Adoptive Immunotherapy

117. Tunable IFNa-Based Gene Therapy Inhibits Glioblastoma Multiforme Growth in a New Syngeneic Mouse Model

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Efficient and targeted delivery of cancer biotherapeutics to the tumor may achieve substantial benefits with limited side effects. Our group previously showed that is possible to exploit a population of tumor associated macrophages (TEM, Tie2 expressing macrophages) to deliver interferon alpha (IFNa) to tumors through a cell and gene therapy approach. Transplant of hematopoietic stem cells (HSPCs) transduced with lentiviral vector expressing IFNa under the control of Tie2 enhancer/promoter induces TEM-specific release of the cytokine in the tumor microenvironment (TME), reprogramming it towards a pro-inflammatory state. This is particularly relevant in the case of poorly immune infiltrated tumors, in which a strong immunoppressive

microenvironment counteracts a possible immune response against cancer cells. In this contest, glioblastoma multiforme (GBM) represents a prototypical example of "cold tumor" with extremely poor prognosis despite available standard therapies. The strong immunosuppressive state of the GBM microenvironment, in addition with its highly vascularized phenotype, makes GBM a suitable candidate for testing our Tie2-dependent IFNa gene therapy strategy. We took advantage of a novel orthotopic model of mouse GBM growing in immunocompetent mice, closely recapitulating several features of the human pathology. Upon GBM challenge, mice transplanted with HSPCs transduced with Tie2-IFNa showed remarkable tumor inhibition and improved survival. Notably, a fraction of treated mice efficiently cleared tumor cells. To further improve our platform we developed an inducible strategy to control the timing and amount of IFNa secreted in the TME. By fusing a destabilizing domain (DD) to a protein of interest (POI) the former confers instability to the latter. Destabilization can be rescued in a reversible and dose dependent manner with the addition of a small molecule specifically binding to the DD. We have designed and *in vitro* tested different fusion proteins of IFNa and the destabilizing domain DHFR with or without the addition of flexible or cleavable linkers and selected them for their capacity to be stabilized in presence of their specific ligand (Trimethoprim) *in vitro*. Through this approach, we have identified effective fusion proteins with low basal activity and high fold induction upon ligand treatment. These novel regulated forms of IFNa are functional *in vitro* and their specific activities are comparable to the wild type form. To validate our inducible strategy *in vivo* we transplanted mice with HSPC transduced with our best DD-IFNa candidate selected *in vitro* and challenged them with GBM. 14 days upon GBM injection, magnetic resonance (MRI) analysis showed comparable tumor growth in control and DD-IFNa transplanted mice. We then started Trimethoprim treatment and followed GBM growth overtime. DD-IFNa treated mice showed strong tumor growth inhibition, and overall improved survival compared to controls, up to full tumor clearance in a fraction of mice. These data indicate that DD-IFNa-based strategy may represent an effective cell and gene therapy approach for GBM treatment. Moreover, the possibility to turn on and off the release of the cytokine allows to best tune delivery to the clinical setting and explore synergy with chemotherapeutic or immunomodulatory drugs. **Co-senior authorship*

118. High Avidity T Cell Receptors Redirect Natural Killer T Cell Specificity While Outcompeting the Endogenous Invariant Chain T Cell Receptor

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TCR gene transfer represents a promising immunotherapeutic approach for redirecting T cells targeting to intracellular antigens. However, two major limitations remain when using T cells: the potential for autoreactivity, if the transgenic TCR chains pair with the endogenous chains, and the occurrence of Graft versus Host Disease in the allogenic setting, as functional native TCRs are retained. To reduce the risk for TCR-chains mispairing and cross-reactivity we hypothesized that NKTs would be a more appropriate platform for TCR engineering. NKTs are indeed a conserved sub-lineage of T cells restricted to the monomorphic CD1d molecule by their invariant TCR (iTCR), consisting of the Vα24Jα18 chain paired with a limited repertoire of TCRβ chains. From ten healthy donors, we isolated, expanded and transduced NKTs with an HLA-A2-restricted Tyrosinase-TCR (TyrTCR, J Imm 2010; 184: 5988). TyrTCR was expressed by 76±8% NKTs (TyrTCR-NKTs) and stable for the 19-days culture period. Unexpectedly, 41±18% of TyrTCR-NKTs downregulated the iTCR, generating 3 distinct subsets: iTCR-TyrTCR+(44±15%), iTCR+TyrTCR+(34±11%) and iTCR+TyrTCR-(19±11%). Confocal microscopy analyses showed that the iTCR was retained in the cytoplasm of iTCR-TyrTCR+NKTs as the ectopic TCR outcompeted the endogenous one for binding to the CD3 complex. Indeed, the percentage of iTCR+TyrTCR+significantly increased after overexpression of the CD3 complex (p=0.0001). While neither CD4 or CD8 expression, nor the TCR Vβ repertoire correlated with the iTCR displacement, the mRNAs of the ectopic TCR chains were overexpressed as compared to the endogenous iTCR, with higher transcript numbers dictating which receptor was expressed on the cell surface. Irrespective of the iTCR downregulation, TyrTCR-NKTs efficiently lysed Tyr-peptide loaded HLA-A2+ targets and eliminated HLA-A2+Tyr+ melanoma cell lines in vitro (Table 1).

TyrTCR-NKTs	C8161A2$^+$Tyr$^-$	M14 wt A2$^-$Tyr$^+$	SK-MEL-5A2$^+$Tyr$^+$	M14 A2A2$^+$Tyr$^+$
Cr-release (% specific lysis, 40:1 E:T)	20±7	22±10	91±5	87±5
Coculture (% of residual tumors)	88±5	77±7	1±2	2±2

Killing activity of TyrTCR$^+$NKTs was paralleled by significant IFNg production. No killing nor IFNg release occurred for non-transduced NKTs. More importantly, TyrTCR-NKTs controlled tumor growth *in vivo* in two (subcutaneous and systemic) melanoma xenograft mouse models, producing improved overall survival (p<0.0001) without toxic effects. In contrast 60% of TyrTCR-Ts treated mice developed lethal graft versus mouse disease. We finally validated the NKTs platform using the low functional avidity MART-1 TCR (*J Imm 2010; 184: 5988*). TyrTCR and MART-1TCR showed similar functionality when expressed in CD8$^+$ T cells, but not in NKTs, with MART-1TCR-NKTs producing inferior IFNg and tumor elimination against HLA-A2$^+$Tyr$^+$ targets as compared to TyrTCR-NKTs. Overall our data show that, as compared to T cells,NKTs represent an advantageous platform for the expression of TCR as they do not require further gene manipulation to down modulate endogenous TCR chains. Our study also suggests that the intrinsic functional avidity of the TCR may be critical to redirect NKTs via HLA class I restricted TCRs.

119. LAG-3, but Not Tim-3, Disruption in TCR Gene Edited Human Memory Stem T Cells Enhance the Anti Tumor Activity Against Multiple Myeloma

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Chronically stimulated lymphocytes infiltrating solid and hematological tumors highly express several inhibitory molecules, such as PD-1, CTLA-4, Lag-3 and Tim-3. Cancer cells up-regulate the ligands of these inhibitory molecules and foster inhibitory pathways in T cells, thus exploiting loss of effector functions of exhausted cells to evade immune attack. The blockade of the interaction between inhibitory receptors (IRs) on T cells and the ligands expressed by tumor cells with monoclonal antibody (e.g. anti-CTLA-4, anti-PD-1) have shown promising results in clinical trials for the treatment of 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. These observations advocate us to develope a strategy to simultaneously redirect T cell specificity by TCR gene editing and permanently disrupt IRs by CRISPR/Cas9 system in long-living memory stem T cells (T_{SCM}) for adoptive cell therapy. Primary T cells were activated with CD3/CD28-conjugated beads and cultured with low doses of IL7 and IL15, to preserve their T_{SCM} phenotype, and electroporated with CRISPR/Cas9 targeting a coding sequence of *TIM-3, LAG3*, and of the TCR α and β chain constant region (*TRAC* and *TRBC1/2*) genes. The efficiencies of NHEJ-mediated inactivation of each gene were assessed by flow cytometry and confirmed at molecular level by ddPCR. To avoid mispairing between the endogenous TCR and the tumor-specific TCR, we simultaneously inactivated TRAC and TRBC1/2 genes. Efficiency of *TRAC* and/or *TRBC1/2* gene disruption was 98%. TCR-disrupted cells could be efficiently (70-85%) transduced with a lentiviral vector encoding for TCR specific for an HLA-A2 restricted peptide from NY-ESO1 (LV-NY-ESO-1). We then combined the TCR gene editing protocol with Tim-3 and LAG-3 disruptions within a multiplexing approach. We obtained very high frequencies of gene disruption, with a median of 76% of TCR/Lag-3-disrupted and 96% of TCR/Tim-3-disrupted T cells. We transduced TCR^{neg}-IR^{neg} T cells with the LV-NY-ESO-1 and obtained a median of 65% of TCR-edited/LAG-3^{neg} cells and 57% of TCR-edited/Tim-3^{neg} cells. More than 90% of edited cells maintained an early differentiated T_{SCM} phenotype. We compared our TCR-edited-$IR^{neg/pos}$ cells in vitro and in vivo against multiple myeloma. In co-colture assays, both TCR/LAG3-disrupted and TCR/Tim-3-disrupted T_{SCM} cells proved highly effective and specific in killing HLA-$A2^{+}$ NY-$ESO1^{+}$ multiple myeloma cells. When tested in immunodeficient mice, limiting doses of TCR-edited-LAG-3^{neg} lymphocytes, but not TCR-edited-Tim-3^{neg}, displayed a significantly enhanced anti-tumor activity compared to TCR-edited-IR-competent cells. In conclusion, by combining the versatility of multiplex gene editing by CRISPR/Cas9 with culture conditions designed to engineer T_{SCM} cells, we can generate innovative tumor-specific cellular products redirected against tumor antigens and resistant to inhibitory signals.

120. Targeting the Tumour Vasculature with CAR T-Cells for Treatment of Solid Tumours

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CAR-T cells have shown remarkable efficacy in haematological malignancies but their use in solid tumours has produced less encouraging results. Both the tumour microenvironment and the difficulty in identifying specific target antigens on solid tumours remain key challenges. An alternative therapeutic approach is to target the tumour vasculature rather than the malignant cells directly. We have shown Clec14a, a cell surface protein involved in angiogenesis, to be upregulated in the vasculature of a range of solid tumours (e.g. ovarian, liver, bladder, prostate, and breast). In contrast, expression in healthy tissues was low or undetectable. We hypothesised that a functional Clec14a-targeted CAR-T cell therapy can act as a vascular disruptive agent causing haemorrhagic necrosis and tumour bulk reduction, which in turn may open further treatment options. The majority of CAR-T cells administered in the clinic have been transduced with a lentiviral or gamma-retroviral vector to permanently express a specific CAR. An alternative approach is electroporation of mRNA to generate transient CAR-T cells which has been shown to elicit an anti-tumour effect in patients with transient persistence of the CAR-T cells. This is an attractive approach to provide additional safety in the clinic, minimising risk of CRS, whilst bypassing the need for 'suicide gene' induction systems. Electroporation of mRNA into T-cells is also being used for delivery of gene editing components to generate off-the-shelf CAR-T cells. A robust manufacturing process for electroporation of mRNA into T-cells is therefore of broad interest with wide applicability in the CAR-T cell field. Towards a clinical trial we have developed a robust, automated and closed manufacturing process for the generation of mRNA-based Clec14a-targeted CAR-T cells utilising an integrated system for T-cell isolation from leukapheresis material, mRNA electroporation and cryoformulation. Following optimisation of transfection parameters we reproducibly achieved 77-98% transfection efficiency (CAR positive) and 80-90% cell viability

in a range of donors. Importantly, T-cell activation was not required enabling a 3-day manufacturing process significantly reducing COGs and time to intervention. In an impedance based killing assay mRNA CAR-T cells from this process were as potent as gamma-retrovirally transduced CAR-T cells with antigen specific activation and T-cell mediated killing seen within 4-hours of exposure to target cells. We found in mice Clec14a-targeted CAR-T cells resulted in a 50-60% reduction in tumour burden, significant reduction in the vasculature of the tumour and loss of Clec14a-expressing vessels in three different solid tumour models, including spontaneous (RIP-Tag2 - beta cell adenomas), orthotopic (pancreatic ductal adenocarcinoma) and syngeneic transplantable (Lewis lung carcinoma) tumours. Large doses (up to 15 million cells per mouse) of Clec14a-targeted mouse CAR-T cells have been infused into over 200 mice without any signs of toxicity. Histopathology studies have also found no evidence of toxicity, and there has been no evidence of selective targeting of these CAR-T cells to healthy tissues such as the lung or liver.

121. Rejection-Resistant Off-The-Shelf T Cells for Adoptive Cell Therapy

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'Off-the-shelf' (OTS) therapeutic T cell products pre-manufactured from healthy donors would be a readily available and less expensive alternative to autologous products with similar therapeutic potency. A major obstacle preventing successful development of OTS therapies is immune rejection by host T- and NK-cells before benefit is produced. Here, we engineered rejection-resistant OTS T cells that recognize and selectively eliminate activated alloreactive lymphocytes while retaining desired anti-tumor activity. We showed that primed and activated alloreactive lymphocytes transiently upregulate a costimulatory receptor 4-1BB on the cell surface. T cells expressing a 4-1BB-specific *alloimmune defense receptor (ADR)* specifically eliminated freshly activated T- and NK-cells while sparing resting lymphocytes. Using this mechanism, ADR-expressing T cells suppressed alloimmune activation and resisted immune rejection by T- and NK-cells in a mixed lymphocyte reaction (MLR) model *in vitro*. In an *in vivo* model of allogeneic rejection, NSG mice pre-engrafted with human PBMCs rejected adoptively transferred allogeneic T cells within 7 days. In contrast, ADR-expressing T cells were protected from immune rejection and, despite the presence of allogeneic PBMCs, were able to persist in the circulation for >7 weeks. We further demonstrated that T cells co-expressing the ADR and a CD19 chimeric antigen receptor (CAR) retained undiminished anti-tumor activity through the CAR *in vitro* and *in vivo*. We established a mouse model of allogeneic cell therapy in which NSG mice were simultaneously engrafted with systemic $CD19^+$ leukemia and human T cells. In this model, subsequent adoptive transfer of unmodified allogeneic CD19 CAR T cells generated from an HLA mismatched donor produced only transient anti-tumor activity, as the CAR T cells were rejected by pre-engrafted allogeneic T cells within 7-10 days, leading to fatal leukemia relapse. In contrast, T cells co-expressing both CD19 CAR and ADR were protected from immune rejection, resulting in long-term persistence (>9 weeks) and durable leukemia eradication in most animals. In subsequent studies using this model, we co-expressed ADR and the CD19 CAR in T cells that had been TCR-edited to ablate their capacity to induce GvHD. These OTS TCR^- CD19 CAR T cells were equally well protected from immune rejection, enabling long-term anti-tumor activity and mouse survival (CR>85% with CAR-ADR T cells vs 0% with CAR only T cells). Thus, we have developed an OTS CAR T cell product that retains uninhibited anti-tumor activity and resists allogeneic rejection from the host immune system *in vivo*. These data support the efficacy of ADR as a first-in-class engineered receptor suppressing immune rejection and demonstrate the feasibility of using ADR to generate highly potent OTS CAR-T cell products with the potential to produce long-term therapeutic benefit even in immunocompetent recipients.

122. A Novel Approach to Targeting NKG2D Ligands Using Engineered T cells Expressing Dimerizing Agent Regulated Immunoreceptor Complexes (DARIC)

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Natural killer group 2D (NKG2D) ligands (NKG2DL) are widely expressed in cancers, and thus may be attractive targets for chimeric antigen receptor (CAR) T cell immunotherapy. However, certain characteristics of NKG2DL expression may compromise traditional CAR designs, specifically: (i) defects in *ex vivo* CAR T cell expansion due to expression on activated T cells; (ii) enhanced risk of normal tissue reactivity; and (iii) progressive T cell exhaustion due to persistent antigen exposure. Early data from NKG2D-CAR T cell clinical trials have demonstrated a promising safety profile with evidence of some clinical responses. However, the accompanying pharmacokinetic data indicating short persistence *in vivo* are consistent with concerns that one or more of the above factors may be limiting their potential efficacy. We have developed a dimerizing agent regulated immunoreceptor complex (DARIC) to overcome limitations of constitutively responsive CAR designs. The DARIC system separates the targeting and signaling functions of a CAR molecule into two distinct polypeptides that incorporate FKPB12 and FRB domains for conditional dimerization in the presence of rapamycin. Here, we describe NKG2D-DARIC T cells that exhibit normal *ex vivo* T cell expansion and enhanced functional activity relative to NKG2D-CAR T cells. We systematically characterized NKG2D-DARIC and standard NKG2D-CAR T cells alongside control epidermal growth factor receptor (EGFR) targeting CAR T cells. We observed comparable *ex vivo* expansion of the EGFR-CAR T cells and the NKG2D-DARIC T cells over time, whereas the NKG2D-CAR T cells had a 6-fold reduced expansion compared to control and NKG2D-DARIC samples. Moreover, NKG2D-CAR T cells were strongly lineage skewed, with nearly all the cells becoming CD8+ at the conclusion of the expansion period. This phenotype was not observed for any other samples. We next characterized functional responses by monitoring cytokine secretion and cytotoxicity during T cell co-culture with the A549 lung adenocarcinoma line that expresses

both NKG2D and EGFR ligands. As expected, the EGFR-CAR T cells and NKG2D-CAR T cells constitutively produced inflammatory cytokines during tumor co-culture, while the NKG2D-DARIC T cells responded only under conditions of rapamycin-induced dimerization. Notably, the NKG2D-CAR T cells produced considerably lower levels of cytokines compared to NKG2D-DARIC and EGFR-CAR T cells. An additional feature of the DARIC architecture is the facile incorporation of additional signaling componentry in parallel with traditional CAR endodomains. We evaluated a panel of additional costimulatory molecules and determined that intracellular domains derived from OX40 or tumor necrosis factor receptor 2 (TNFR2) could be incorporated into the NKG2D-DARIC T cells without significantly impacting *ex vivo* T cell expansion or phenotype. Moreover, each of these domains greatly enhanced cytokine responses and, surprisingly, the TNFR2 domain had the property of eliminating the minor immunosuppressive impact that rapamycin treatment has in comparison with the non-immunosuppressive rapamycin analog AP21967. Taken together, these findings support the development of the NKG2D-DARIC T cells as a regulated, potent, and adaptable platform that addresses several of the complexities inherent to the targeting of NKG2D ligands and may enable the broad application of this target in solid and liquid tumor indications.

123. Exploiting Clonal Tracking of WT1-Specific T Cells to Generate a Library of Tumor-Specific T Cell Receptors (TCR), for TCR Gene Editing of Acute Leukemia

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Recent encouraging clinical results obtained with engineered T lymphocytes have opened new opportunities for adoptive T cell therapy for cancer. Unfortunately, two main issues are still present: the difficulty in identifying appropriate tumor-specific antigens, and the limited number of high avidity T cell receptors (TCRs) against shared oncogenic antigens. Here, we aim at the identification of a panel of novel tumor-specific TCRs, to be exploited by TCR gene transfer and TCR gene editing. We focused on Wilms' Tumor 1 (WT1), a tumor-associated antigen widely expressed on a variety of hematological and solid tumors, elected as a high priority antigen by the National Cancer Institute (NCI). We designed and implemented an innovative protocol for the rapid isolation of WT1-specific T cells and for the characterization of a library of tumor-specific TCRs restricted to different human leukocyte antigen (HLA) alleles. We repetitively stimulated T cells from healthy donors (HDs) with autologous antigen-presenting cells, including immortalized B cells, pulsed with overlapping peptides spanning the entire WT1 protein. T cell recognition was assessed by flow cytometry in terms of CD107a expression and IFNγ production. Recognized peptides were mapped by a deconvoluting grid and their HLA restriction assessed by using a panel of cell lines harboring the HLA alleles of interest. Tumor-specific TCRs were identified by TCR αβ sequencing. We achieved successful expansion of tumor-specific T cells from 14 consecutive HDs, with an average of 4 rounds of *in vitro* stimulation. Upon identification of the immunogenic epitopes, we assessed the ability of WT1-specific T cells to recognize naturally processed peptides and their on-target specificity upon co-culture with antigen-expressing targets, including primary leukemic blasts. TCR αβ sequencing at different time points enabled the longitudinal clonal tracking of the tumor-specific T cells and the correct pairing of TCR α and β chains, without the need of performing single cell analysis. We identified 20 clonotypes recognizing several tumor-associated peptides that were restricted by more than 5 HLA alleles, including *HLA-A*02:01*. Newly identified TCRs were then expressed as transgenes via genome editing. Briefly, simultaneous editing of endogenous TCR α and β chain genes was achieved by using CRISPR/Cas9 technology (editing efficiency >90%), followed by transduction of edited T cells with lentiviral vectors encoding WT1-specific TCRs (transduction efficiency >95% CD8+ T cells). Phenotypic characterization of engineered T lymphocytes showed a major enrichment of cells exhibiting a T stem cell memory phenotype. Functional validation of edited T cells is currently ongoing. Preliminary results of a 6-hour co-culture experiment show that TCR-edited T cells kill fresh WT1+ leukemic blasts harvested from HLA-matched patients with an efficiency up to 70%, at an effector to target cell ratio of 5 to 1, while no killing of controls is observed. Comparative analysis of both safety and efficacy profile of TCR edited T cells will be discussed.

Nonclinical Studies and Assay Development

124. A Novel Non-Integrating DNA Vector for the Persistent Genetic Modification of Embryonic and Hematopoietic Stem Cells

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Introduction: The capacity of stem cells to differentiate into all types of specialised progeny, holds great promise for the future of gene therapy and regenerative medicine. The most efficient way to genetically modify a cell population is by targeting the originating population which can propagate infinitely and can pass on genetic modifications to their progeny. One example is the targeting of hematopoietic stem cells (HSC) for the genetic correction of hematopoietic diseases. Typically, the modification of these cells is done by using integrating viral vectors. Although vectors based on modified viruses are the most effective gene delivery systems in use today, their efficacy at gene transfer is, however, tempered by their potential integration and genotoxicity. An ideal vector for the genetic modification of cells should deliver

sustainable therapeutic levels of gene expression without compromising the viability of the host cell or their progeny in any way. Permanently maintained, episomal and autonomously replicating DNA vectors, might provide the most suitable method for achieving these goals. Here we propose a non-viral, non-integrating and autonomously replicating S/MAR DNA vector system based on minimally sized-antibiotic free Nanoplasmids™ as a novel technology to persistently genetically modify mESC cells and their progeny without causing any molecular or genetic damage. **Results:** Murine embryonic stem cells (mESCs) were stably labelled with GFP-S/MAR DNA nanoplasmids, which yielded robust and persistent levels of transgene expression. The S/MAR nanoplasmids remained episomal and did not modify the stem cells' properties, as demonstrated by the expression of pluripotency markers such as Alkaline Phosphatase, Nanog, Oct4 and SSEA-1. The behaviour of the vectors during differentiation was then evaluated *in vitro* in random differentiation experiments, in which GFP-expressing representatives of the three germ layers were obtained. Additionally, S/MAR-labelled mESC were differentiated into hematopoietic precursors, in which we observed sustained expression of our DNA vectors throughout the process without observing transgene silencing. Finally, the suitability of S/MAR Nanoplasmids was challenged *in vivo* by generating chimeric mice, whose transgenic organs, including the hematopoietic tissues, showed high levels of transgene expression. Our data demonstrates that S/MAR Nanoplasmids can sustain episomal transgene expression from embryonic stem cells to fully differentiated hematopoietic tissues without silencing, vector loss or integration. **Conclusions:** For the first time, we have shown that a non-viral episomal vector based on mammalian chromosomal elements is capable of genetically modifying embryonic stem cells while avoiding vector loss or differentiation-mediated transgene silencing. We demonstrate that this DNA vector system provides robust and sustained transgene expression in pluripotent cells during hematopoietic differentiation, without damaging or altering the stem cells' properties. Our vector system represents an alternative tool for 1) the modification of HSC and the treatment of hematopoietic diseases, such as Fanconi Anemia; 2) for gene therapy approaches that rely on a robust and continuous expression of transgenes; as well as 3) a cell source for replenishment of HSC in diseases in which they are affected or absent, such as bone marrow failure syndromes.

125. Failure to Mobilize Peripheral Blood Hematopoietic Stem Cells Upon Readministration of AMD3100 in Nonhuman Primates

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Background: AMD3100 (plerixafor) is a small molecule CXCR4 antagonist used to mobilize hematopoietic stem cells (HSCs) into the peripheral circulation. It is most commonly used to facilitate peripheral blood HSC collection in patients who mobilize poorly with G-CSF alone or in sickle cell disease (SCD) patient where G-CSF is contraindicated. For poor mobilizers, upfront AMD3100 addition to G-CSF is recommended during the first mobilization episode. A number of these patients will still collect inadequate CD34+ cells for autologous HSC transplantation, and therefore undergo repeat mobilization attempts including AMD3100 administration. Many experimental gene therapy protocols require recurrent mobilization to obtain sufficient numbers of HSCs and AMD3100 is used or considered for this indication. The number of patients re-exposed to AMD3100 is therefore expected to increase. Thus, it is important to establish the effect of recurrent AMD3100 administration on HSC mobilization. Since mobilization using repeated G-CSF administration is reported to result in lower CD34+ yields, we sought to determine whether similar results are observed with AMD3100. Our group developed a nonhuman primate (NHP) transplant model using rhesus macaques to study therapeutic modalities for β-hemoglobinopathies. In the process, NHPs were mobilized with single agent AMD3100 repeatedly and the effect on multi-lineage peripheral blood cell mobilization was investigated. **Methods:** AMD3100 administration was at 1mg/kg (SQ). 5 adult NHPs received a 1st dose of AMD3100. 3 animals received 2nd doses, after 6-14 weeks. One animal received a 3rd dose, 4 weeks after the 2nd. Peripheral blood samples were taken at multiple time points post dose. Complete blood count and flow cytometry were undertaken to characterize mobilization of CD34+ HSPCs as well as the prevalence of the long-term repopulating HSCs within this population, previously defined by our group to be CD34+CD45RA-CD90+ (CD90+). **Results:** Peak white cell count was 2.5 hours post dose (range 2-3 hrs). Following 1st dose all animals mobilized CD34+ cells well, with the CD90+ subpopulation particularly enriched within these. Following subsequent AMD3100 administration, both CD34+ mobilization and CD90+ enrichment were significantly lower. Similar results were obtained on paired analysis of response to 1st and 2nd doses of AMD3100 in 3 NHPs, reaching statistical significance for CD34+ mobilization. (*Figure*) **Conclusions:** We have demonstrated a failure to remobilize CD34+ cells, and also the long-term repopulating HSC population of CD90+ cells, with repeated AMD3100 administration in NHPs. This effect persists to 14 weeks following 1st dose of AMD3100 -longer than the 2-4 weeks between mobilization attempts recommended by ASBMT. Possible mechanisms include loss of mobilized cells; loss of sensitivity to AMD3100; failure to reverse CXCL12 gradient on repeated dosing; or development of inactivating antibodies. We are currently conducting studies to investigate all above hypotheses. It is vital that clinical response to repeat AMD3100 dosing is established and our group is currently investigating this phenomenon. If such remobilization failure persists in humans then alternative agents -single and adjunctive - must be fast-tracked into clinical practice to allow repeat mobilization for transplantation and gene therapy protocols.

Figure

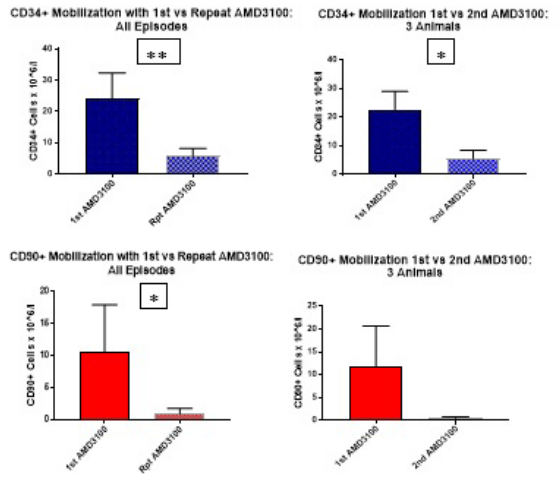

Figure 1. Comparison of CD34+ and CD90+ mobilization with first and subsequent AMD3100 administration. Statistical significance: * $p<0.05$; ** $p<0.01$ (t-test).

126. DNA In Situ Hybridization Protocol for the Detection of Integrated Lentiviral Vector in Preclinical and Clinical Samples

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Lentiviral vectors (LVVs) are commonly used to transduce both mature immune cells and hematopoietic stem and progenitor cells with novel genetic payloads encoding molecules such as chimeric antigen receptors (CAR) and therapeutic proteins for disease correction. Following transduction, the LVV is semi-randomly incorporated as a provector into the genome, transcribed and translated into protein by the engineered cells. After adoptive transfer of these engineered cells, collected tissues can be evaluated by RNA in situ hybridization (R-ISH) to determine the frequency and distribution of cells that were actively transcribing the transgene at the time of sample collection. For example, R-ISH may be utilized to track CAR mRNA-expressing cells in preclinical and clinical samples when an appropriate reagent to detect the mature CAR protein is not available. The R-ISH protocol utilizes a probe that is complementary to provector mRNA and because mRNA may be distributed in the cytoplasm or nucleus, the R-ISH signal can be located anywhere in the cell. This becomes problematic in densely cellular tissues because robust cytoplasmic R-ISH signal may make it difficult or impossible to specifically identify and enumerate the engineered cells. In these circumstances, detection of integrated provector via DNA in situ hybridization (D-ISH) may be preferable. The D-ISH protocol utilizes a probe that is the same sense as provector mRNA and therefore only detects incorporated provector DNA and the D-ISH signal is localized to the nucleus. Using D-ISH, engineered cells can be readily detected and enumerated in even densely cellular tissues such as neoplasms or bone marrow. Therefore, we adapted the ACDBio RNAscope® R-ISH protocol to detect integrated provector DNA. Complementary probes against sequence in the LVV backbone were subsequently designed in collaboration with ACDbio, including one probe anti-sense to the LVV mRNA (for R-ISH) and another probe the same sense as the LVV mRNA (for D-ISH). Using these probes, both the R-ISH and D-ISH protocols were subsequently compared on a sample of preclinical and clinical samples. In NSG mice implanted with RPMI-8226 xenografts and treated with anti-huBCMA CAR T cells, the R-ISH signal within the xenografts was robust, overlying both the nuclei and cytoplasm of adjacent CAR T cells so that it was difficult to determine the cell of origin. In contrast, D-ISH analysis of a serial xenograft section revealed 1-5 provector signals directly over the nuclei of infiltrating CAR T cells, thus allowing for easy enumeration (see figure). We used the same strategy to evaluate a bone marrow core biopsy from a patient treated with the LentiGlobin BB305 Drug Product (an investigational gene therapy consisting of autologous CD34+ hematopoietic stem cells transduced with the BB305 LVV). Like the xenograft experiment, the bone marrow R-ISH signal was predominantly cytoplasmic, rendering the cells of origin unclear and engineered cell enumeration difficult. In contrast, the D-ISH protocol provided a clear nuclear provector signal, allowing for identification and enumeration of engineered cells with integrated provector. Additional potential applications of a LVV provector-specific D-ISH protocol include assessment of unintended germline and/or vertical transmission during safety studies, as well as a method for assessing the potential role of LVV-mediated insertional mutagenesis in oncogenesis during preclinical or clinical studies.

127. Biodistribution and Tolerability of HMI-102, a Novel AAVHSC15 Encoding Human Phenylalanine Hydroxylase, in Cynomolgus Monkeys

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A six-month GLP study to assess the biodistribution and tolerability of HMI-102, a recombinant AAVHSC15 gene transfer vector encoding human phenylalanine hydroxylase (*PAH*), was conducted in cynomolgus monkeys. The AAVHSC15 capsid is a natural Clade F adeno-associated virus (AAV) variant that was isolated from CD34+ human peripheral blood stem cells from healthy adults. HMI-102 is an investigational AAV vector-based gene therapy under development for the treatment of patients with phenylketonuria (PKU), a rare disease caused by inherited mutations in the *PAH* gene that result in the absence or deficiency of PAH activity. PAH, which in humans is expressed in hepatocytes, is a critical enzyme for the formation of tyrosine (Tyr) from phenylalanine (Phe), an essential amino acid obtained exclusively from the diet. This study consisted of three groups, each with 2 male and 2 female cynomolgus monkeys, and each group received either vehicle or HMI-102 at a low or a high dose as a 30-minute intravenous (IV) infusion. The dose range was selected to

approximate the expected clinical dose range. Animals were sacrificed at 3 months (low dose) and at 6 months (vehicle and high dose). A concurrent study with the same manufactured lot was conducted in the mouse model of disease, the *Pah*^{enu2} mouse, and timepoints were selected to allow for direct comparison. This also allowed comparison of the pharmacodynamic activity of HMI-102 in mice to the potential exaggerated pharmacology in nonhuman primates expressing supranormal levels of human PAH. A key endpoint was also to assess the allometric scaling of AAVHSC15 between mouse and cynomolgus monkey to allow for dose extrapolation to humans. Endpoints in this study included both safety and biodistribution. Safety was assessed by physical examinations, clinical observations, body weight, food consumption, ophthalmoscopic examinations, ECG's, immune response to capsid and transgene, clinical pathology and histopathology. Biodistribution (vector genome and human *PAH* mRNA levels) were assessed in liver biopsies taken at 1 month and 3 months post-dose, and comprehensive tissue collections at 3 months and 6 months post dose. HMI-102 was well tolerated at the doses tested for up to 6 months. Vector genome levels per cell in the liver were similar to the levels found in the livers of *Pah*^{enu2} mice receiving the same doses of HMI-102. The doses utilized resulted in normalized Phe levels in *Pah*^{enu2} mice within one week, with concomitant increases in Tyr levels. In cynomolgus monkeys, treatment with HMI-102 did not alter blood Phe or Tyr concentrations, as expected given the known allosteric regulation of PAH. The results of this study support the planned Phase 1/2 gene therapy clinical trial of HMI-102.

128. Development of an Objective Image-Based Flow Cytometry Sickling Assay for Quantification of Amelioration of Sickle Cell Disease Phenotype in Erythroid Differentiated CD34+ Cells

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Sickle Cell Disease (SCD) is caused by a single point mutation in the human β-globin gene that leads to the production of abnormal sickle hemoglobin (HbS) in erythroid cells. Under low oxygen conditions, HbS can polymerize and cause red blood cells (RBCs) to assume the characteristic "sickled" shape that is responsible for much of the pathophysiology in SCD. Early clinical results with *ex vivo* gene therapy have shown promise in SCD [Ribeil, 2017], and as these and other therapies advance, there is a need to objectively quantify the degree of amelioration of disease phenotype in gene-modified cells in a laboratory setting. Here, we describe a robust and objective assay that can quantify the hypoxia-induced sickling of SCD RBCs differentiated from CD34+ hematopoietic stem and progenitor cells (HSPCs). Moreover, this assay can be used to assess the relative level of correction of sickling in RBCs differentiated from SCD HSPCs transduced with a lentiviral vector (LVV) encoding an anti-sickling β-globin. Transduced and cell lot-matched untransduced control cells were cultured in a two-phase erythroid differentiation protocol to generate RBCs, with the second phase of culture occurring in 2% oxygen. Cells were fixed, stained with thiazole orange, and images of the cells collected on the Amnis ImageStream imaging flow cytometer. Fixed erythroid differentiated cells were found to be stable for up to 3 months. Images of the erythroid differentiated RBCs were then analyzed using a stringent gating strategy to determine the proportion of sickled cells in the cell population, and to quantify the relative amelioration of disease phenotype in RBCs derived from transduced cells. Assessment of assay readout precision spanning two operators, two sampling time points, and two instruments resulted in a 5.8% coefficient of variation (CV), indicating reliable performance of the method and ImageStream analysis. Assessment of overall assay precision spanning six cell culture runs with two operators and three to six replicates of test article per cell culture run resulted in a 4.0% CV for untransduced cells and 9.2% CV for cells transduced with LVV encoding an anti-sickling β-globin. Transduction with LVV encoding an anti-sickling β-globin results in a decrease in the proportion of sickled cells as compared to the untransduced controls across cells from six subjects with SCD, and the reduction in sickling was found to be specific to transduction with LVVs that lead to increased expression of anti-sickling hemoglobins. This relative decrease is correlated with vector copy number, the percentage of transduced cells, and the amount of protein expressed. This assay is robust, precise, and suitable for the *in vitro* characterization of the anti-sickling properties of LVV encoding an anti-sickling β-globin in CD34+ cells from subjects with SCD. Moreover, these data demonstrate a marked reduction in sickled *in vitro* derived RBCs driven by transduction with LVV encoding an anti-sickling β-globin. Ribeil, *et al.* The New England Journal of Medicine, 2017.

129. The Pi3k/Akt Pathway Impacts Early RAAV Transduction Events and is Activated by Insulin Receptor Signaling

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Our lab previously reported a 3-5 fold increase in transgene expression by co-administering insulin with AAV vector. Based on these early results some aspect of the insulin receptor (INSR) pathway impacts AAV transduction. These preliminary studies also focused on determining which event in transduction process is involved: uptake, intracellular trafficking and/or stable gene expression. Based on testing different time periods for co-administration of insulin with AAV vectors and measuring vector genomes in transduced cells the proposed mechanism is vector uptake. This is further supported given that endocytosis of the liganded insulin receptor uses the same mechanism that has been reported for AAV. Therefore a brief spike (2 hr) in insulin is sufficient for enhancing transduction. Given, the expansive network of signaling associated with the INSR it is not surprising that insulin impacts AAV transduction. However, to verify that INSR signaling does indeed impact AAV transduction, we are inhibiting points along the INSR pathway starting with the Pi3k/Akt pathway. Pi3k/Akt 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. In human liver cells, Hep3B and HepG2, phosphorylated Akt was measured via Western Blot following insulin treatment. Biologically, Hep3B cells downregulate phosphatase and tensin homolog, a modulator of the

dephosphorylation of phosphatidylinositol triphosphate (PIP_3) to phosphatidylinositol diphosphate (PIP_2). Due to this downregulation baseline Akt phosphorylation is naturally increased. Despite this innate increase in Akt activation, treating Hep3B cells with insulin resulted in a 2.5 fold increase in phospho-Akt. Using the Pi3K inhibitor, Zstk-474, phospho-Akt levels were reduced in both insulin treated (3 fold drop) and untreated (2 fold drop). Therefore it is clear that the Pi3k/AKt pathway is functional in Hep3B cells and that this pathway responds to insulin treatment and Pi3k inhibition. Next we tested co-administration of insulin with AAV transduction combined with Pi3k inhibitor. AAV2-CMV-eGFP was co-administered with insulin to cultured Hep3B cells for 2 hrs. AAV and insulin was removed and GFP levels were measured 72 hrs later. GFP+ cells were assessed by FACS using side scatter analysis. These cell culture studies were done with low glucose (1 g/dL) and no FBS to reduce signaling events from effectors in FBS. As expected, there was a 2.5 fold increase in transduction when using insulin. This insulin enhanced AAV2-CMV-eGFP transduction was reduced 3-fold with the Pi3K inhibitor. The Pi3K inhibitor was also tested on AAV2-CMV-eGFP without insulin co-administration and while there was a trend that showed a mild 1.5 fold decrease in GFP+ cells this was not significant. This study clearly demonstrates that inulin activates the Pi3k/Akt pathway in human liver cell lines and that this pathway impacts AAV transduction. Additionally, since insulin was only administered for 2 hrs and transduction was measured 72 hrs later, the activation of this pathway impacts early transduction events in this model. Also, based on previous data from this lab, insulin was given after AAV treatment and had no impact on transduction. This suggests that the activation of the Pi3k/Akt pathway does not impact transcription of the transgene. Since the Pi3k/Akt pathway is common to many receptor pathways other ligands need to be tested including ones that inactive this pathway. Additionally, the link between the Pi3k/Akt pathway and endocytosis needs to be explored to be definitive that insulin and the Pi3k/Akt pathway impacts uptake of rAAV.

130. Comparison of ddPCR and qPCR in Quantification of Advanced Therapy Products in Biological Samples

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Droplet digital PCR (ddPCR) is a new methodology that is expected to be an alternative to quantitative real-time PCR (qPCR). The advantages of ddPCR over qPCR include better reproducibility, higher sensitivity, higher tolerance to PCR inhibitory substances, and the capability for absolute quantification without using standard curves. Also, ddPCR is more suited for automation, which significantly increases processivity. In the advanced therapy field, researchers often need to perform time-consuming and labor-intensive mass qPCR to quantify products, such as plasmids, virus vectors, and cell-based therapy products (CTP), in biological samples, which may contain high amounts of PCR inhibitory substances. ddPCR can be a savior for such applications. However, to use ddPCR in place of qPCR for the quantification of CTP, the ddPCR assay needs to overcome some technical problems, such as the selection of the target sequence and the need for human cell-derived gDNA to be digested with restriction enzyme before ddPCR, which may inhibit absolute quantification. Here, we have tested whether ddPCR is a better alternative to qPCR in detection of gene therapy products (GTP) by comparing their reproducibility, sensitivity, and the tolerance to inhibitory effects, and have attempted to establish a ddPCR assay to quantify human gDNA. To compare ddPCR and qPCR in detection of GTP, we used the herpes simplex virus-1 (HSV-1) genome or a synthetic oligo to analyze the coefficient of variation (CV) of quantified copy numbers, limit of detection (LOD), and inhibitory effects on the detected copy numbers under the existence of matrices using the QX100 Droplet Digital PCR System (Bio-Rad) and the CFX96 Touch Real Time PCR System (Bio-Rad). To establish an assay to quantify human gDNA, we designed primers/probe set to target DUF1220, a multiple copy gene, in order to achieve higher sensitivity than with targeting a single copy gene. Human gDNA, digested with HaeIII, Mse I, or CviQ I, was tested for the DUF1220 assay. For the HSV-1 assay, the CVs of quantified copy numbers in the quantitative range for ddPCR and qPCR were under 5% and about 20%, respectively. The LOD of ddPCR was under 50 copies, which was not reliably achieved by qPCR. Matrices that are known to contain inhibitory substances did not significantly affect copy number quantification in ddPCR, unlike in qPCR, where the existence of matrices completely inhibited reaction or significantly lowered the quantified copy number. For the quantification of human gDNA, the DUF1220 assay demonstrated a linear standard curve (R^2=1.00) with the serial dilution of digested human gDNA, which suggests that the assay can perform quantification. However, when each aliquot of gDNA was digested with a different enzyme, each quantified copy number varied, which suggests that the DUF1220 assay cannot perform absolute quantification. Our results suggest that ddPCR has advantages over qPCR in GTP quantification due to its better reproducibility, higher sensitivity, and higher tolerance to inhibitory effects. Taken together with its suitability for automation, ddPCR is useful for the quantification of GTP. On the other hand, in establishing a ddPCR assay to quantify human gDNA, the assay targeting multiple copy genes had difficulty performing absolute quantification, probably because every enzyme digested fragments may not carry only a single copy of the target sequence. Although the cell numbers derived from quantitated human gDNA are of the most interest to researchers in a biodistribution study, cell number quantification by ddPCR is difficult due to its lack of absolute quantification capability for human gDNA. Our results only suggest that ddPCR allows comparison of the amount of human gDNA between samples. Whether ddPCR allows relative quantification may require further analyses.

Measuring and Mitigating Genotoxicity of Genome Editing

131. Self Cleaving Guide RNAs for Selective Expansion of Precisely Gene Edited Hepatocytes In Vivo

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Transplantation of precise recombinant adeno-associated virus (rAAV) gene edited cells is an attractive approach for cell-based therapies, especially for genetic disorders. However, gene editing *in vivo* by homologous recombination is inherently inefficient. One strategy to achieve a higher overall efficiency of gene editing is to selectively expand cells that have acquired the desired targeting event *in vivo* after transplantation. This can be achieved by linking the desired genetic modification to a selectable gene disruption cassette in cis, such that selection and expansion can only occur if proper gene targeting has been achieved in that cell. Using a Factor 9 (F9) expressing Generide rAAV vector targeted to the albumin locus we previously showed that an shRNA embedded in a microRNA within an intron can protect hepatocytes from a hepatotoxic drug and result in high transgene levels. While this approach can work, shRNAs usually can only knock down a gene by ~ 80-90%, thus limiting its utility for drug protection regimens. In contrast, complete gene knockouts can be achieved by CRISPR-Cas9 gene editing. In order to generate selectable CRISPR-mediated gene knockouts, it is necessary to restrict its endonuclease activity to only cells that have proper gene targeting. This strategy requires that the guide RNA (gRNA) expression is driven by a tissue-specific polymerase 2 promoter. Here we show that flanking gRNAs with self-cleaving ribozymes permits proper processing of active gRNAs from polymerase 2 transcripts. In *Fah*$^{-/-}$ mice, loss of Hpd or Hgd, genes upstream of FAH in tyrosine degradation, provides a selective advantage for hepatocytes. We observed strong in vivo selection of the Albumin-F9 Generide rAAV vectors harboring self-cleaving gRNA (scgRNA) against Hpd and Hgd. In *Fah*$^{-/-}$ mice treated with these vectors and then taken off NTBC, we observe clonal expansion of cells expressing human F9. Insertion/deletion analysis show that hepatocytes with the integration can expand to 60% of the liver mass and the mice display superphysiologic levels of human F9. In order to broaden this paradigm, we engineered a system that renders hepatocytes resistant to the hepatotoxic drug acetaminophen. A scgRNA designed to knock out NADPH-cytochrome P450 reductase (Cypor) was incorporated into the albumin-human F9 gene ride vector. Loss of Cypor prevents the conversion of acetaminophen into its toxic metabolite. Acetaminophen treatment resulted in the expansion of Cypor null hepatocytes up to ~20% of the liver mass. To track the progression of selection, the humanF9 was replaced with luciferase. We found that luminescence increased over the selection process. The use of an easily visualized biomarker will allow us to target other lower expressed hepatocyte specific genes with a gene ride vector and track the expansion of hepatocytes with the integration. Targeting of lower expressed genes is one way to modulate the expression levels of the transgene that is delivered.

132. A Novel Preclinical Genotoxicity Assay, CAST-Seq, Enables New Insights in DNA Repair Dynamics and Chromosomal Aberrations in CRISPR-Cas Edited Human Hematopoietic Stem Cells

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Therapeutic genome editing with designer nucleases has shown great success in the last few years, leading to their application in several clinical trials. Despite their potential, engineered nucleases can lead to genotoxic side effects by introducing mutations and/or chromosomal aberrations through activity at unintended sites, so called off-target sites. Computational prediction tools and recently developed unbiased methods to identify such off-target activity unveiled these risks but had major limitations, including insufficient sensitivity and/or specificity as well as failure to detect particular gross chromosomal aberrations. To overcome these limitations, we established a new assay termed CAST-Seq that allows us to detect chromosomal aberration by single targeted LM-PCR. Unlike other unbiased methods, CAST-Seq is capable of detecting chromosomal aberrations derived from on- and off-target activity of designer nucleases, including large deletions, inversions and translocations, with unmatched sensitivity in clinically relevant human cells. To this end, primary human hematopoietic stem cells were treated with CRISPR-Cas or TALEN designer nucleases targeting various loci. Using less than 1 µg of genomic input DNA, we were able to detect off-target sites and chromosomal aberrations that were neither predicted *in silico* nor identified using other unbiased methods, confirming the high sensitivity of CAST-Seq. In addition, we were able to detect on-target activity mediated chromosomal aberrations, such as homology-mediated translocations, acentric and dicentric translocations between homologous chromosomes, and large chromosomal deletions. Finally, validation of the results by digital PCR gave new insights in the DNA repair kinetics in human stem cells. In conclusion, we have developed highly sensitive assays in clinically relevant cells that allowed us to expand our understanding of the DNA repair dynamics and to detect hitherto unidentifiable chromosomal aberrations in gene edited human stem cells.

133. Optimizing Nuclease Specificity via Catalytic Domain Engineering Enables Complete Gene Modification with No Detectable Off-Targets

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Engineered nucleases have gained broad appeal for their ability to mediate highly efficient genome editing. However the specificity of these reagents remains a concern, given the potential mutagenic consequences of off-target cleavage. Although diverse strategies have been described for improving the specificity of designed nucleases, almost all such approaches have focused on initial target binding as the principle determinant of specificity, leaving downstream steps such as the cleavage event itself largely unexplored. In the studies described below, we have developed a new approach for improving the specificity of zinc finger nucleases (ZFNs) that modifies the FokI nuclease domain with the goal of reducing cleavage rate, in order to allow sufficient time prior to catalysis for dissociation from suboptimal targets. Our studies proceeded in four stages. In the first, we examined several hundred single-residue substitutions in the FokI domain of a previously characterized ZFN dimer[1] for impact on cellular specificity, and identified over twenty variants that improved on-target preference. This included point mutations of three distinct residues that reduced off-target modification to below the limit of detection of standard, sequencing-based indel analysis (>1000-fold) while retaining full on-target activity. Next, to enable more accurate assessment of the specificity enhancement afforded by this approach, we developed a new, more sensitive protocol for sequencing-based indel analysis that uses oversampling to discern bona-fide indels from protocol artifacts. This allowed indel detection at levels as low as 0.001% (100-fold lower than conventional sequencing-based protocols), which revealed a single FokI point mutation that could reduce off-target cleavage >3000-fold. We then characterized our variant ZFNs in a series of biochemical binding and cleavage studies, which confirmed that the FokI domain mutations altered neither affinity nor binding preference of the ZFNs, but instead reduced catalytic rate. Finally, we combined our strategy of engineering the FokI domain with a second specificity improvement strategy - removing a conserved, well-characterized zinc finger phosphate contact - to generate ZFNs targeted to the TCR alpha and B2M genes that can introduce indels into the targeted loci in T cells at levels exceeding 98%, with no detectable off-target activity. These results establish a new approach for optimizing ZFP specificity that will enable the development of highly specific ZFNs for virtually any gene target.

[1] Nat Biotechnol. 2016 Apr;34(4):424-9

134. Evaluation of Homology-Independent CRISPR-Cas9 Off-Target Assessment Methods

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Introduction: CRISPR-Cas systems are widely used for genome editing, in both in research and therapeutic settings. To bring CRISPR to the clinic, CRISPR-Cas guides must have high rates of editing at the on-target sites, while minimizing off-target editing. Typically, a two-pronged approach is used to comprehensively identify potential off-target sites and select a highly specific guide. In the first, a homology-dependent approach, computational tools are used to nominate potential off-target sites based on the presence of a PAM sequence and the degree of homology between the adjacent DNA sequence and the guide. In the second, a homology-independent approach, genome-wide assays and next-generation sequencing (NGS) based assays are used to empirically nominate genomic sites edited by a guide. Potential off-target sites nominated by any of the methods are then deep sequenced after editing, using amplicon or hybrid capture NGS to measure possible editing activity. Cell-based and biochemical homology-independent approaches for nominating off-targets have been developed, and studies have suggested that biochemical methods may be more sensitive. However, a head-to-head comparison study of these methods paired with confirmatory deep sequencing has not previously been performed. Here, we compared the performance of the cell-based assay GUIDE-seq and the biochemical assays CIRCLE-seq and SITE-seq. **Methods and Results:** We used a panel of eight guides previously shown to have a range of off-target activities to evaluate the methods in terms of sensitivity of nominating potential off-target sites. We confirmed that GUIDE-seq and CIRCLE-seq yielded semi-quantitative data with a strong correlation between the sequencing read count for a given site and the degree of guide homology. In contrast, we found that SITE-seq was less quantitative, with low correlation between the sequencing read count for a given site and the degree of guide homology. While most GUIDE-seq nominated sites were also detected by CIRCLE-seq, CIRCLE-seq produced two orders of magnitude more nominated off-target sites than GUIDE seq. Hybrid capture followed by deep-sequencing of greater than 75,000 nominated off-target loci in edited cells revealed that both GUIDE-seq and CIRCLE-seq performed equally well in capturing true off-target sites of a given guide. The number of sequence-confirmed false positives nominated by CIRCLE-seq was significantly more than GUIDE-seq. The data also suggest that the false negative rate is low, which is an important consideration when using the assay in clinical applications. **Conclusions:** Our work provides a comprehensive comparative assessment of these methods benchmarked to an extensive NGS assessment. Our data support the use of GUIDE-seq as an efficient homology-independent off-target nomination method, especially in therapeutic programs leveraging *ex vivo* CRISPR editing. The data show that CIRCLE-seq also performed well, albeit with more false positives, indicating that CIRCLE-seq will be a valuable homology-independent method in situations that prevent the use of GUIDE-seq. The false positive rate of these methods highlights the critical need for confirmation of any off-target sites with NGS.

135. Engineering Potent, Small, Chimeric, Synthetic, RNA-Guided Nucleases (sRGN) from Four Uncharacterized Cas9 Genes

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Adeno-associated viruses (AAVs) and lipid nanoparticles (LNP) are among the methods of choice for delivery of nucleases for *in-vivo* genome editing. The widely used and highly active *Streptococcus pyogenes* (Spy) Cas9 nuclease is specific for an advantageously short and non-degenerate PAM sequence. However, the large size of SpyCas9, in combination with required sgRNA and expression elements, presents a challenge to the 4.5 kb DNA packaging limit of AAVs, as well as to the synthesis of long mRNA templates and their stable formulation into LNPs. On the other hand, the best-characterized smaller Cas9s, such as *Staphylococcus aureus* Cas9, frequently recognize degenerate and longer PAMs, which significantly reduce the number of addressable genomic target sites. To address these issues, we evaluated four related, previously uncharacterized Cas9 nucleases of ~1050 amino acids in length. Surprisingly, most were found to be specific for a common, non-degenerate, small PAM motif. Using multiple protein engineering approaches, we altered these genes to generate novel, chimeric, synthetic, RNA-guided nucleases (sRGNs) and demonstrated efficient editing in human cells. Analyses using all 60 possible, single-nucleotide mismatched off-targets of a DNA substrate indicated high overall specificity for each clone. Importantly, the different clones displayed a localized signature of higher specificity for different mismatches at different sites along the target. Thus, we have generated a set of novel, small sRGNs, from which improved nucleases can be selected for particular targets of interest. LNP packaging and *in-vivo* performance vs. SpyCas9 will be reported in a separate abstract. Because these sRGNs were not derived from Spy or S. aureus Cas9s, we are working toward assessing these alternative nucleases regarding the potential for lower occurrence of pre-existing antibodies in human populations. All in all, these engineered, chimeric, synthetic, RNA-guided nucleases are thus expected to be valuable additions to the canon of known genome-editing nucleases that can be employed for human gene therapy applications.

136. In Vivo Validation and Tracking of CRISPR-Cas9 Off-Targets Predicted In Vitro by CircleSeq in Rhesus Macaques

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Cas9 nucleases can be programed by gRNAs to induce specific DNA breaks. However, Cas9 has been shown to tolerate mismatches between gRNAs and unintended off-target sites. Accurate methods of CRISPR/Cas9 off-target characterization are crucial prior to applying the system therapeutically. Here, we aim to investigate the predictive power of CIRCLE-Seq (CS), an in vitro off-target prediction method that can be performed on genomic DNA. We compare CS results to the more commonly used in silico predictions (ISP), which rely on sequence similarity to the gRNA, to determine which of is more accurate at predicting in vivo edits. To date, CS has been validated in cell lines and mouse models (following in vivo editing of liver via adenoviral injection). Here, we utilize our rhesus model to ask whether top off-targets identified via CS and ISP can be detected in blood cells collected from animals following autologous transplantation with CRISPR/Cas9-edited HSPCs, and if the edits can be followed long-term. First, we performed CS to test its efficacy on DNA from primary rhesus cells focusing on a gRNA designed against the CD33 gene and being developed for CD33 knockout in HSPC to facilitate CAR-T therapy of AML(Kim et al, CELL, 2018). We tested the reproducibility of CS by running replicates with the CD33 gRNA on the same animal and found it to be reproducible($R > 0.92$). We then ran CS with the CD33 gRNA on DNA from 3 macaques and found high correlation among the predicted sites across all animals($R > 0.80$). The CS predicted sites showed little correlation with the ISP sites. We pilot tested the predictive power of CD33 off-target sites identified with CS versus ISP by sequencing the 15(out of 870) CS sites with the highest read counts, and the 15(out of 2586) top scoring ISP sites via targeted Illumina sequencing on an aliquot of the edited CD34+ infusion product (IP) prior to autologous transplantation. 4/15 CS sites were edited in the IP. 2 of the edited sites were also in the top 15 of ISP, the 3rd ranked 1258th, and the 4th was not predicted by ISP. Likewise, 4 of the ISP sites were found to be edited. Of these, 2 were among the top 15 by CS read count and 2 were in the top 30. We next tuned our CS site ranking by creating a "site score" taking into account both the CS read number and similarity to the gRNA, using the following formula: Site Score = (on-target read count/off-target read count) * Levenshtein distance. We then sequenced the 15 CS sites with the highest site score. 5/15 were edited in the IP, capturing one more relevant site than CS read count alone. In total, we have identified 7 bona fide off-target sites in IP between CS and ISP. Before proceeding to large scale in vivo sequencing, we asked whether these edits were present in HSPC and blood cells post transplant. We sequenced 5 of the bona fide sites in blood cells collected 1 month after transplantation and found 3 of the 5 sites to be edited in vivo, at levels of 4% to 6% with concurrent on target editing of 62%. Some of the sites being validated had sequence changes close to the Illumina

limit of detection, thus we concluded site screening might benefit from error-corrected sequencing. We are performing large-scale multiplexed error-corrected targeted sequencing with custom AmpliSeq-HD panels of the top 500 sites ranked by CS site score and the top scoring 500 ISP sites. These sites will be tested in blood lineages and HSPC from 3 CD33-edited macaques, followed for as long as two years post-transplant, looking for any evidence of clonal expansions of off-target edited sites and will represent the first longitudinal in vivo follow-up of off-target editing. The resulting data will also provide insights into the variability in off-target effects between animals due to polymorphisms.

137. Abstract Withdrawn

CAR T Cell Therapies for Cancer

138. Control of Natural Killer Cell Expansion, Anti-Tumor Activity and Safety with Small Molecule-Regulated Molecular Switches and Chimeric Antigen Receptors

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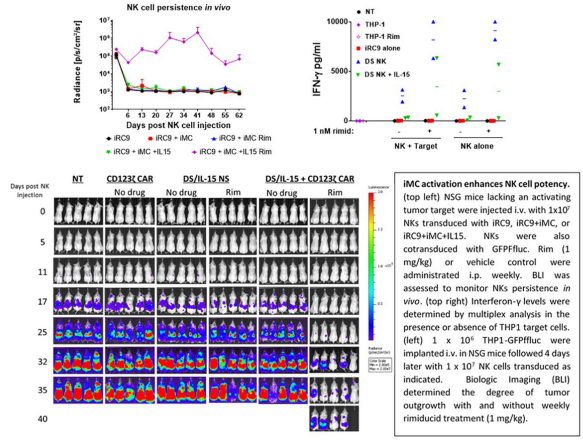
The innate anti-tumor activity of Natural Killer (NK) lymphocytes can be used as an allogeneic cell therapy with reduced risk of GvHD relative to αβ T cells. Despite their potential, adoptive NK cell immunotherapies have been limited by poor expansion *in vivo*. Previously, we described a Chimeric Antigen Receptor-T (CAR-T) cell strategy that relies on rimiducid-based dimerization of inducible MyD88/CD40 (iMC) to regulate T cell expansion and survival. Here, we apply iMC to NK cell growth and anti-tumor efficacy along with a Chimeric Antigen Receptor (CAR) to further drive antigen-specific tumor killing *in vitro* and *in vivo.* Further, we incorporate an orthogonally regulated safety switch, rapamycin-inducible Caspase-9 (iRC9), to reduce toxicity risk.CD56^{+} NK cells were selected from peripheral blood of human donors, activated and transduced with γ-retrovirus encoding iRC9-2A-ΔCD19 (marked negative control), iRC9-2A-ΔCD19-2A-iMC (dual-switch NK) or iRC9-2A-IL-15-2A-ΔCD19-2A-iMC (dual-switch plus IL-15 NK). iMC expression and activation with 1 nM rimiducid enhanced the growth potential of NK cells. In cocultures with THP1 leukemia cells at increasing Target:Effector (T:E) ratios, presence and activation of iMC increased tumor killing. Coexpression of a first-generation CAR targeting CD123 or BCMA with dual-switch expression vectors further enhanced anti-tumor efficacy *in vitro* with performance similar to CAR-T cells derived from the same donors. Cytokine and chemokine production was elevated (10 to >1000 fold) in an iMC-dependent fashion with a pattern of activated expression distinct from dual-switch CAR-T cells. To study NK growth and anti-tumor activity *in vivo*, immunodeficient NSG mice were engrafted with dual-switch NK cells with or without autocrine IL-15 expression in the presence or absence of THP-1 tumor targets. In tumor-free animals only dual-switch/IL-15 NK cells with weekly rimiducid stimulation expanded and persisted *in vivo* (up to 7 weeks). In THP-1 tumor-bearing animals, rimiducid-treated dual-switch/IL-15 NK cells without a CAR exhibited enhanced but modest tumor control *in vivo;* however, coexpression of a first-generation CAR targeting CD123 or BCMA led to rimiducid-dependent control of tumor outgrowth *in vivo* beyond six weeks. Persistence of dual-switch CAR-NK was observed at day 53 in the bone marrow and spleens of rimiducid-treated mice. Conversely, temsirolimus-mediated activation of the iRC9 safety switch rapidly (< 24 hours) ablated dual-switch NK cells *in vivo*. This regulated platform provides solutions to several of the challenges facing NK cell-based therapy as an off-the-shelf cellular therapy for cancer.



iMC activation enhances NK cell potency. (top left) NSG mice lacking an activating tumor target were injected i.v. with 1x10^{7} NKs transduced with iRC9, iRC9+iMC, or iRC9+iMC+IL15. NKs were also cotransduced with GFPffluc. Rim (1 mg/kg) or vehicle control were administrated i.p. weekly. BLI was assessed to monitor NKs persistence *in vivo*. (top right) Interferon-γ levels were determined by multiplex analysis in the presence or absence of THP1 target cells. (left) 1 x 10^{6} THP1-GFPffluc were implanted i.v. in NSG mice followed 4 days later with 1 x 10^{7} NK cells transduced as indicated. Biologic Imaging (BLI) determined the degree of tumor outgrowth with and without weekly rimiducid treatment (1 mg/kg).

139. UCARTCS1A: Allogeneic CAR T-Cells Targeting CS1 as Treatment for Multiple Myeloma

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Data accumulated over the past years using autologous T-cells expressing chimeric antigen receptors (CARs) have positioned CAR technology as a major breakthrough in cancer treatment, with long-term durable remissions achieved in patients with B cell leukemia. Multiple Myeloma (MM) is a B-cell neoplasia of clonal malignant plasma cells in the bone marrow which still remains incurable in most of the cases, even if current available therapies can improve patient's overall survival. Nevertheless, promising clinical activity has been reported for autologous CAR T-cells targeting B-Cell Maturation Antigen (BCMA) in patients with relapsed/refractory disease. Most CAR T-cell therapy products are currently generated from autologous cells, with the limitation that they have to be manufactured in a "per patient" basis, and remain still difficult to implement for lymphopenic and critically ill patients. In the present work we will present UCARTCS1A as a new therapy for MM, an off-the-shelf product composed of allogeneic CAR T-cells engineered from healthy donors and targeting the CS1 antigen (also known as SLAMF7).

MM tumor cells express indeed high levels of CS1, which displays limited expression in normal tissues. UCARTCS1A cells are engineered to lack expression of the TCRαβ receptor, in order to limit graft-versus-host disease when infused into patients. This is performed by inactivation of the *TCRα constant* (*TRAC*) gene using TALEN®, a gene-editing technology already in the clinic. Since CS1 is also expressed on normal activated CD8+ T-cells, the *CS1* gene is also inactivated so as to minimize fratricide of CS1-specific CAR+ T-cells during manufacturing. Preclinical efficacy results against MM cell lines and primary MM tumor cells generated with double knock-out (*TRAC*/*CS1*) CS1-specific universal CAR T-cells will be presented, showing increased cytotoxic activity of gene-edited CAR T-cells against MM cell lines compared to non-engineered T-cells expressing the same CAR. In addition, the CD8+ population is preserved in CS1 KO T-cells, while a progressive loss of CD8+ T-cells is observed in non-edited cells during expansion of CAR+ cells. UCARTCS1A was shown to specifically lyse primary MM tumor cells and showed degranulation in presence of CS1+ target cells, as well as significant antigen specific proliferation. Cytokine release was as well measured in supernatants of UCARTCS1A cells co-cultured with primary MM tumor cells, showing efficient antigen driven cytokine release. In addition, *in vivo* activity was evaluated in an orthotopic mouse model, showing that intravenous UCARTCS1A treatment allowed to control tumor progression and extend overall survival, as well as to maintain undetectable M-protein levels in sera. Similar results were obtained in immunodeficient mice in which primary MM xenografts were implanted in human fetal bone chips. Our results support therefore further development of these allogeneic CS1-specific CAR T-cells as a universal "off-the-shelf" product for the treatment of MM patients. Manufacturing of GMP grade UCARTCS1A is currently ongoing, and a Phase I dose escalation clinical trial is planned to be initiated in 2019.

140. Base Editors Generate Allogeneic CAR-T Cells with No Detectable Genomic Rearrangements and Reduced Genotoxicity

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Autologous, patient-derived chimeric antigen receptor-T cell (CAR-T) therapies have demonstrated remarkable efficacy in treating some hematologic cancers. While these products have led to significant clinical benefit for patients, the need to generate individualized therapies creates substantial manufacturing challenges and financial burdens. Allogeneic CAR-T therapies were developed as a potential solution to these challenges, having similar clinical efficacy profiles to autologous products while treating many patients with cells derived from a single healthy donor, thereby substantially reducing cost of goods and lot-to-lot variability. Most first-generation allogeneic CAR-Ts use nucleases to introduce two or more targeted genomic DNA double strand breaks (DSBs) in a target T cell population, relying on error-prone DNA repair to generate mutations that knock out target genes in a semi-stochastic manner. Such nuclease-based gene knockout strategies aim to reduce the risk of graft-versus-host-disease and host rejection of CAR-Ts. However, the simultaneous induction of multiple DSBs results in a final cell product containing large-scale genomic rearrangements such as balanced and unbalanced translocations, and a relatively high abundance of local rearrangements including inversions and large deletions. Furthermore, as increasing numbers of simultaneous genetic modifications are made by induced DSBs, considerable genotoxicity is observed in the treated cell population. This may significantly reduce the cell expansion potential from each manufacturing run, thereby decreasing the number of patients that can be treated per healthy donor. Base editors (BEs) are a class of emerging gene editing reagents that enable highly efficient, user-defined modification of target genomic DNA without the creation of DSBs. Here, we propose an alternative means of producing allogeneic CAR-T cells by leveraging base editing technology to reduce or eliminate detectable genomic rearrangements while also improving cell expansion. We show that, in contrast to a nuclease-only editing strategy, concurrent modification of three genetic loci by base editing produces highly efficient gene knockouts with no detectable translocation events by Uni-Directional Targeted Sequencing (UDiTaS). Further, coupling nuclease-based knockout of the *TRAC* gene with simultaneous BE-mediated knockout of two additional genes yields a homogeneous allogeneic T cell population with minimal genomic rearrangements, enabling the targeted insertion of a CAR transgene at the *TRAC* locus. Taken together, we demonstrate that base editing alone or in combination with a single nuclease knockout and viral CAR insertion is a feasible strategy for generating allogeneic T cells with minimal genomic rearrangements compared to nuclease-alone approaches. This method addresses known limitations of multiplex-edited T cell products and represents a promising new development towards the next generation of precision cell based therapies.

141. Point-of-Care Production of CD19 CAR-T Cells in an Automated Closed-System: Report on First Clinical Experience

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Introduction CD19 CAR-T cell products were recently approved as therapy for B-lineage malignancies. We initiated an IIT trial of CAR-T cell delivery to the patient is via a point-of-care manufacturing process. **Patients and methods** A total of 23 pts with relapsed/refractory B-cell lineage malignancies (7 female, 16 male, median age 11 y) were screened, 16 pts were enrolled for a trial, 7 were eligible for compassionate use of CD19 CAR-T cell therapy. Eleven patients had relapsed B-ALL after haploidentical HSCT, 10 pts refractory relapse, 1 induction failure, one patient had refractory B-cell lymphoma, 11 patients had previous blinatumomab infusion. Fifteen patients had >5% blast cells (median-

65%), 7 pts had minimal residual disease (MRD) >0,1% in BM, two had skeletal involvement, one CNS involvement. The CliniMACS Prodigy T cell transduction (TCT) process was used to produce CD19 CAR-T cells. Automatic production included CD4/CD8 selection, CD3/CD28 stimulation with MACS GMP T Cell TransAct, transduction with lentiviral (second generation CD19.4-1BB zeta) vector (Lentigen, Miltenyi Biotec company) and expansion over 10 days in the presence of serum-free TexMACS GMP Medium supplemented with MACS GMP IL-7/IL-15 combination. Final product was administered without cryopreservation to the patients after fludarabine/cyclophosphamide preconditioning. All patients received tocilizumab at 8mg/kg before CAR-T cell infusion. **Results** All production cycles were successful. Median transduction efficacy was 60% (20-80). Median expansion of T cells was x 46 (18-51). CD4/CD8 ratio in the final product was 0,73. The CAR-T cells were administered at a dose of $1*10^5$/kg of CAR-T cells in 6 pts, $5*10^5$/kg in 12 pts, $1*10^6$/kg in 5 pts. The cytokine release syndrome (CRS) occurred in 13 (56%) pts: grade I - 10 pts, grade II- 2 pts, grade IV - 1 pt. Neurotoxicity occurred in 8 (35%), grade I in 2 pts, grade II - in 5 pt, grade III - in 1 pt, grade V - 1 pt. In a patient with grade V neurotoxicity concomitant culture-positive K.pneumonia sepsis was documented. Six patients were admitted to the intensive care unit (ICU) - 3 for management of the CRS and 3 dues to severe condition caused by sepsis. Four patients died before day 28 (3 due to sepsis, 1 due to fatal brain edema and sepsis, on brain autopsy Klebsiella pneumoniae emboli were detected), 2 of them had blast clearance on day 14, 2 died before first evaluation and 19 patients were evaluable for response at day 28. Three pts had persistent leukemia. In 15 (83%) cases MRD-negative remisson were achieved. Patient with lymphoma had a PET/CT major response. Disease relapse/progression after initial response was registered in 2 cases. At the moment of reporting 17 pts are alive, 15 in complete remission with a median follow up of 3 months (0,08-0,9 m). **Conclusion** Production of CAR-T cells with the CliniMACS Prodigy TCT process is a robust option that provides a point-of-care manufacturing approach to enable rapid and flexible delivery of CAR-T cells to patients in need. Robustness and consistency of this approach provides a solid basis for multi-center academic trials in the field of adoptive cell therapy.

142. Design and Characterization of Persistent CAR-T Cells Targeting Multiple Tumor Antigens Simultaneously

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Introduction: Clinical data from diverse cell therapeutics highlight two critical issues: First, in many indications cell therapeutics fail to persist after initial expansion. This is particularly true in the case of solid tumors. Second, tumors rapidly respond to cell therapeutics by reducing or eliminating target antigen expression. Antigen loss has been widely reported in the context of cell therapies targeting both clonal hematologic cancers and heterogeneous solid tumors. Here we present a novel solution to the critical issues of CAR T cell persistence and antigen loss. **Results & Discussion: 1** - A stabilized form of the CD19 extracellular domain (ECD) was cloned in frame with antigen binding domains (eg. scFv, VHH) targeting antigens of interest. For example, an scFv targeting HER2 was linked together with the CD19 ECD to create a CD19-anti-Her2 fusion protein (FP). The purified soluble FP was used to bridge CAR-T cells specific for CD19 (CAR19s) to Her2+ tumor cells, effectively forming a cytotoxic synapse. We have generated FPs that target EGFR, Clec12a, BCMA, CD20 and many other tumor antigens. FPs mediated selective tumor cell killing at very low concentrations, eg. 10pm for the CD19-anti-Her2 FP and 8.3pm for a CD19-anti-Clec12a VHH FP. Thus, FPs can coat any target tumor cell with CD19, allowing for robust killing. **2** - CAR T cell secretion of the the CD19-anti-Her2 FP was evaluated *in vitro*. The CAR19 and FP were encoded in a lentiviral vector. The transduced T cells expressed both CAR19 and secreted the FP at 10ng/ml (140pm), and effectively killed Her2-positive tumor cells *in vitro*. Next we used an adenoviral vector to transduce the target tumor cells with the CD19-anti-Her2 FP expression construct, and then added the CAR19 T cells, which eliminated the CD19-coated tumor cells. Finally, a soluble, half-life extended form of CD19-anti-Her2 FP was used to coat SKOV3 cells with CD19, and the CAR19 T cells were then added to kill the coated tumor cells. Thus three methods of FP delivery are effective: T cell secretion (lentiviral), tumor cell secretion (oncolytic viral) and systemic delivery (biologics). **3** - The CD19-anti-Her2 FP was evaluated *in vivo* using the Her2+ SKOV3 model and two methods of delivery: CAR-T cell expressed, and systemic injection (using the biologic form). In both models tumor cell killing *in vivo* was robust and complete eradication of established tumors was achieved. Long term tumor control was observed with no tumor relapse in the treated groups. **4** - Next we added an scFv to EGFR, thus creating an anti-EGFR-CD19ECD-anti-Her2 FP. This FP binds either or both antigens, as demonstrated using *in vitro* binding and cytotoxicity assays. The dual antigen binding FP addresses the potential of antigen loss in heterogeneous tumor types such as solid tumors. We add a third domain in an indication-specific manner, eg. an anti-IL-13Ra binding domain. The resulting FP therefore can bind EGFR or HER2 or IL13Ra, and all binding events display CD19 on the surface of the tumor cell (eg. glioblastoma) and can be attacked by CAR19 T cells. This addresses the issues of tumor heterogeneity and antigen loss relapse. **5** - In these examples the persistence of CAR19 T cells will be enhanced by the presence of normal B cells. Clinically, eg. in a solid tumor patient, normal B cells will present a non-tumor dependent, self-renewing source of antigen due to the presence of peripheral B cells and ongoing B cell hematopoiesis from the bone marrow. This addresses the issue of CAR persistence. **Summary** We have created a robust and highly modular system for attacking tumors with multiple antigens simultaneously and in a manner that supports CAR-T cell persistence independently of tumor antigen expression. This technology will support CAR19 attack against diverse cancer indications, including hematologic and solid tumor cancers.

143. Enhancing Chimeric Antigen Receptor-Modified Gamma Delta T-Cell Killing of T-Cell Leukemia by Modulating Antigen Expression in the Effector Cells

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Chimeric antigen receptor (CAR)-modified T cells have demonstrated efficacy against B-cell leukemias. However, redirecting the cytotoxicity of T cells to malignant T cells is more challenging. Some limitations to using CAR T-cell therapy in the setting of T-cell malignancies include fratricide, which can limit the expansion and total number of the modified cells, and memory cell formation of CAR T cells, which can result in life threatening T-cell aplasia. To overcome the latter, we propose utilizing gamma delta (gd) T cells instead of alpha beta (ab) T cells. Vg9/Vd2 gd T cells are predicted to persist *in vivo* for a finite time (weeks to months) and do not develop into memory cells. In addition, gd T cells are not actively involved in the generation of graft vs host disease. Therefore, with repeated infusions, it is possible that CAR-modified gd T cells could be useful in autologous or allogeneic clinical settings. We developed a serum-free protocol for gd T-cell expansion from PBMCs, resulting in a population of up to 90% gd T cells. Using an anti-CD5-scFv-CD28-CD3z CAR, we optimized the transduction efficiency of the gd T-cell product. Similar to our previous findings and that of others for ab T cells, the CD5-CAR gd T cells down regulate endogenous CD5 surface expression. This mechanism of modulating endogenous CD5 expression allows for CAR expression without robust fratricide. Although gd T cells have intrinsic abilities to kill T-cell leukemia, cytotoxicity is enhanced against CD5+ target cells after transduction compared to cytotoxicity of non-transduced gd T cells. Although endogenous CD5 expression is modulated by expression of the anti-CD5-CAR, we recently showed elimination of the targeted antigen expression from the effector cells using CRISPR-Cas9 results in increased CAR expression in effector cells. Therefore, to reduce fratricide and increase CAR expression, we tested various methods of manipulating CD5 expression in gd T cells. Electroporation methods for editing gd T cells were found to be inefficient. We designed and tested anti-CD5-based decoy CARs, which contain the scFv and transmembrane portions of the CAR but not the intracellular activation domains. Expression of the decoy-CAR virtually eliminated CD5 surface expression. Decoy-CAR-modified cells showed no differences in cytotoxicity compared to that of naïve cells. However, gd T cells expressing both the anti-CD5-decoy-CAR and the anti-CD5-CAR exhibited over 1.5-fold increase in cytotoxicity than that of CD5-CAR only cells. Furthermore, the cytotoxicity of gd T cells expressing both the anti-CD5-decoy-CAR and the anti-CD5-CAR was 4-fold higher against CD5+ target cells compared to that against CD5- target cells, suggesting CD5-specific cytotoxicity. To more fully understand the mechanism of the enhanced killing, the Jurkat T-cell line was used to investigate modulation of CD5 expression by the anti-CD5-decoy-CAR and the anti-CD5-CAR. These results show the anti-CD5-decoy-CAR Jurkats exhibit reduced activation, measured by CD69, when further modified with the anti-CD5-CAR in a dose-dependent manner, which in cytotoxic cells would result in reduced fratricide. Therefore, we propose gd T cells may also display decreased activation upon anti-CD5-CAR modification, and this may be one mechanism of the enhanced cytotoxicity observed in gd T cells. The anti-CD5-decoy-CAR may provide a mechanism to modulate endogenous CD5 expression while minimizing activation of the effector cells, thereby eliminating the potential issues of fratricide and allowing for increased CAR expression, augmenting anti-tumor cytotoxicity.

144. T-cells Expressing an Anti-Signaling Lymphocytic Activation Molecule F7 (SLAMF7) CAR and a Suicide Gene Exhibit Anti-Tumor Activity and Can be Eliminated On-Demand

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Multiple Myeloma (MM) is hematological disorder manifested by clonal proliferation of malignant plasma cells. Current therapies for MM have been ineffective due to short remission, relapse, and death, so better therapeutic strategies for MM are needed. Chimeric antigen receptors (CARs) are artificial fusion protein with antigen-recognition domains and T-cell signaling domains. T-cells expressing CARs targeting the MM surface antigen B-cell maturation antigen (BCMA) have proven clinical efficacy. However, clinical studies by our group recently reported a loss of BCMA expression on MM cells after CAR T-cells infusions thus warranting an investigation of new antigen targets for MM. SLAMF7 is a heavily investigated target for MM. SLAMF7 is uniformly expressed on MM cells; SLAMF7 is also expressed on a variety of other leukocytes including some CD8+ T-cells, dendritic cells and most natural killer (NK) cells. Despite being a promising therapeutic target for MM in preclinical studies by different groups, anti-SLAMF7 CAR T-cells pose a risk of cytopenia, especially NK cell deficiency. We designed constructs encoding both an anti-SLAMF7 CAR and a suicide gene known as ICas9 (IC9). The IC9 suicide gene system is a clinically proven suicide gene system that eradicated T-cells in humans. The IC9 suicide gene contains the inducible caspase 9 gene fused to FK binding protein-12 (FKBP12). Binding of the small molecule Rimiducid (AP1903) to FKBP12 leads to dimerization and activation of caspase 9, leading to apoptosis of anti-SLAMF7 CAR T-cells. Our CAR constructs were encoded by a Ɣ-retroviral vector that harbored the IC9 gene, a 2A-ribosomal skip site, followed by the CAR sequence of a single chain variable fragment (ScFv) derived from the Luc90 (anti-SLAMF7) antibody, a CD8α hinge and transmembrane domain, a CD28 costimulatory domain and a CD3 zeta T-cell activation domain. We transduced patient T-cells with the CAR constructs and assessed the expression and function of T cells expressing the Luc90-CD828Z CAR and IC9. We found that our engineered Luc90 CARs were expressed on T-cells after transduction, and we found a higher ratio of CD4+: CD8+ T-cells, which was possibly due to CAR T-cell killing of CD8+ T cells. Importantly, T-cells expressing Luc90 CARs demonstrated SLAMF7-specific cytokines release, degranulation, cytotoxicity and proliferation. Based on in vitro experiments we selected construct IC9-Luc90-CD828Z for extensive testing. Our murine experiments showed that T-cells expressing both

IC9 suicide gene and Luc90-CD828Z CAR can eliminate both solid tumors or disseminated SLAMF7-expressing human MM cell from immune-deficient NSG mice. Next, we demonstrated a significant and exclusive elimination of T-cells expressing IC9 suicide gene + Luc90-CD828Z CAR in vitro and in vivo by the AP1903 dimerizer. We cultured normal peripheral blood mononuclear cells (PBMC) with: untransduced T-cells, T-cells expressing the anti-CD19 CAR Hu19-CD828Z (control), the Luc90-CD828Z CAR, or the IC9 suicide gene+ Luc90-CD828Z CAR. In this experiment, we evaluated the IC9 suicide gene+Luc90-CD828Z CAR T cells with and without AP1903. We found that addition of the AP1903 to cultures of PBMC and T cells expressing IC9 suicide gene+Luc90-CD828Z CAR, eliminated the CAR T-cells and resulted in prevention of NK cell elimination. In summary, we constructed and tested an anti-SLAMF7 CAR construct containing a suicide gene. CARs expressing this construct specifically recognized SLAMF7 in vitro and eradicated tumors from mice. Furthermore, we demonstrated that IC9 acted as an efficient safety switch that eliminated the CAR T-cells.

Use of Immune Modulation in Gene Therapy

145. Direct Type I Interferon Signaling in Conventional Dendritic Cells and T Help are Required for Cross-Priming AAV Capsid-Specific CD8+ T Cells

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AAV vectors have now been used for *in vivo* gene transfer to successfully treat several human diseases. However, broadening this approach to a more diverse set of disorders, especially those that require systemic delivery, has largely been stymied by $CD8^+$ T cell responses against the viral capsid. We have made progress toward defining mechanisms that govern anti-capsid $CD8^+$ T cell activation. Previously, we found that anti-capsid $CD8^+$ T cell priming requires the cooperation of two distinct dendritic cells (DC) subsets, plasmacytoid DCs (pDCs) and conventional DCs (cDCs). Specifically, TLR9 sensing of the vector's DNA genome occurs in pDCs, while cross-presentation of AAV-vector derived antigen to naïve $CD8^+$ T cells is carried out by cDCs. The segregation of innate sensing from cross-presentation implies that cDC licensing signals, which are required for effective T cell priming, must be delivered *in trans* from pDCs to cDCs. Type I Interferons (T1 IFNs) are abundantly produced by pDCs downstream of TLR9 signaling and are considered key mediators of anti-viral immunity. Our prior studies showed that mice deficient in the T1 IFN receptor (IFNAR-1) fail to generate a $CD8^+$ T cell response to capsid and ruled out an indirect effect of T1 IFN via activation of NK cells. Hence, to test for a requirement for a direct effect of T1 IFN signaling in cDCs, we generated a CD11c-Cre x IFNAR-$1^{fl/fl}$ mouse model (CD11c-$IFNAR^{-/-}$) in which the IFNAR-1 gene is selectively deleted in cDCs but not pDCs. In CD11c-$IFNAR^{-/-}$ mice treated with AAV2-SIIN, the absence of T1 IFN signaling in cDCs substantially mitigated $CD8^+$ T cell responses against the viral capsid (n=7/group; AAV2-SIIN has an engineered capsid for detection of anti-capsid $CD8^+$ T cells using an H2-K^b-SIINFEKL tetramer). This result supports the model that T1 IFN is the critical mediator that translates innate sensing in pDCs to a licensing signal in cDCs that permits anti-capsid $CD8^+$ T cell priming. To test whether this information can be exploited for therapeutic intervention, WT C57BL/6 mice were treated with a blocking antibody for αIFNAR-1 one day prior to i.m. injection of $1x10^{11}$ vg AAV2-SIIN. Anti-capsid $CD8^+$ T cells were significantly reduced on day 7 and day 14 p.i. in αIFNAR-1 treated mice compared to isotype control treated. Moreover, the anti-capsid $CD8^+$ T cell response never rebounded in αIFNAR-1 treated mice after 2, 3 & 4 weeks, underscoring its importance during the early priming phase. Although we've demonstrated that T1 IFNs are distinctly essential for cDC licensing, the canonical mechanism for cDC licensing is through CD40-CD40L interaction with $CD4^+$ T cells. To determine whether this pathway is also involved in cDC licensing in response to AAV, WT mice were treated with an αCD40L blocking antibody 1 day prior to AAV2-SIIN. We observed a significant reduction in anti-capsid $CD8^+$ T cells in αCD40L treated mice, which averaged 0.48% $tetramer^+$ compared to 2.1% in isotype control treated mice (n=5/group, with 4 of 5 mice having undetectable responses). Furthermore, MHC class $II^{-/-}$ mice, which completely lack $CD4^+$ T cells, had a similar reduction in anti-capsid $CD8^+$ T cell responses (n=7/group). Therefore, T help is another requirement for DC licensing that results in cross-priming of CD8+ T cells against AAV capsid and represents an alternative target for preventing this response. Taken together, direct T1 IFN signaling in cDCs as well as $CD4^+$ T cell help through CD40-CD40L are critical licensing signals during the early stages of $CD8^+$ T cell priming against the AAV capsid. Leveraging these pathways may minimize $CD8^+$ T cell responses that have previously curtailed clinical efficacy for many AAV applications.

146. Tolerogenic ImmTOR™ Nanoparticles Enhance Vector Transduction, mRNA Synthesis and Transgene Expression after Initial and Repeated Administrations of AAV-Based Gene Therapy Vectors through Immunological and Non-Immunological Mechanisms

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Achieving clinically effective and stable expression of therapeutic transgenes is a key challenge for gene therapy. In particular, transgene expression following liver-directed systemic delivery of AAV vectors in pediatric patients is expected to wane over time, and the formation of neutralizing antibodies prevents AAV re-administration. We have previously shown that tolerogenic nanoparticles encapsulating rapamycin (ImmTOR™ or SVP-Rapamycin) have the ability to block the humoral and cellular responses against the capsid, thereby enabling repeat administration of AAV vectors (Meliani et al., Nat. Commun., 2018). Here we further demonstrate that co-administration of ImmTOR™ with AAV-based vectors also enhances

transgene expression after the first dose of AAV vector in naïve mice. This beneficial effect of ImmTOR™ on first dose transgene expression appears to be largely independent of its immunomodulatory effects on adaptive immunity. Admixing ImmTOR™ and AAV prior to injection is required for enhanced transgene expression after the first dose, but is not required for inhibition of the antibody response to AAV or for repeated administration of AAV vectors. Moreover, the first dose benefit of ImmTOR™ is also observed in beta 2-microglobulin- and Rag2-deficient mice. However, the partial or complete absence of adaptive responses in these mice enhances transgene expression, compared to wild type mice, even without ImmTOR™, especially after repeated vector administrations, thus confirming that the adaptive immune response is a crucial impediment for repeat AAV dosing. Mechanistically, our data suggests that ImmTOR™ can affect multiple aspects of AAV biology at first dose, including trafficking to the liver, inhibition of acute inflammation, and autophagy. The physical association of ImmTOR™ and AAV, which is time- and dose-dependent and can be blocked in vitro by pre-exposure of AAV to low levels of neutralizing antibodies, may facilitate trafficking of AAV to the liver, which results in increased vector copy numbers and transgene mRNA expression in the liver. The combination of ImmTOR™ and AAV also inhibits AAV-induced NFκB activation in the liver of transgenic NFκB reporter mice. Finally, ImmTOR™ appears to enhance autophagy in vivo, which is consistent with a recent report that autophagy induced by mTOR inhibitors, such as rapamycin, increases AAV transduction, nuclear localization, and transgene expression in vitro and in vivo (Hősel et al. Hepatology, 2017). This multi-pronged mechanism of ImmTOR™ action makes it an attractive candidate to enhance systemic gene therapeutic applications, particularly in those clinical indications where repeat vector dosing may be necessary. The first dose benefit of adding ImmTOR™ to AAV gene therapy is immediate, dose-dependent and not mouse strain-specific. It requires direct interaction of ImmTOR™ particles with AAV virions and is characterized by increased vector copy number in liver cells and elevated synthesis of transgene-encoded mRNA. The rapid and enhanced transgene expression may enable therapeutic benefit at lower doses of AAV and faster onset of transgene-directed therapeutic effects. Importantly, the inhibition of the formation of neutralizing antibodies by ImmTOR™ enables productive expression of the transgene with repeat administration.

147. Combination Therapy Reduces Existing Anti-AAV Antibody by Logs and Allows for Safe and Efficacious Redosing

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Previous studies have shown the efficacy of repeat adeno-associated viral vector (AAV) administrations is inhibited by anti-AAV antibodies (Ab) that develop after initial vector dosing. Treating patients with high-titer Ab also poses safety risks, as Ab-antigen complexes can cause hypersensitivity reactions resulting in adverse events such as anaphylaxis, nephritis, or arthritis. Regardless, such repeat dosing may be necessary for some patients. While protocols are developing to prevent the initial formation of Ab, many patients will have already been exposed to AAV through clinical trials, commercial treatments, or wild type infections. Here we tested the ability of immune suppression coupled with therapeutic plasma exchange (TPE) to reduce anti-AAV8 Ab following an initial dose to levels allowing for safe and efficacious redosing of vector. We have shown that AAV8 expressing canine myotubularin 1 (cMTM1) from the muscle-specific desmin promoter can successfully treat X-linked myotubular myopathy (XLMTM) dogs (Mack *et al* Mol Ther 2017). Here, two 11-week old XLMTM dogs were systemically treated with 8e13 vector genome (vg)/kg of the aforementioned vector with only a 4-week corticosteroids course—current standard of care for high-dose AAV in patients—for immune modulation. As expected, high-titer (titers > 1e5) anti-AAV8 IgG2 Ab developed. ~4 months after the 1st AAV dose, 1.4e14 vg/kg of the same vector was re-administered. For redosing safety and to reduce anti-AAV Ab, rapamycin was started 38 days prior to the second dose and maintained for 2 weeks after redosing, prednisolone was started 24 days prior and tapered beginning 2 weeks after, and a canine anti-canine CD20 Ab was infused 32 days prior. Finally, each dog went through three rounds of TPE: 1.5x the dogs' plasma volume was exchanged for saline and donor plasma 4 days, 2 days, and 30 minutes prior to redosing. The potential exists that additional rounds could reduce Ab titers even further. Baseline anti-AAV8 total Ab levels as determined by ELISA were 128 (Dog #1) and 216 (Dog #2) ng/mL, corresponding with titers of 100 and 315, respectively. Ab peaked on day 87 (#1: 6.0e5, #2: 3.0e5 ng/mL). A 25-45% reduction was achieved with pharmaceuticals/biologics, with an additional log decrease achieved with TPE. At the time of redosing, Ab concentrations were 4.3e4 (#1) and 1.5e4 (#2) ng/mL; titers were within 1.5 logs of baseline. Vector copy number was 0.52 ± 0.25 vg/diploid genome (dg) in 3 muscle biopsies per dog 25 days after 1st AAV. That dropped to 0.10 ± 0.05 vg/dg on day 98, but then rebounded to 0.59 ± 0.17 vg/dg 25 days after redosing. This increase in intramuscular vector copy number suggests that despite residual anti-AAV Ab, redosing successfully led to new transduction. Additional quantitation of vector expression is ongoing to confirm this conclusion and will be presented at the meeting. Neurology assessments, which score gait, exercise tolerance, and muscle tone (10 is normal), increased in Dog 2 after redosing (low 8 baseline; scores of 8-9 after 1st AAV; 8-10 after 2nd AAV). Dog 1 maintained scores of 9 throughout. Limb reflex scores increased for both dogs after redosing (1.33 ± 0.18 baseline, 1.74 ± 0.11 after 1st AAV, 1.91 ± 0.13 after redosing, $p<0.01$). Neither dog had overt hypersensitivity responses to the 2nd infusion of vector. Together, these data suggest that anti-AAV8 Ab can be overcome for safe and efficacious repeat vector dosing.

148. Toll-Like Receptor Agonists Have Distinct Effects on Activation of Dendritic Cell Subsets and Antibody Formation in Muscle AAV Gene Transfer

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The effects of innate immune signaling, including through toll-like receptors (TLRs), on dendritic cell (DC) activation and frequency during AAV gene transfer is not well-defined. Promotion of adaptive immune responses by activated DCs, such as enhancement of follicular helper T cells by TLR9 activation of monocyte-derived DCs (moDCs), was previously shown to promote antibody formation during AAV gene transfer. Like TLR9, TLR7 is found in the endosomes of many of the same subsets of DCs and couples with the same adaptor signaling molecule, myeloid differentiation factor 88, which increases production of pro-inflammatory cytokines and surface levels of costimulatory/presentation molecules and causes nucleated cells to become resistant to viral infection. TLR7 agonists are approved for treatment of basal cell carcinoma and are being tested for use in a wide variety of other therapies. With continued development of both TLR7 agonist and AAV gene therapies, there is additional potential for overlap in patients, making investigation of their possible interactions increasingly important. Additionally, during AAV gene transfer, patients may be exposed to TLR7-stimulating RNA viruses (e.g., influenza virus), which could potentially hamper gene therapy if TLR7 activation enhances antibody formation similarly to TLR9. Therefore, we asked whether activation of endosomal TLRs, by adenine analog CL264 (TLR7 agonist), imidazolquinolone compound R848/resiquimod (dual TLR7/8 agonist), or class B CpG oligonucleotides ODN1826 (TLR9 agonist) could augment antibody formation upon intramuscular administration of AAV1 expressing human clotting factor IX (AAV1-hFIX) in mice. The TLR9 agonist robustly enhanced antibody formation within 2 weeks, initially eliminating systemic hFIX expression. In contrast, the TLR7 and dual TLR7/8 agonists did not promote antibody formation. A transient reduction of circulating hFIX was nonetheless noted after AAV treatment with the TLR7 agonist but not the dual TLR7/8 agonist. Concurrently, we investigated the effects of these TLR agonists on splenic and draining lymph node (dLN) DCs, including: moDCs, conventional DCs ($CD11b^+$ or $CD8\alpha^+$), and plasmacytoid DCs (pDCs). When administered AAV only, mice displayed increased surface levels of MHCII on splenic moDCs, $CD11b^+$ DCs, and $CD8\alpha^+$ DCs. Among other changes, TLR9 stimulation increased frequencies of dLN moDCs and $CD11b^+$ DCs. Treatment with the dual TLR7/8 agonist resulted in both increased dLN moDCs and expanded and activated dLN pDCs. Lastly, the TLR7 agonist increased splenic $CD8\alpha^+$ and dLN pDCs but failed to increase moDCs. Thus, these TLR ligands have various effects on DC populations, yet only the TLR9 agonist enhanced the humoral immune response against AAV-expressed hFIX. These new findings and literature data indicate that TLR9 is unique among TLRs in its ability to stimulate B cell activation in muscle gene transfer. Our new findings further show that antibody formation cannot merely be explained by expansion of moDCs via TLR activation but is likely also influenced by effects on other subsets of DCs, such as pDCs. Although the TLR7 agonists had somewhat diverse effects on DC populations and may therefore shape immune responses differently from each other, neither TLR7 agonist induced antibody formation against the transgene product in muscle AAV gene transfer.

149. LV.Insulinb9-23/Anti-CD3 Combined Therapy Inhibits Recurrence of Autoimmunity in NOD Mice after Allogeneic Pancreatic Islets Transplant

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Type 1 diabetes (T1D) is an autoimmune disease resulting in the complete destruction of pancreatic beta-cells by auto-reactive T cells targeting islet-associated antigens and induction of antigen (Ag)-specific tolerance represents a therapeutic option. We previously showed that systemic administration of a single dose of lentiviral vector (LV), enabling stable or transient expression of insulin B chain 9-23 (InsB9-23) in hepatocytes, arrests beta-cell destruction in NOD mice at advanced pre-diabetic stage, by generating InsB9-23-specific $FoxP3^+$ T regulatory cells (Tregs). Here we show that LV.InsB in combination with a suboptimal dose (1X 5μg) of anti-CD3 mAb (combined therapy CT5) reverts overt diabetes, prevents recurrence of autoimmunity and maintains insulin independence when provided the day after syngeneic or allogeneic pancreatic islet transplantation in 50% and 40% of treated mice, respectively. Moreover, we investigated whether an optimized CT (1X25μg of anti-CD3 mAb plus LV.InsB-CT25) could arrest recurrence of autoimmunity and possibly suppress allo-response to transplanted islets. To obtain stabilization of disease progression and to establish the CT-driven tolerogenic program prior to islet transplantation, we transplanted allogeneic islets 7 days after CT25. Results indicate that 100% of diabetic NOD mice treated with CT25 and transplanted with Balb-c islets (at glycemia <500mg/dL) remained normoglycemic for 100 days, displayed a reduced T cell responsiveness to InsB9-23 stimulation and an increased frequency of Tregs in pancreatic inflitrates and lymphnodes. Histological analysis showed that the transplant under the kidney capsule was lost and cured mice showed a reduced insulitis compatible with their glycemic level. Overall, results indicate that optimized CT25, via induction of Ag-specific $FoxP3^+$ Tregs and control Ag-specific effector T cells, represents a curative treatment for T1D when associated with allogeneic islets transplantation to restore activity of the endogenous beta cell mass.

150. Transient Immunosuppression and Adeno-Associated Virus (AAV) Vector Gene Transfer of Programmed Death Ligand-1 (PD-L1) Prevents the Development of Chronic Cardiac Transplant Rejection

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The accepted treatment for end-stage heart failure is transplantation. Immunosuppressive therapies diminish the incidence of graft loss due to acute graft rejection, leaving chronic rejection (CR) as the main impediment to long-term transplant survival. CR in cardiac allografts is characterized by interstitial fibrosis, vascular occlusion, cardiac hypertrophy, and progressive dysfunction of the graft. In the mouse vascularized cardiac allograft model, prolonged allograft survival can be achieved by transiently depleting recipients of CD4+ T cells or by disrupting CD40-CD40L interactions. However, allografts in mice treated with anti-CD40L monoclonal antibody (mAb) remain free of CR, while grafts in mice transiently depleted of CD4+ T cells develop CR. Programmed death-1 (PD-1) is an immune checkpoint receptor on cytotoxic T cells. Upregulation of the PD-1 receptor plays a key role in the debilitating process of T cell exhaustion, as well as being an important factor during the normal immune response to prevent autoimmunity. In this study, AAV gene transfer of programmed death ligand-1 (PD-L1) into cardiac allografts was employed to assess the impact of intragraft PD-L1 expression on both acute and chronic rejection of the transplanted organ and donor-reactive T cell responses. To evaluate efficacy of PD-L1 gene transfer in the acute and chronic rejection mouse cardiac allograft models, allografts were transduced by perfusion with self-complimentary AAV vectors that encoded a mouse PD-L1 gene (scAAV9-PD-L1) or a Null vector encoding a scrambled mouse PD-L1 gene sequence (scAAV9-Null) and heterotopically transplanted into fully-MHC disparate recipients that either received no immunosuppression (acute rejection model) or inductive anti-CD4 mAb therapy on days -1, 0, and 7 transplant (chronic rejection model). Graft function was monitored by palpation, and overexpression of PD-L1 in transduced allografts was verified by quantitative RT-PCR. In the ***acute rejection model***, all recipients rejected the grafts by day 9 post-transplant. Gene transfer of PD-L1 to allografts failed to alter the splenocyte cellular composition or abrogate the dominant Th1 response associated with acute rejection. Cellular infiltrate, myocyte necrosis, and hemorrhage—all hallmarks of acute rejection—were observed in both the scAAV9-PD-L1 or scAAV9-Null transduced allografts, indicating that PD-L1 gene transfer did not alter the course or pathological characteristics of acute rejection. Conversely, in the ***chronic rejection model***, PD-L1 overexpression in combination with inductive anti-CD4+ mAb treatment significantly attenuated cardiac hypertrophy, improved graft function, and significantly reduced the percentage of graft-infiltrating lymphocytes compared to the control group. Donor-reactive T and B cells also remained in a hyporesponsive state in the PD-L1-gene transferred recipients. Furthermore, morphometric trichrome analysis revealed a significant reduction in interstitial fibrosis in the PD-L1-overexpressing allografts compared to the Null vector transduced grafts. Strikingly, allografts transduced with scAAV9-PD-L1 combined with transient depletion of CD4+ T cells exhibited comparable collagen deposition to allografts in mice treated with inductive anti-CD40L mAb therapy, which remain free of CR. These findings demonstrate that combined transient immunosuppression and AAV-mediated PD-L1 cardiac gene transfer holds great promise in preventing the development of chronic allograft rejection, and provides a new treatment strategy to overcome a critical challenge to long-term transplant success.

151. In Vivo Chemoselection of Antibody-Secreting Hematopoietic Cells

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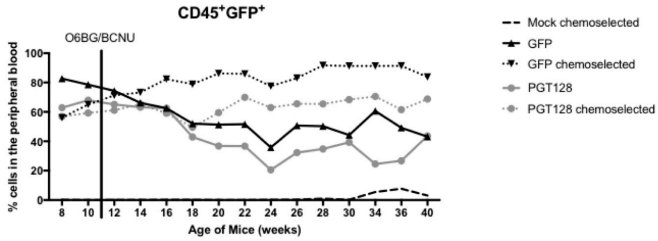
Despite efficacy of the antiretroviral therapy (ART) for the treatment of HIV-infected patients, replication-competent latent viral reservoirs persist in sanctuaries, leading to viral rebound after treatment interruption. Alternative treatment approaches are necessary to address this challenge. Broadly neutralizing antibodies (bNAbs) represent an encouraging and safe novel treatment strategy, as recently demonstrated in clinical trials. However, sustained antibody expression still has to be achieved. We have previously established that in vivo secretion of bNAbs by gene-modified hematopoietic stem and progenitor cells (HSPCs) represent a promising alternative to address these issues and we are now investigating approaches to reach higher therapeutic concentrations of secreted antibody. We have modified human HSPCs $CD34^+$cells with lentiviral vectors conferring not only antibody secretion but also resistance to O6-benzylguanine (O6BG) and bis-chloroethylnitrosourea (BCNU) chemotherapy through expression of the $MGMT_{P140K}$ protein. The cells, either modified or unmodified for control, were injected into irradiated NOD-SCID-gamma (NSG) neonate mice which underwent chemoselection with O6BG and BCNU at 11 weeks following the infusion. We found that treatment with the chemoselection drugs stabilized the engraftment of the gene-modified GFP-expressing human cells in the peripheral blood relative to non-chemoselected mice until 40 weeks, duration of the experiment. Indeed, after 24 weeks, the engraftment in the antibody-secreting group reached a plateau averaging 65% of $CD45^+GFP^+$cells in the chemoselected mice, which was higher than in the non-chemoselected mice averaging 30% of $CD45^+GFP^+$cells (see figure). Longitudinal and necropsy analyses revealed that the engraftment and differentiation of HPSCs into $CD3^+$T cells, $CD20^+$B cells and $CD14^+$monocytes were unaffected by the treatment of mice with the chemoselection drugs relative to the untreated mice. The $CD34^+$derived hematopoietic cells secreted anti-HIV PGT128 bNAb in the plasma at a slightly higher concentration than in the non-chemoselected mice despite engraftment of human $CD45^+$cells below 10%. At 22 weeks of age, the mice were challenged with HIV-1 virus and the antiviral antibody function is now being investigated. Future experiments will also assess $MGMT_{P140K}$ expression in the modified cells and trafficking to the tissues of interest. The indefinite renewal potential of HSPCs and ability to differentiate into lineages migrating to tissues hosting the viral reservoirs is a relevant advantage to address sustained antibody

secretion and delivery to the sanctuaries. In vivo chemoselection with O6BG and BCNU drugs further support the persistence of the engraftment of the gene-modified cells rather than an increase in the engraftment, as well as increased antibody concentrations. Such strategy highlights the promising potential of using HSPCs as a means to improve therapeutics delivery and importantly, could be applied to other infectious or non-infectious diseases whose treatments require sustained antibody expression.Figure 1: Selected mice from mock (unmodified cells), GFP (GFP expression) and PGT128 (GFP and PGT128 antibody expression) groups were chemoselected at 11 weeks. Gene-modified $CD45^+GFP^+$cells were quantified in the peripheral blood by flow cytometry at the indicated time points.



supportive environment, the MPFUs generate expanding neodermal islands that close the wound from the inside out in a manner similar to natural healing. Following pre-clinical validation in a swine model of wound healing, efficacy of AHSC to close burn, acute traumatic, and chronic wounds was evaluated clinically. Digital photography, functional analysis, and biopsied tissue analysis including confocal fluorescent microscopy, environmental scanning electron microscopy, multiphoton second harmonic generation microscopy, and RAMAN spectroscopy demonstrated regeneration of full-thickness skin (Figures 1B-D). *Ex vivo* processing of the patient's skin and activation of its regenerative cellular populations during AHSC processing provides an opportunity to manipulate AHSC including genetic modification. Preliminary gene transfer results using fluorescent protein lentiviral reporter constructs demonstrated the ability to transduce cellular elements of murine-AHSC (Figure 1E). These data demonstrate AHSC is able to close complex cutaneous defects with full-thickness, functional skin, which may be genetically manipulated to potentially treat debilitating genetic cutaneous defects.

Regenerative Medicine I

152. Novel Commercially Available Autologous Homologous Skin Construct Yields Neo-Generation of Full-Thickness Skin Following Burn, Acute Traumatic, and Chronic Cutaneous Wounds That Can Be Genetically Modified

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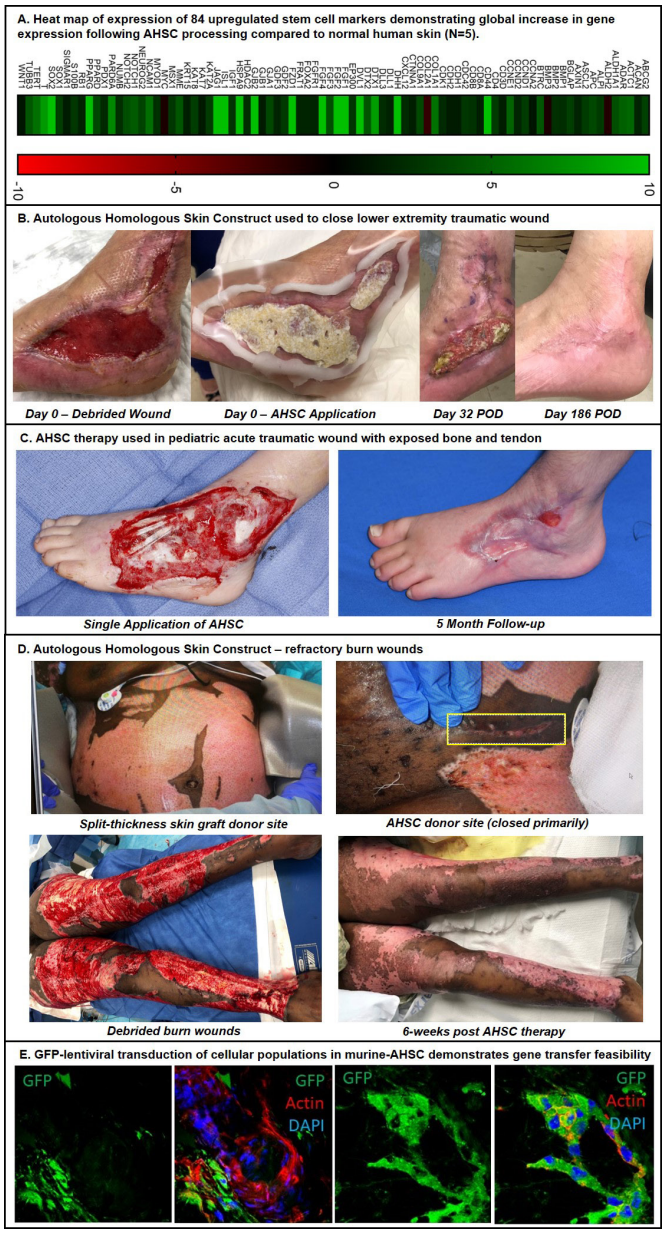
Despite advancements in tissue engineering, current therapies cannot regenerate fully-functioning skin. Established therapies for cutaneous wounds like split-thickness skin grafts (STSG) and advanced skin substitutes are limited by incomplete healing, scarring, contraction, and lack the function of normal skin. Burn, acute traumatic, and chronic wounds affect approximately 40,000, 700,000, and 2 million Americans, respectively costing the healthcare system >$30 billion. An Autologous Homologous Skin Construct (AHSC) comprised of minimally polarized functional units (MPFUs) is created from a small piece of full-thickness skin. AHSC processing activates cutaneous endogenous regenerative cellular niches seen by increased RNA expression on stem cell associated gene array (Figure 1A). AHSC is not cultured *ex-vivo*, rather it is returned to the wound bed following processing where using the endogenous wound bed signaling and



153. Morphological and Functional Rescue of Retina in a Laser Induced RPE Injury Swine Model Using iPSC-Derived RPE Patch

Aaron C. Rising, Yichao Li, Juan Amaral, Haohua Qian, Irina Bunea, Kristi Creel, Arvydas Maminishkis, Sheldon Miller, Kapil Bharti

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Purpose: Geographic atrophy (GA) is an advanced stage of age-related macular degeneration (AMD) that results in severe visual impairment and blindness in the elderly. GA is caused by degeneration of photoreceptors, which is triggered by retinal pigmented epitheial (RPE) atrophy. The purpose of this study was to perform a pre-clinical Good Lab Practices (GLP) efficacy analysis of human induced pluripotent stem derived RPE (iRPE) patch. **Methods:** Yucatan pigs were used to test a degradable PLGA scaffold with iRPE cells (N=6), without cells (N=5) or iRPE-suspension without scaffold (N=6). To induce an AMD-like phenotype, laser injury of the RPE was performed in the pig visual streak 2 days prior to the surgical intervention. Multi-focal electroradiograph (mfERG) and optical coherence tomography (OCT) were used to assess longitudinal changes over the course of the project (baseline, 14 , 35 and 54 days). For the mfERG, 7 different waveform components (N1 and P1 amplitude, N1 and P1 width, N1P1, scalar product and area under the curve) were calculated and grouped together for linear mixed effect modeling (LME). Eyes were histologically processed to determine basic morphological changes in the ONL and RPE and other retinal features. **Results:** mfERG results indicate the PLGA-iRPE implant improve significantly over both the implant without cells and cell suspension (LME: vs implant no cells, p =0.03 and vs suspension p=0.05). 6 of the 7 waveform components of the PLGA-iRPE were higher than the implant without cells at the final time point. iRPE-suspension showed no differences to the impant without cells (p=0.18). OCT results showed the retina of the PLGA-iRPE had moderately better morphology as compared to those without cells. The retina in the iRPE-suspensions did not reattach, but it did in the two other groups. Histologically the areas above the PLGA-iRPE implant showed improved morphology. **Conclusions:** Our results show in the pre-clinical GLP study that a PLGA-iRPE scaffold can improve functionality in a laser induced AMD model as compared to both an empty scaffold and a iRPE-suspension. Additionally morphological improvements were evident from both the OCT and histological assessments in the PLGA-iRPE when compared to the other treatments. These promising results will provide the necessary pre-clinical work for a phase I IND-enabling study.

154. Platelet-Based Cell Therapy Ameliorates CNS Inflammation in Mice with Neuropathic Gaucher Disease by Reversing Leukocyte Recruitment

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Gaucher disease is caused by mutations in the *GBA1* gene encoding acid β-glucosidase (GCase). There has no effective treatment for neuronopathic Gaucher Disease (nGD) due to the difficulty of therapeutics to cross the blood-brain-barrier (BBB). Platelets are one of blood components that contain secretory macromolecules, including certain lysosomal enzymes, cytokines and chemokines that are involved in hemostasis, angiogenesis and inflammation. We have recently shown that weekly platelet transfusion achieved CNS benefits as indicated by significantly extended life-span, improved neurological function and reduced pro-inflammation and neuronal necroptosis in a nGD (4L;C) mouse model. However, GCase carried by normal platelets is not sufficient to provide such CNS benefits

as diseased BBB was not penetrable for 10kDa dextran (vs. GCase ~60kDa) and no significant increase of enzyme activity was detected in brain parenchyma after platelet transfusion. Interestingly, we detected 2.5-fold increase in the percentages of leukocyte-platelet aggregates (CD45+CD61+) in peripheral blood from 4L;C mice compared to normal controls, indicating a higher initiation rate of leukocyte transmigration in Gaucher mice. Indeed, we observed significantly higher percentages of brain leukocytes (CD45+, ~2-fold), CNS macrophage ($CD45^{high}CD11b+$, 2.1-fold) and T cell subpopulation (CD45+CD3+, 4.3-fold) in the diseased mice than those in normal controls by flow cytometry analysis. These data indicate elevated level of leukocyte recruitment and subsequent pro-inflammation in Gaucher brain. In line with high immune cell infiltration, we found that levels of certain chemokines such as *Ccl2*, *Rantes*, and cytokines such as *Il-1β*, *Il-6* and *Tnf-α* were dramatically elevated in diseased brain, and significantly reduced by platelet transfusion. Consequently, the severe astrogliosis in nGD brain was significantly ameliorated with normalized astrocyte morphology in certain brain regions including brain stem and thalamus of platelet-treated mice by immunohistochemistry staining of glial fibrillary acid protein (GFAP). In addition, the elevation of two adhesion molecules involved in endothelium activation (*Icam1* and *Vcam1)* found in nGD brain was significantly reduced after platelet transfusion. Interestingly, after quantifying cytokine/chemokine concentrations in circulation with Luminex assay, we found that elevated RANTES and KC1 protein levels observed in 4L;C mice were normalized in plasma from mice treated with platelet transfusion. Importantly, purified platelets from 4L;C mice demonstrated significantly higher levels of KC1, IL-6 and CCL2 than those from normal controls. Taken together, these results suggest that hypoactive platelets in Gaucher disease may partially contribute to CNS inflammation. Transfusion of healthy platelets reduced activation status of BBB-forming endothelium, as well as normalized pro-inflammation-related cytokine/chemokine profiles in blood, which may reverse high leukocyte recruitment from blood to CNS in diseased mice. These studies not only provide a proof-of-concept with platelet transfusion as a new treatment approach for neuropathic lysosomal storage disease, but also shed lights on the pathogenesis of CNS involvement in nGD.

155. Genome Edited Airway Stem Cells as a Durable Cell-Based Therapy to Treat Cystic Fibrosis

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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) Cl^- channel cause CF. CF results in chronic lung infections that cause lung failure and can only be treated by lung transplantation, which is limited by a short supply and poor prognosis after transplant. Although CFTR modulators have benefited many patients, they cannot treat about 10% of patients who produce no CFTR (e.g premature stop codons). The ideal therapy for CF is a gene therapy that corrects CF causing mutations in airway stem cells and generate a durable layer of corrected airway cells. I*n 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. In this study, we describe the development of autologous gene corrected primary Krt5+ airway basal stem cells as a cell therapy for CF. We use CRISPR/Cas9 and AAV to insert the cDNA of CFTR in exon 1 of primary Krt5+ basal stem cells (basal cells) obtained from CF and non-CF patients and address almost 100% of CF mutations. A major challenge in using CRISPR/cas9 for CF is the achievement of high frequency correction in physiologically relevant airway stem cells. We have achieved high frequency correction of the F508del mutation in primary airway basal cells (up to 60%) and restore CFTR function (up to 99%). In this study, we extend these techniques to further address the more challenging task of inserting the full-length CFTR cDNA. In preliminary studies, we have inserted CFTR cDNA with a selection marker (truncated CD19) in 5-10% primary Krt5+ airway basal stem cells and further purified the edited cells to obtain a 50-80% edited population. After differentiation in air-liquid interface, we observed restoration of CFTR mediated Cl- transport in the differentiated epithelial sheets using 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 upper and lower airways of animal models.

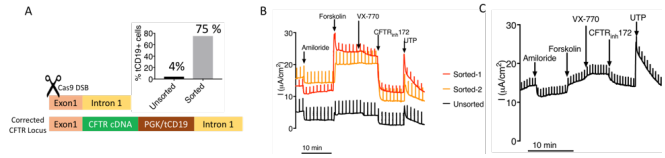

Figure 1: A. *In preliminary studies,* CFTR cDNA with tCD19 was inserted into exon 1 of primary F508del homozygous bronchial cells. Edited cells were sorted by flow cytometry (FACS) to obtain 75% corrected cells. B. FACS sorted cells displayed forskolin and $CFTR_{inh}$-172 responses similar to non-CF samples (not shown) in ussing chamber assays C. Uncorrected CF cells from the same donor as B did not exhibit the same responses to forskolin or $CFTR_{inh}$-172

156. Messenger RNA-Correction of MAGT1-Deficient T- and NK Cells from Patients with XMEN Disease

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'X-linked immunodeficiency with magnesium defect, Epstein-Barr virus (EBV) infection, and neoplasia' (XMEN) disease is a primary

immunodeficiency disease due to the deficiency of the magnesium transporter 1 encoded by the *MAGT1* gene. Mutations in *MAGT1* abrogates the expression of "Natural-Killer Group 2, member D" (NKG2D) receptor in natural killer (NK) and CD8 T cells which is essential for the recognition and the killing of virus-infected or transformed cells. Chronic elevated EBV viral load increases risks of secondary lymphoid expansion and recurrent lymphomas in XMEN patients. There is no specific treatment for XMEN disease and to date, hematopoietic stem cell transplant for these patients have resulted in high mortality that may be compounded by uncontrolled infections. We sought to develop an approach to functionally correct XMEN patient immune cells by *MAGT1* mRNA transfection. Our objective was to optimize a scalable, clinically applicable therapeutic approach for the purpose of treatment of severe infections in these immunodeficient patients. Peripheral blood mononuclear cells were collected by leukapheresis from a MAGT1-deficient XMEN patient (c.598del) and healthy donors (Protocol 94-I-073). We performed selective activation and expansion of T cells with CD3/CD28 beads in RPMI medium supplemented with fetal calf serum and 200 UI/mL rhIL2 for 1 week. NK cells were also bead-isolated and expanded for 10 days. XMEN cells were electroporated with MAGT1 mRNA 400 ug/mL and *in vitro* cultured for up to 28 days. We observed restored MAGT1 expression by western blot at 6h and 24h post-EP. To determine if corrected MAGT1 was functional, we performed serial FACS analysis for restoration of NKG2D expression in T (CD4+, CD8+) and NK cells following EP. We detected that >90% of CD8+ T cells and NK cells showed restored NKG2D expression at 48h that persisted in 40.5% and 8% of cells at day 7 and day 14 respectively. In addition, Mg flux was restored in CD8 T cells after EP. To analyze the cytotoxic function of mRNA-corrected XMEN cells, we co-cultured CD8 T and NK cells with L1210 (mouse lymphocytic leukemia cell line) and K562 (human chronic myelogenous leukemia cells) target cells respectively at several effector:target ratios and demonstrated improved cytotoxicity following MAGT1 mRNA transfection. To determine if mRNA-transfected PBMC maintain their restored function following cryopreservation, we cryopreserved transfected cells at 24h following EP, which were then thawed two weeks later for analysis. We confirmed that mRNA-corrected lymphocytes that had undergone cryopreservation exhibited the same NKG2D expression kinetic and restored cytotoxic function following thaw, an attractive feature for potential clinical utility of cryopreserved cell products. In summary, we provide the first demonstration of highly efficient correction of T and NK cells by mRNA transfection to restore protein expression and improve cytotoxicity. This transient approach is devoid of genotoxicities, an important safety feature. Retained functional correction following cryopreservation will allow repeat dosing of mRNA-corrected autologous cells for control of intractable infections. This mRNA-based therapy may offer a new therapeutic approach for XMEN patients.

157. Rescue of Autophagic Flux in a Mouse Model of Danon Disease by Hematopoietic Stem and Progenitor Cell Transplantation

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Purpose: Danon disease is a lethal familial cardiomyopathy associated with impaired autophagic flux due to mutations in the gene encoding lysosomal associated membrane protein type 2 (LAMP-2). Most Danon disease patients die from progressive heart failure in the second and third decade of life without heart transplantation. There are currently no specific treatments for this deadly disease. We investigated the potential for hematopoietic stem and progenitor cell (HSPC) transplantation to rescue autophagic flux in Danon disease. **Methods:** We isolated HSPCs from wild-type (WT) transgenic GFP mice, and systemically transplanted irradiated LAMP-2 knockout (KO) mice. A year post-transplantation, we investigated the fate and function of HSPCs by confocal and fluorescence microscopy, electron microscopy, and by western blotting. **Results:** We found that a single systemic transplantation of WT-HSPCs rescued autophagic flux in the LAMP-2 KO mouse model. Following transplantation, the HSPCs engrafted and then differentiated into CD68-positive macrophages in the heart of the transplanted LAMP-2 KO recipient mice. HSPC-transplanted LAMP-2 KO mouse hearts exhibited the presence of LAMP-2-positive lysosomes within cardiac myocytes, demonstrating the transfer of wild-type lysosomes from WT-HSPC-derived macrophages to LAMP-2 KO mouse cardiac myocytes in vivo. Moreover, HSPC-transplanted LAMP-2 KO mouse hearts showed rescue of autophagic flux and decreased accumulation of autophagic vacuoles, the major hallmarks of Danon disease, compared to non-transplanted LAMP-2 KO mice. **Conclusion:** Our results demonstrate the WT-HSPC-mediated rescue of autophagic flux in Danon disease mice and supports a new potential therapeutic strategy for treating Danon disease.

158. Cdx2 Cells for Cardiac Regeneration

Sangeetha Vadakke-Madathil[1], Gina Larocca[1], Koen Raedschelders[2], Jesse Yoon[3], Sarah Parker[2], Joseph Tripodi[3], Vesna Najfeld[3], Jennifer E. Van Eyk[2], Hina Chaudhry[1]

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The extremely limited regenerative potential of adult mammalian heart has prompted the need for novel cell-based therapies that can restore contractile function in heart disease. However, the lack of an appropriate cell-type that can differentiate to cardiomyocytes *in vivo* persists as an ultimate unmet need. Our prior study demonstrates that experimental myocardial injury in pregnant mice triggers the flux of fetal cells via the maternal circulation into the injured heart where they undergo differentiation into diverse cardiac cell fates. One subpopulation of placenta-derived cells, which was unique and highly prevalent in the maternal hearts, expressed Caudal-related homeobox2 (Cdx2); a

trophoblast stem cell marker involved in placental development. To understand the intriguing role of murine placental Cdx2 cells as a novel source for cardiac regeneration, we utilized a lineage-tracing strategy to label fetal-derived Cdx2 cells with enhanced green fluorescent protein (Cdx2-eGFP). Cdx2-eGFP cells from end-gestation placenta were characterized and assayed for cardiac differentiation *in vitro* and *in vivo* using a mouse model of myocardial infarction. We observed that Cdx2-eGFP cells differentiated into spontaneously beating cardiomyocytes and vascular cells *in vitro*, suggesting a multipotent nature compared to the extremely limited differentiation ability of Cdx2 negative cell population from the placenta. When administered via tail vein to infarcted wild-type male mice, Cdx2 cells selectively and robustly homed to the heart and differentiated to cardiomyocytes and blood vessels, significantly improving the contractility noted by magnetic resonance imaging. Proteomics and immune transcriptomics studies of Cdx2-eGFP cells compared to embryonic stem (ES) cells reveal that they appear to retain 'stem'-related functions of ES cells, but exhibit unique signatures supporting roles in homing and survival, with an ability to evade immune surveillance, critical for cell-based therapy. Advancing towards a translational role, we recently found that CDX2 expressing cells can be isolated from human term placenta. Our results herein may represent a paradigmatic shift in the way we approach early embryonic lineages and cell fate choices and will establish the translational potential of placental Cdx2 cells for cardiac repair.

Cellular Engineering; Novel Cell Types and Approaches

159. Fully-Closed System Cell Sorting of CD90⁺ Results in More Efficient Hematopoietic Stem/Progenitor Cell Transduction

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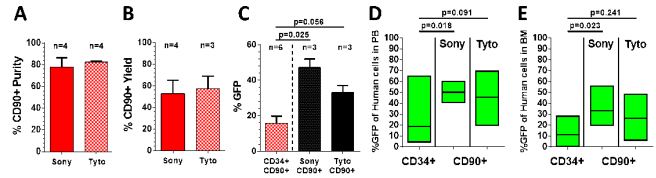
Hematopoietic stem cell (HSC) gene therapy is a viable treatment option for many hematological diseases and disorders. Most if not all approaches currently target CD34⁺ cell fractions, a heterogeneous mix of mostly progenitor cells and only very few long-term engrafting HSCs. We recently described a novel HSC-enriched CD34 subset (CD90⁺CD45RA⁻) that is exclusively responsible for rapid short-term recovery, robust long-term multilineage engraftment as well as entire reconstitution of the bone marrow stem cell compartment (Radtke et al. 2017, STM). Most importantly, we demonstrate that this CD34 subset reduces the number of target cells, modifying reagents and costs by more than 10-fold without compromising the long-term efficiency of gene-modification in our pre-clinical nonhuman primate (NHP) stem cell transplantation and gene therapy model. Here, we aimed to develop a clinical protocol to reliably purify and efficiently gene-modify human HSC-enriched CD90⁺ cell fractions. Large-scale enrichment of CD34⁺ cells from GCSF-mobilized leukapheresis products was initially performed on the Miltenyi CliniMACS Prodigy according to previously established protocols (Adair et al. 2017, Nat. Comm.). Yield, purity, and feasibility of CD90 sorting was then tested on two different systems comparing the jet-in-air sorter FX500 from Sony with the cartridge-based closed-system sorter MACSQuant Tyto from Miltenyi Biotech. Sorted CD90⁺ cells were transduced with a lentivirus encoding for GFP and the multilineage differentiation as well as engraftment potential tested using established *in vitro* assays and the NSG mouse xenograft model. Sort-purification of CD90⁺ cells was similarly efficient in purity and yield using either the FX500 or Tyto (**Figure A,B**). Both strategies reliably reduced the overall target cell count by 10 to 15-fold without impacting the cells viability and differentiation potential. Interestingly, the transduction efficiency of sort-purified CD90⁺ cells was significantly improved compared to bulk-transduced CD34⁺ cells and CD34 subsets (**Figure C**). Similarly, significantly higher levels of GFP⁺ expression in the peripheral blood and bone marrow were observed after transplantation of gene-modified CD90⁺ compared to bulk CD34⁺ cells in NSG mice (**Figure D,E**). In summary, purification of human CD90⁺CD34⁺ cells significantly increased the targeting efficiency of HSCs with multilineage engraftment potential. These findings should have important implications for current HSC gene therapy and gene editing protocols, allow more targeted gene modification and thus also reduce unwanted off target effects. Our approach further reduced the overall costs for gene modifying reagents, can be combined with a closed transduction system, increase the portability and ultimately make HSC gene therapy GMP-facility independent. **Figure: A)** Purity and **B)** yield of CD90⁺ cells after sort-purification on the FX500 (Sony) and Tyto (Miltenyi). **C)** Transduction efficiency of bulk CD34⁺ cells, CD34 subsets and sort-purified CD90⁺ cells. Frequency of GFP⁺ human CD45⁺ cells in the **D)** peripheral blood and **E)** bone marrow after transplantation of gene-modified bulk CD34⁺ or sort-purified CD90⁺ cells.



160. Analysis of T-Cell Vector Integration Sites for a Murine Gamma-Retroviral Vector Encoding the Anti-CD19 Chimeric Antigen Receptor Used in the Production of Axicabtagene Ciloleucel

Edmund C. Chang, Martha G. Sensel, John M. Rossi

Kite, A Gilead Company, Santa Monica, CA

Currently licensed engineered chimeric antigen receptor (CAR) T-cell products use replication-incompetent viral vectors to deliver the CAR transgene. Although the vector cannot replicate, vector integration sites (VIS) in such therapies are a topic of concern because of the theoretical risk of insertional mutagenesis, ie, dysregulated activation of oncogenic

genes at the site of vector integration in the host chromosome. To address this concern, VIS were assessed in CAR T cells manufactured from healthy donor T cells transduced with a replication-incompetent murine gamma retroviral vector engineered to express the anti-CD19 CAR construct used in the manufacture of axicabtagene ciloleucel. CAR T-cell products from 3 donors were analyzed. VIS were identified in the T-cell products by ArcherDX Anchored Multiplex PCR (AMP™) targeted sequencing using primers directed against the retroviral long terminal repeat (LTR). Amplicons spanning the integration junction were mapped to the human genome reference by the Basic Local Alignment Search Tool (BLAST®) and quantified by their unique molecular sequence tags. The analysis utilized 5 independent cell aliquots per donor product, each containing approximately 500,000 total T cells (ie, both transduced and nontransduced) from a culture containing approximately 2 x 10^8 total T cells. Results showed 6819, 10864, and 11578 unique VIS, respectively, for 3 donors analyzed, which paralleled the percent transduction for each donor (38.6%, 56.4%, and 57.2%, respectively). Across chromosomes, VIS were found preferentially near transcriptional start sites (TSS), which is consistent with VIS mapping for other murine gamma retroviral vectors reported in literature. Furthermore, we observed strong distance association between VIS and T-cell-related genes, as expected of transcriptionally active chromatin at the time of vector integration, consistent with previous reports in the literature. Across donors, only 8% to 9% of integrations mapped to exons and no specific VIS integrant was over-represented, indicating that no transduced T-cell clone acquired a growth advantage during manufacturing resulting from the integration event. The likelihood of detecting a given VIS in all 5 aliquots was < 1%, indicating no predominance of a single VIS; conversely, 64% of all VIS were found in only a single aliquot. These findings indicate that capturing the complete VIS diversity would require sequencing of an entire product, and that genotoxicity cannot be adequately assessed by integration site mapping alone. Taken together, these VIS characterization studies indicate that T-cell transformation due to murine gamma retroviral insertional mutagenesis would be an extremely rare event that likely requires the contribution of multiple additional factors beyond the integration site of the viral vector. Nevertheless, a risk monitoring approach is being used in clinical trials and the post-approval setting to characterize adverse events, such as secondary malignancies and presence of replication competent retrovirus (RCR), that have the potential to be related to genotoxicity.

161. Ultra-Short Manufacturing of Quiescent Chimeric Antigen Receptor T Cells for Adoptive Immunotherapy

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The recent success of immunotherapy using chimeric antigen receptor modified T cells (CART) in ALL highlights the potential of these cytotoxic "drugs" for cancer therapy. All current CART therapies rely on the prior activation, genetic modification and expansion of patient-derived T cells. 6-14 days of ex vivo culture are routinely used to generate large numbers of cells for adoptive transfer. Our group has shown that the commonly used CD3/CD28 stimulation, while a potent mitogenic signal for T cells, promotes progressive T cell effector differentiation over time in culture. This differentiation is associated with reduced engraftment and long-term persistence of T cells required for durable anti-tumor efficacy. Since cell division is not a prerequisite for lentiviral vector-mediated gene delivery, we hypothesized that lentiviral transduction of quiescent T cells without prior activation will enhance engraftment and persistence of CART cells that is associated with long-term leukemia control. Here, we describe a novel approach that can generate potent CD19-specific CART cells using lentiviral vectors that can be infused within 24 hours of T cell collection. In a murine xenograft model of acute lymphoblastic leukemia, CD19-specific CART cells generated using this ultrashort manufacturing process exhibit potent, dose-dependent anti-leukemic activity associated with persistent engraftment and durable anti-leukemic activity (Fig. 1). CART cells manufactured using our highly abbreviated process also exhibit a greater fraction of naïve-like and central memory T cells when compared with standard anti-CD3/CD28 microbead-based manufacturing. The ultrashort manufacturing approach described has the potential to markedly reduce the ex vivo manipulations required for CART cell manufacturing, providing a fast, simple and less costly method for achieving a potent CART cell immunotherapy.

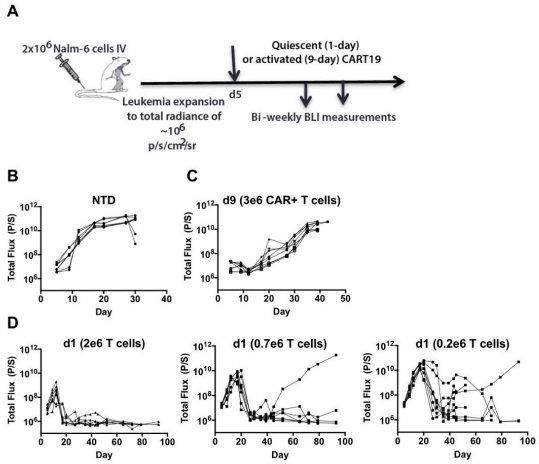

Fig. 1. (**A**) Schematic of the xenograft model and CART19 cell treatment in NSGs IV-injected with 2x10^6 NALM6 cells. Serial quantification of disease burden by bioluminescence imaging of (**B**) control cells (NTD), (**C**) day 9 CART19 (3x10^6 CAR+), and (**D**) day1 CART19 (2x10^6, 0.7x10^6, 0.2x10^6) show the disease progression.

162. Intraocular Cell Implant Technology to Treat Diseases of the Eye

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Treating diseases of the back of the eye remains a challenge due to not only a lack of existing therapies, but also due to the lack of localized, sustained delivery options. Take for example ciliary neurotrophic factor (CNTF), a member of the IL-6 cytokine family. CNTF has been shown to promote both photoreceptor and retinal ganglion cell survival in laboratory studies and in multiple animal models of disease. However, practical delivery of CNTF to the eye has been a significant challenge due to both the extremely short half-life of this

drug as well as the eye's blood-retina barrier. To address the need for sustained, long-term and localized drug delivery, Encapsulated Cell Therapy (ECT) has been developed. ECT is an intraocular implant utilizing a proprietary, immortalized, human, non-tumorigenic retinal pigment epithelial cell line, genetically engineered to constitutively produce therapeutic proteins for a minimum of two years or longer. This presentation will review the manufacturing, regulatory and pre-clinical development of ECT delivery of CNTF and discuss preliminary data from two completed Neurotech clinical trials. Data from both macular telangiectasias and glaucoma clinical trials suggest that sustained, intraocular delivery of CNTF may slow down or even arrest retinal disease progression and prevent vision loss in both diseases, providing a novel therapy for patients currently without effective treatment options.

163. CAR-T Cell Manufacturing with CliniMACS Prodigy

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The remarkable success of CAR-T cell therapy for the treatment of acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma, and its approval by FDA as a new therapeutic modality for these diseases are drastically increasing academic and industrial interest in using this approach for the treatment of other types of cancer. One integral component for the success of this promising therapy relies on reproducible CAR-T cell manufacturing. With more applications and growing numbers of patients, the reproducible manufacturing of high-quality clinical-grade CAR T cells with less demands on technical specialty has become an even greater challenge. The manufacturing process for CAR-T cells is complex; it includes T cell isolation, activation, transduction, expansion, formulation and cryopreservation. Over a decade ago we established a robust modular CAR-T production platform that has allowed us to successfully manufacture more than 350 CAR-T cell products supporting 12 CAR-T cell clinical trials and allowed us to obtain FDA breakthrough designation for our clinical trial in patients with ALL (NCT01044069). In order to further increase the throughput in CAR T cell production at our center and to enable reproducible manufacturing between multiple point of care for multi-center trials, we are evaluating the CliniMACS Prodigy system, which incorporates all steps of the manufacturing process from T cell selection to end of process CAR T cell formulation. To date we have performed a total of 10 Prodigy runs to generate T cells expressing CARs encoded by gammaretroviral vectors starting from either healthy donors or from cryopreserved apheresis products derived from patients with ALL, chronic lymphocytic leukemia (CLL) and prostate cancer. Among the 10 runs, 1 run was aborted due to temperature failure, 6 runs were conducted with a TCT program without spinoculation and 3 runs were conducted with TCT program with spinoculation. During the 10 to 14 days of production period, we were able to generate 1.9E9 to 5.6E9 total viable T cells, with a medium of 2.8E9. We found that spinoculation is essential to enable transduction of T cells with gammaretroviral vectors, with a medium transduction efficiency of 46.3% using TCT program with spinoculation (vs 2% without spinoculation). We further evaluated the *in vivo* anti-tumor activity of the CAR-T cells generated from Prodigy in comparison to the CAR-T cells generated with our current manufacturing platform using either PC3 tumor model for our PSMA specific CAR-T cells or NALM6 leukemia model for our CD19 specific CAR-T cells. In our PC3 model, tumor bearing NSG mice were treated with 150,000 or 300,000 CAR-T cells. PSMA-CAR-T cells from both platforms showed comparable and significant anti-tumor activity. In our NALM6 model, tumor bearing NSG mice were treated with either 50,000 or 200,000 CD19-CAR-T cells. Interestingly, CAR-T cells generated from the Prodigy platform showed superior anti-tumor activity than the CAR-T cells generated with our current manufacturing platform. Correspondingly, we found that CAR-T cells generated from Prodigy displayed a significant higher level of CCR7+, CD62L+ and CD27+ central memory phenotype. These encouraging preliminary results indicate that Prodigy could be an exciting alternative manufacturing platform for our future CAR-T cell clinical trials.

164. Highly Efficient and Selective CAR-Gene Transfer Using CD4- and CD8-Targeted Lentiviral Vectors

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Chimeric antigen receptor (CAR) modified T cells have revealed promising results in the treatment of cancer but still need to overcome various hurdles including a complicated manufacturing process. Receptor-targeted lentiviral vectors (LVs) delivering genes selectively to T cell subtypes may facilitate and improve CAR T cell generation but so far resulted in lower gene delivery rates than conventional LVs (VSV-LV). To overcome this limitation, we studied the effect of the transduction enhancer Vectofusin-1 on gene delivery to human T cells with CD4- and CD8-targeted LVs, respectively, encoding a second generation CD19-CAR in conjunction with ΔLNGFR as reporter. Vectofusin-1 significantly enhanced the gene delivery of CD4- and CD8-LVs, without a loss in target cell selectivity and killing capability of the generated CAR-T cells. Notably, delivery rates mediated by VSV-LVs were substantially reduced by Vectofusin-1. Interestingly, a transient off-target signal in samples treated with Vectofusin-1 was observed early after transduction. However, this effect was not caused by uptake and expression of the transgene in off-target cells but rather resulted from cell-bound LV particles having ΔLNGFR incorporated into their surface. The data demonstrate that gene transfer rates in the range to those mediated by VSV-LVs can be achieved with receptor-targeted LVs.

165. An Automated Manufacturing Solution for Patient Specific CAR-T Cell Therapies

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Autologous cell therapies, such as chimeric antigen receptor T-cell (CAR-T) immunotherapies, are becoming a promising treatment option for difficult diseases. However, as a greater number of clinical trials begin globally, the challenge of manufacturing autologous cell therapies remains significant. Closed and automated systems are a solution to produce cost effective and robust therapies to enable clinical success. To accomplish a successful CAR-T process translation from a manual process to an automated patient scale system, we utilized a platform that automates cell seeding, activation, transduction, real time process monitoring, feeding, washing and concentration, and harvesting. In these processes, 100 million peripheral blood mononuclear cells (PBMC) were activated with CD3/CD28 beads, transduced with HER-2 lentivirus, and expanded with a defined feeding strategy and IL-2 supplements. After harvest, cells were analyzed for cell yield, viability, transduction efficiency, and an array of cell phenotype, potency and functionality via FACS and killing assays. Automated runs using the above process yielded an average of 2 x 109 cells with a viability > 90% with a population of 73% CD4+ T cells and 20% CD8+ T cells. Harvested cells yielded approximately 80% NGFR+ cells with a higher detection of NGFR in the CD4+ fraction than in CD8+. Both CD4+ and CD8+ subsets also demonstrated T cell phenotype such as naïve T cells, T stem cell memory, T central memory, T effector memory, and T effector cells. Cell health was demonstrated by low levels of senescent T cells and anergic cells and low expression of the exhaustion marker, PD-1, and the apoptotic marker, Caspase-3. In addition, cells from automated runs showed the specific killing of NGFR+ tumor line and were correlated with high levels of effector cytokines: TNF-alpha (~34%) and IFN-gamma (20-25%) as compared to a manual control. In summary, this system is a viable solution to translate labor-intensive CAR-T manual process into a fully automated system for commercial success, thus allowing scalability, reduction of manufacturing cost, and better process control to yield high quality CAR-T cells.

AAV Vectors

166. Developing an Adeno-Associated Virus-Based Protein Display Platform for Modular Delivery of Gene Editing Machinery

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Adeno-associated virus (AAV) has emerged as a promising vehicle for the delivery of gene-editing machinery, primarily due to its nuclear entry ability and low immunogenicity. However, AAV's genomic carrying capacity (4.7kB) is too small to package the well-characterized *Streptococcus pyrogenes* Cas9 (spCas9) gene along with standard promoters. AAV can fully package smaller Cas9 variants, but no room is left to spare for repair DNA templates. Additionally, AAV-delivered transgenes can persist episomally in cells for several years, resulting in prolonged Cas9 expression and increased off-target editing. To resolve these concerns, we have developed an AAV-based delivery system displaying Cas9 protein on the surface of the capsid. This delivery approach will result in transient presence of Cas9 in target cells limiting off-target editing, and free up space within the AAV capsid for repair template delivery and inclusion of reporter genes. To attach Cas9 to the surface of AAV, we have functionalized the AAV capsid and Cas9 proteins with coiled-coil binding motifs. These motifs exhibit specific heterodimerization, allowing for AAV and Cas9 to be synthesized separately and avoiding the need for a combined purification protocol. We have established that both Cas9 and AAV retain their structural and functional properties with these coiled-coil motifs attached through gene editing activity and infectivity assays. Successful Cas9 attachment to AAV is demonstrated through column chromatography. The Cas9-AAV assemblies are currently being characterized to determine the copy number of Cas9 proteins bound per viral capsid. Cas9-AAV will be evaluated for infectivity and genome editing capacity *in vitro* and screened for immunogenicity through serum neutralization assays. Finally, this platform will be tested for *in vivo* genome editing in a mouse model. This coiled-coil display approach is modular and versatile in design, allowing for the display of multiple proteins in addition to Cas9. In the future, our engineered AAV-protein display platform may be used to create multifunctional viral therapeutics capable of targeting tissues and delivering a variety of functional protein domains.

167. Epidemiological Deep Sequencing of Signature-PCR Amplicons from AAV-Positive Tissues Reveals Significant Inter-Patient and Intra-Tissue Capsid Diversity

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We previously performed a large-scale molecular epidemiological study of adeno-associated virus (AAV) in a cancer patient population receiving care at West China Hospital (Chengdu, China; N=413) to determine whether AAV positivity correlated with tumor incident and progression. Our conclusions were that, 1) there is no significant difference in AAV detection in patient matched tumor and adjacent non-lesion tissues in incidence (~80%) and abundance, and 2) no specific AAV sequences were overrepresented in tumor samples as compared with normal tissues, suggesting that tumor tissues did not contain stable integration and clonal expansion of AAV genomes.

Rather, both tumor and non-tumor tissues showed a high degree of sequence variability. Notably, the latter findings were drawn from analysis of low-throughput TOPO vector screening of "signature" PCR amplicons that span a known hypervariable region within the AAV capsid sequence.

Intrigued by the diversity of sequences obtained in the signature region among tissues, we proposed to profile the signature-PCR amplicons by deep sequencing in order to define the diversity of AAV proviral sequences in our tissue cohort. Resected tumor samples reflecting six different carcinoma types (breast carcinoma, rectal cancer, hepatocellular carcinoma, gastric carcinoma, lung squamous cell carcinoma, and lung adenocarcinoma), diagnosed by radiological and histopathological examination were used in this study. In addition, patient-matched neoplastic masses and adjacent non-lesion tissues were specifically selected to further investigate any correlation between AAV infection and tumorigenesis. In total, amplicon libraries of 190 samples were analyzed by HiSeq sequencing, yielding approximately 2-3 million reads per library. Reads were then mapped to a reference list containing the signature regions of 14 contemporary AAV capsids (AAV1, AAV2, AAV2-3-hybrid, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh.8, AAVrh.10, AAVrh.39, and AAVrh.43). Majority of reads mapped to the AAV2-3-hybrid signature sequence, coinciding with our previous analysis. Interestingly, all tissue samples yielded reads mapping to at least five different serotypes, suggesting that individuals positive for AAV have undergone multiple infection cycles. Furthermore, we discovered intra-sample and inter-patient variation in reads, suggesting that AAV is indeed very diverse. Our new findings are in fact supported by our recent single molecule, real-time sequencing of proviral sequences that spanned the full AAV capsid ORF. In this previous study, we reported significant diversity within individual tissues. Here, the depth of coverage, although only over a short 250-bp fragment, is >200-fold over what was achieved previously. Importantly, we observed some sequence differences in the signature region between a few patient-matched tumor and non-tumor samples, but there was no consistent trend; again, providing evidence that AAV prevalence in our cohort of tumor tissues is a correlative, not a causative factor for cancer.

In summary, this body of ongoing work critically adds to the epidemiological profile of AAV in humans, highlights the evolvability of the virus, and provides important and informative findings that may be helpful for rAAV-based clinical studies, gene therapeutics, and its overall safety for human application.

168. Self-Deletion of AAV-CRISPR Reduces Off-Target Gene Editing In Vivo

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Significance/Background: Adeno-Associated Virus (AAV) has been a promising method for delivering CRISPR-based gene editing reagents *in vivo* with high efficiency and specificity. However, persistent expression can introduce off-target cutting. We previously reported a self-deleting AAV-CRISPR system that has been shown to reduce *S. Aureus* Cas9 (*Sau*Cas9) protein upwards of 79% in mouse liver. We have further investigated the potential benefits of this self-deleting in reducing off-target cutting *in vivo*. **Methods:** Potential gene candidates were initially screened for off-target sites *in silico* using COSMID. A gRNA targeting the mouse olfactory family of genes was selected based on the high frequency of 1 and 2 base mismatches. Off-target activity was initially screened *in vitro* using a firefly luciferase single-strand annealing assay (SSA), confirming off-target activity. AAV encoding S*au*Cas9 driven by a small liver specific promoter along with a gRNA targeting the *Olf207* gene were packaged into AAV8. Self-editing gRNAs were similarly packaged along with an EmGFP reporter. AAV were co-injected into 6-8 week male C57BL/6 mice and followed for four weeks. Off-target sites predicted *in silico* were analyzed for indel formation using next-generation sequencing. **Results:** Our SSA data revealed that the Olf1 gRNA targeting the *Olf207* gene had significant off-target cutting at multiple 1 and 2 mismatch gRNA sites. Although our previous work showed that "Self-5" had a much higher indel rate than "Self-1" based on the SSA, SaCas9 protein levels were reduced at a similar rate *in vivo*. Interestingly, Self-1 had a higher indel rate at the SauCas9 locus than Self-5 upon ICE analysis. Deep sequencing revealed 10 true off-target sites that had indels above background. Mice that were co-injected with either Self-1 or Self-5 all had significantly reduced off-target cutting, with Self-5 having the largest reduction at most sites. Although on-target editing of the *Olf207* gene was decreased, the on-target cutting of mice injected with a self-editing gRNA were significantly increased. **Summary/Conclusions:** We previously reported a self-deleting AAV-CRISPR system that is able to reduce Cas9 protein expression *in vivo,* while maintaining high endogenous on-target editing. We have shown that this system is capable of reducing off-target editing *in vivo* while also increasing the on-target specificity. This will allow for a more controlled

169. Characterization of Novel Human-Derived AAV2 Variants with Brain Transduction Profiles that are Comparable to AAV9

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Recombinant adeno-associated viruses (rAAVs) are promising vectors for human gene therapy, due to their excellent safety profiles and therapeutic transgene delivery efficacies. To date, AAV serotype-2 (AAV2) is the most commonly used and best characterized serotype in clinical studies. Nevertheless, the development of novel AAVs with improved transduction efficiencies, unique tissue specificities, and reduced host immunities, continues to be a major focus of the field.

Using single molecule, real-time (SMRT) sequencing of proviral capsid libraries from >800 human surgical specimens, a pool of new AAV capsids that are closely related to AAV2 were discovered. Among these AAV capsids, approximately 27% exhibited higher packaging efficiencies than prototypical AAV2. We screened a selection of these capsids for *in vivo* tissue tropism in mice by combining together multiple capsids that each package unique barcoded transgenes, and injected them as a pool into animals via three routes of administration: intravenous, intramuscular, and intracranial. Whole DNA and RNA were isolated from livers, muscles, or brains of treated animals, and detection of barcoded transgenes was performed by high-throughput sequencing to quantify both vector genomes and transcript abundances in tissues. In this way, we have identified a class of AAV2 variants that can confer brain transduction levels that rival that of AAV9 after intracranial injection. But unlike AAV9, they exhibit relatively low muscle and liver transduction profiles. In addition, these AAV2 variants also showed high transcript abundances (transgene RNA) per vector genomic copies (transgene DNA) in tissues. This indicates that the selected capsids are more efficient at intracellular trafficking, endosomal escape, and/or nuclear import. Preliminary characterization of these novel capsids reveals multiple function-defining residues located around the three-fold protrusion and five-fold interface that may confer their unique properties. Overall, we have identified a novel population of AAV2 variants from natural isolates with high brain transduction profiles, which will help expand the toolkit of vectorized AAVs and are potentially suitable for clinical utility. #Co-corresponding authors

170. The Effect of Glucocorticoids on Global Transduction Efficacy of Adeno-Associated Virus Vectors

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Adeno-associated virus (AAV) vector mediated gene delivery has been used in clinical trials for patients with eye diseases and hemophilia. AAV vectors have also been broadly studied in central nervous system (CNS) and muscular disorders. Most patients with CNS or muscular diseases need systemic administration of AAV vector for global targeting *via* high-dose of AAV vector administration. However, clinical studies have demonstrated that administration of high doses of AAV vectors may induce a higher risk of treatment related adverse effects, especially for the liver. Glucocorticoids are a well-known potent anti-inflammatory and immunosuppressive regimen, and have been proposed to prevent these side effects when systemic administration of AAV vectors is required in patients. In some clinic trials with AAV vectors, glucocorticoids (prednisolone and dexamethasone) have been used to prevent liver toxicity and rescue transgene expression by blocking the immune response. Furthermore, glucocorticoids have the ability to modulate vascular permeability during inflammation. To date, there are no studies about whether the administration of glucocorticoids impacts global transduction after systemic application of AAV vector. Therefore, it is important to elucidate whether glucocorticoids have an effect on the whole body transduction of AAV after systemic administration. In this study we first investigated the impact of dexamethasone on AAV9 global transduction after systemic injection. When a low-dose of AAV9 (2×10^{10}vg) was injected into the mice pre-treated with dexamethasone, the global transduction and vector bio-distribution were not significantly different in most of the tissues other than the liver and the heart when compared to the control mice. Furthermore, an enhanced liver transduction was only observed in the mice with a pre-injection of dexamethasone when we attempted to investigate whether the administration timing of the first dose of dexamethasone affected the AAV9 transduction. Amuch higher dose of AAV vector has been proposed for brain or whole body muscular transduction with a systemic administration of AAV vector at a minimum of 1×10^{13}vg of AAV/kg in clinic trails. Therefore, AAV9 vectors were used at a high dose (2×10^{11}vg/mouse) for systemic administration in mice. Both the transgene expression and the AAV vector genome copy number were significantly decreased in the majority of murine tissues, such as heart, kidney, and brain. When the mice was given dexamethasone treatment prior to AAV injection. Next, we applied dexamethasone at different time points for optimizing the strategy of AAV administration (2×10^{11} vg). No effect on the global transduction was observed when dexamethasone was administered 2-hours after AAV vector injection. The study on the kinetics of AAV virus clearance demonstrated that dexamethasone slowed down the clearance of AAV9 in blood after systemic application. The mechanism study showed that dexamethasone inhibited the enhancement of AAV9 vascular permeability mediated by the serum proteins. These findings indicate that dexamethasone is able to inhibit the vascular permeability of AAV and compromise the therapeutic effect after systemic administration of AAV vector. In conclusion, this study provides valuable information to design future clinical studies when glucocorticoids are needed to be compatible with the systemic administration of AAV vectors in patients with CNS and muscular diseases.

171. Classical Mouse Genetics Identifies a Locus on Chromosome 15 Responsible for the Exceptional CNS Gene Transfer Properties of AAV-PHP.B in C57BL/6J Mice

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The AAV gene therapy field has been evolving to the development of new and better capsids capable of crossing the blood-brain barrier (BBB). This ability will allow for systemic delivery that enhances the potential for therapeutic applications of gene therapy for central nervous system (CNS) diseases. Recent reports have shown that new capsids selected from a specific species don't necessarily transpose their characteristics to other species. The AAV9 derived capsid AAV-PHP.B was initially selected from Cre transgenic mice in a C57BL/6J genetic background and showed exceptional ability to transduce the mouse CNS. When compared to AAV9, AAV-PHP.B is ~60 times more efficient in transducing the mouse brain, but it's only 2-fold higher in

rats and shows comparable efficacy in marmosets and cats. Here we show that AAV-PHP.B also does not retain the same properties across mice with different genetic backgrounds. Unlike a previous report showing the CNS tropic properties of AAV-PHP.B are limited to C57BL/6J mice, we show that is capable of efficient CNS gene transfer in the inbred strains FVB/NJ, DBA/2J, 129S1/SvImJ, AKR/J and also the outbred strain CD-1. In addition, we show that AAV-PHP.B is ineffective for systemic CNS gene transfer in BALB/cJ, BALB/cByJ, A/J, NOD/ShiLtJ, NZO/HILtJ, C3H/HeJ or CBA/J mice. The apparent gene transfer deficit seems limited to CNS as liver transduction is observed in all tested mouse strains, albeit at different levels. The variability in CNS tropism across mouse strains is a unique opportunity to use the power of classical genetics to uncover the molecular mechanisms AAV-PHP.B engages to transduce CNS at high efficiency. For this purpose, we used CB6F1/J mice backcrossed to BALB/cJ for QTL mapping, which presented some animals with about 50% transduction efficiency and others with zero, identified a 6 Mb region in chromosome 15 with a LOD score ~20 with SNPs in the coding region of 9 different genes. Studies in mice from the Collaborative Cross strains as well as transcriptomics analysis of CNS are ongoing to identify the gene or genes involved in the mechanism used by AAV-PHP.B to cross the BBB.

172. AAV-Mediated Protein Trans-Splicing in the Retina

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Retinal gene therapy with AAV vectors is safe and effective, yet it is limited by AAV cargo capacity of about 5 kb. To overcome this limitation we explored the use of intein-mediated protein *trans*-splicing to reconstitute large proteins in the retina. Inteins work as independent peptides fused to C- and N-termini of two host proteins (i.e. the two halves of a large protein) and mediate their association in a multistep autocatalytic process.To test protein *trans*-splicing in the retina, we generated two AAV vectors separately encoding each of the two halves of either the reporter EGFP protein or large therapeutic proteins flanked by split-inteins. These include ABCA4 and CEP290, respectively defective in Stargardt disease (STGD1) and Leber congenital amaurosis 10 (LCA10), two severe and common inherited blinding diseases. We identified in each protein optimal splitting points for the generation of AAV-intein constructs which take into account both amino acid residue requirements for *trans*-splicing to occur, as well as the preservation of the native protein domains. Upon co-administration of both AAV split-intein vectors, full-length proteins were reconstituted in the mouse and pig retina as well as in human retinal organoids derived from induced pluripotent stem cells. Importantly, the levels of large protein reconstitution achieved reduced lipofuscin accumulation and retinal degeneration in the mouse models of STGD1 and LCA10, respectively. Our data support the use of intein-mediated protein *trans*-splicing in combination with AAV subretinal delivery for gene therapy of inherited blindness due to mutations in large genes.

173. Effective Transduction of Rhesus Macaque Lung and Human Enteroids with AAV1-CFTR

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Several gene therapy human and non-human primate studies have shown that AAV delivery of CFTR is safe. A challenge is to deliver enough CFTR to be therapeutic. Given that the turnover of airway cells may make gene transfer with recombinant AAV-based vectors transient, repeat dosing of AAV1 will be required. The goal is to assess whether repeat dosing of AAV1CFTR leads to widespread gene transfer and CFTR expression. To test the ability of AAV1 to transduce human cells, normal human enteroids were grown on permeable supports, infected with AAV1 containing del264- or del27-264-CFTR and Isc measured. These versions of CFTR increased endogenous CFTR via transcomplementation. Significant increases in forskolin-activated currents were detected indicating that transduction occurred after apical and/or basolateral exposure. To test whether repeat dosing of AAV1 is effective, we sprayed into the airways of 4 healthy Rhesus monkeys, 2 doses of 10^{13} vg of AAV1Δ27-264-CFTR at 0 and 30 days, respectively, followed by a single dose of 10^{13} vg of AAV1GFP at day 60. Monkeys were sacrificed at day 90. There were no adverse events related to the study indicating that triple dosing with AAV1 vectors is safe. Sera from treated animals were analyzed for anti-AAV1 neutralizing antibodies. There was a significant rise in the titer after the first dose in all 4 animals as the animals transitioned from a preimmune state to post-vector exposure. An elevated anti-AAV1 titer was established in all treated monkeys 30 days after the first dose and increased further 30 days after the second dose. By the third dose all four monkeys had escalating titers. A positive T cell response was noted after the second dose in one animal and after the third dose in all the animals. Thus, AAV1 antibodies were induced in sera upon reexposures to vector but no adverse events occurred. Samples were taken from 17 different lung regions and the vector genomes measured in lung using vector-specific real-time PCR. rAAV1CFTR and rAAV1GFP vector DNA was detectable in all lung sections and animals respectively. The lung cells were estimated to contain between 1 and 60 particles per cell of AAV1CFTR. Interestingly, vector genomes were detectable in the liver with a tendency to detect more AAV1GFP containing genomes compared to those containing CFTR. Despite the presence of AAV1,

there was no liver toxicity detected in any of the animals. GFP protein expression detected by western blot was detected in the lungs of all animals. CFTR protein expression detected by western blot was significant higher compared to an uninfected control. All lung sections assessed by confocal microscopy showed increased CFTR staining compared with uninfected monkey and were positive for GFP staining indicating widespread gene transduction by AAV1GFP. Our results show that the AAV1 serotype transduces both human and monkey airway cells. Given that significant numbers of vector genomes from AAV1CFTR virus were present in monkeys four months after the first instillation coupled with CFTR and GFP transduction suggests that repeat dosing of AAV1 based vectors is feasible.

174. Assessing Purity and Structures of AAV Vector Genomes by High Performance Size Exclusion Chromatography

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Product purity is a critical quality attribute for therapeutic biologics including gene therapy products with the potential to impact safety and efficacy, thus assurance of control, including release testing, is critical. The AAV vector genome is delivered to the cell nucleus in gene therapy and can be persistent for a long period, much longer than that for capsid proteins. Thus, it is desirable to assess the vector genome purity for AAV products. The relative purity of intact AAV that quantify the empty and full capsid ratio can be determined by Analytical Ultracentrifugation (AUC), cryo-electron microscopy, and ion-exchange chromatography. In addition, the presence of vectors with fragmented genomes and non-transgene-related DNA contaminants, often referred to as partially-filled capsids, can be resolved by AUC. Capsid protein purity can be determined by SDS-PAGE or SDS-CGE. While a few studies have been published to analyze AAV genome by capillary and gel electrophoresis, no consensus method has been established for AAV genome purity. Here, we report our work to assess AAV vector genome purity and structure with high sensitivity and high reproducibility by Size Exclusion Chromatography (SEC). AAV vector genome is a single stranded DNA (ssDNA). Once released from AAV vectors, the single-stranded DNA may anneal spontaneously to form double-stranded DNA (dsDNA dependent on the denaturing conditions. At low temperature where denaturation is incomplete, ssDNA with different secondary structures can be detected by SEC in addition to dsDNA. Under a complete denaturing condition at high temperature, all ssDNA was converted to dsDNA. However, we found there were certain AAV populations isolated from anion-exchange chromatography that displayed an increased resistance to denaturation, suggesting these AAV vectors may contain ssDNA with a compact structure. Genome content of these AAV vectors determined by ddPCR was lower than that determined by spectrophotometry or SEC quantification, indicating the presence of compact DNA structures and the need for further denaturation for improved PCR efficiency. Upon complete denaturation, all released vector genome forms dsDNA, which can be separated by SEC from impurities including host cell residual DNA, plasmid DNA, and genome fragments. Analysis of the released DNA from AAV by SEC provides a quantitative assessment of the size purity of the AAV vector genome. The percentage of DNA fragments determined by this method correlates well with the percentage of partially-filled capsids determined by AUC. AUC analysis is time-consuming, requiring advanced techniques and knowledge for system operation and data analysis, and often consuming a large sample size. In contrast, purity analysis by SEC is fast, reproducible, highly sensitive with improved limit of quantitation (LOQ), and can be automated to a certain degree for high throughput analysis. The SEC method developed here has been used for quick assessment of the partially-filled capsids to support product development and process optimization, and as an orthogonal method for AAV product characterization. In addition, vector genome titers can be determined using the peak area detected by this SEC method with UV absorbance at 260 nm.

175. Widespread Axonal Transport of a Modified AAV2 Capsid in the Non Human Primate Central Nervous System

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Background: Axonal transport of adeno-associated virus (AAV) is an essential feature of neurological gene therapy. We previously reported that a modified AAV serotype 2, incapable of binding to its receptor heparan sulfate proteoglycan (AAV2-HBKO), distributes extensively after thalamic injection in the non-human primate (NHP). **Objective:** The goal of this study was to evaluate the efficiency of axonal transport of this AAV2 variant encoding green fluorescent protein (GFP) after putaminal injection in the NHP. **Results:** Putaminal infusions of AAV2-HBKO resulted in widespread axonal transport to the cortex, deep cerebellar nuclei, and several subcortical regions. The presence of GFP-positive fibers in the substantia nigra pars reticulata, a region that receives projections from putamen, indicated anterograde transport of AAV2-HBKO. GFP-positive cell bodies in the substantia nigra pars compacta indicated retrograde transport of AAV2-HBKO. A 10-fold lower dose of AAV2-HBKO was infused into in the left hemisphere of a separate subgroup of animals compared to the right hemisphere. Both GFP immunoreactivity and bovine growth hormone (BGH) mRNA levels were higher in the right hemisphere than the left hemisphere. GFP immunoreactivity revealed a titer-dependent effect on axonal transport directionality where the high dose led to enhanced transgene expression in retrograde-linked rather than anterograde-linked structures. **Conclusion:** Our results demonstrate AAV2-HBKO undergoes extensive, dose-dependent bidirectional axonal transport, and support its use in treatment of neurological diseases with substantial cortico-striatal pathology.

176. In Vivo Characterization of a Novel Adeno-Associated Virus Natural Isolate that Targets Brain Endothelial Cells

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Adeno-associated viruses (AAVs) are among the most effective vector candidates for gene therapy due to their low immunogenicity and non-pathogenic nature. However, despite allowing for efficient gene transfer, the AAV vectors currently used in the clinic can be hindered by preexisting immunity to the virus and restricted tissue tropism. To find vectors that overcome these issues, we explored the genetic variation of AAV in natural hosts by using AAV single genome amplification to discover five novel AAV sequences from clades A, D, E, and the primate outgroup. We assessed the biological properties of the five natural isolate-derived AAV vectors in mice after intravenous (IV) and intracerebroventricular (ICV) delivery. We assayed expression of the reporter transgene (enhanced green fluorescent protein) and vector biodistribution in heart, brain, spinal cord, liver, and skeletal muscle of C57BL6 mice. Our results show both clade-specific and variable transduction patterns of the new variants compared to their respective prototypical clade member controls. Fluorescence microscopy of tissues showed notable transduction of heart tissue by the novel clade A variant after IV delivery; after ICV delivery, this variant showed higher transduction levels in rostral regions of the brain than AAV6.2. The novel clade D variants showed lower levels of transduction than AAV7 in all tissues sampled. AAV8 and our novel clade E variant showed comparable levels of robust transduction in all tissues assayed for both delivery methods. Interestingly, the novel primate outgroup clade vector showed high levels of transduction in CD31-positive brain vascular endothelial cells after IV delivery. The potent transduction of this cell type has the potential for the treatment of a variety of neurovascular diseases.

177. Development of AAV3-GRE Vectors for their Potential Use in Gene Therapy of Hemophilia

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Various AAV serotype vectors and their variants have been used, or are currently being used, for gene therapy of hemophilia. AAV2 vectors yielded therapeutic levels of hF.IX, but the expression was short-lived due to capsid-specific CTL response. AAV8 vectors were successful, but hF.IX levels declined over time. A second trial with AAV8 vectors yielded inconsistent results, AAVrh10 vectors failed to lead to expression of therapeutic levels of h.FIX. AAV5 vectors yielded modest levels of expression of hF.IX, but high vector doses were required. Although the use of a capsid-modified AAV8 vector led to therapeutic levels of hF.IX at a lower dose, it is clear that AAV8 vectors, although highly efficient in transducing *mouse* liver, are not optimal for *human* liver. We have reported that AAV3 vectors possess a natural tropism for human hepatocytes, and thus, may prove to be more efficacious in gene therapy of hemophilia. In addition to the development of next generation of capsid-modified AAV3 vectors, our recent efforts have focused on improving the transduction efficiency through vector genome-modifications, thereby further reducing the vector dose, and mitigating the risk of host immune response [https://www.ncbi.nlm.nih.gov/ pubmed/27431826]. We have reported that the AAV2 inverted terminal repeats (ITRs) contain a 20-nt sequence, termed the D-sequence, which shares partial homology to glucocorticoid response element (GRE) [https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(16)32820-9]. We have also reported that GR enhances transgene expression from AAV2 ITR-GRE vectors [https://www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(16)38147-3]. Since AAV3, which has lower prevalence of neutralizing antibodies in the general population, has a D-sequence which differs from AAV2 in the last 10 nts, and since bioinformatic analysis identified a slightly offset partial-GRE motif, which is conserved between the two serotypes, we investigated whether GR also plays a role in augmenting transgene expression from AAV3 vectors. To this end, human Huh7 cells were transduced with scAAV2 or scAAV3 vectors expressing the EGFP reporter gene, each with their native ITRs and D-sequences, under identical conditions. Transgene expression was compared in the absence or presence of Dexamethasone (DexM) or Prednisolone (PredL). At 10 μM final conc, ~10-fold increase with DexM, and ~13-fold increase with PredL was observed in transgene expression from scAAV2 vectors. Similarly, ~4-fold increase with DexM, and ~5.0-fold increase with Pred was also also observed from scAAV3 vectors. Cyanine5-labeled oligonucleotides of AAV2 and AAV3 D-sequences were compared qualitatively for GR binding affinity by electrophoretic mobility-shift assays (EMSA) using partially purified GR. The results showed competitive binding of GR with both AAV2 and AAV3 D-sequences. These results corroborated that despite a slightly offset, the GRE motif provided sufficient binding to GR in AAV3 ITRs to allow it to be positively regulated by glucocorticoids, suggesting that the GR pathway can be exploited to improve transduction efficiency of AAV3 vectors as well. Genome-modified AAV3 ITR-GRE vectors expressing either hF.IX or hF.VIII are currently being generated. By combining these with capsid-modified next generation AAV3 vectors, we aim to utilize the GR-GRE interaction to achieve therapeutic levels of hF.IX and hF.VIII for gene therapy of hemophilia B and hemophilia A, respectively.

178. Optimized AAV6 Vectors Stimulate Cellular and Humoral Immune Responses Against a Self-Antigen

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Adeno-associated virus (AAV) vectors have been widely used for clinical-stage gene therapy for diseases ranging from Leber's congenital amaurosis to hemophilia B. More recently the use of AAV for cancer vaccines has been studied; however, much of this research is performed using a foreign, strong immunogen, namely chicken ovalbumin (OVA). Here we focused on the ability to use an AAV vaccination against

melanoma using the endogenous melanocyte protein—tyrosinase (Tyr). We have shown that using optimized AAV6 (AAV6-Opt) vectors expressing amino-terminal (TyrI) or the carboxy-terminal (TyrII) halves of the gene can be used in relatively small AAV titers to stimulate antigen specific cell-mediated and humoral responses. Furthermore, we have shown that activated T-lymphocytes from immunized animals sufficiently kill B16F10 melanoma cells using an in vitro killing assay. **Methods:** Vaccination was carried-out through one intramuscular (i.m.) injection in the tibialis anterior of C57BL/6 mice. A Tyr-specific cellular immune response was estimated using IFNγ and TNFα production measured by ELISpot. The humoral response was determined by the presence of antigen specific antibodies in mouse serum measured by indirect ELISA or Immunoblotting. Killing activity of tyrosinase-specific T-cells against B16F10 was measured on a Live Cell Imager (Essen Biosciences) IncuCyte S3. To determine the role of dendritic cells (DCs) in AAV-based vaccination, AAV6-Opt encoding GFP was used to show the direct targeting and expression of antigens in mouse DCs. Vectors were injected i.m into C57BL/6 mice. Three days post-infection, inguinal draining lymph nodes (LN) were harvested and DCs were isolated using FACS sorting for CD11c+/MHCII+ cells. The presence of GFP expression in LN DCs was analyzed by wide-field fluorescence microscopy. **Results:** From these experiments, it was determined that AAV6-Opt-Tyr vaccinations produce more robust cellular immune responses when compared to AAV6-WT-Tyr vaccinations. The vaccination with AAV6-Opt leads to significantly higher IFNγ and TNFα-producing splenocytes than the WT vector. It also results in a strong humoral response that increases antibody production through six weeks post-infection. We confirmed that AAV vectors directly target DCs and initiate expression of the transgene after i.m. injection. Direct AAV infection and consequent expression of the transgene in DCs could be further evidence that AAV can induce an MHC class I driven immune response both directly and by cross-presentation. Our data show our optimized AAV6 vector can be used to elicit a DC-based cell-mediated and humoral immune response against an endogenous antigen. Our future directions will include targeting a combination of melanoma antigens, as well as using immune checkpoint blockade, to show a synergistic effect and improved tumor killing in vivo.

179. Functional and Histological Improvements Comparing 4 Micro-Dystrophin Constructs in the mdx Mouse Model of DMD

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Introduction: Duchenne muscular dystrophy (DMD) is the most common severe childhood form of muscular dystrophy. More than 2000 mutations of the *DMD* gene are responsible for progressive loss of muscle strength, loss of ambulation, and ultimately respiratory and cardiac failure. In order to evaluate the 4 different micro-dystrophin constructs, a head-to-head comparison was evaluated to compare functional and histological benefit across constructs. The constructs include the use of the MHCK7 promoter in comparison to a less active MCK promoter with the same micro-dystrophin transgene that contains the N-terminus and spectrin repeats R1, R2, and R3, respectively (AAVrh74.MHCK7.micro-dystrophin; AAVrh74.MCK.micro-dystrophin), a mini-dystrophin construct that contains the nNOS binding site (AAVrh74.DV.minidystrophin), and a micro-dystrophin containing the C-terminus (AAVrh74.MHCK7.microdys.Cterm). The study to be presented demonstrates efficacy following intramuscular delivery of 4 unique AAVrh74.micro-dystrophin constructs in the mdx mouse model of DMD. **Methods:** We designed 4 constructs of AAVrh74 vector containing human micro-dystrophin transgene driven by a muscle-cardiac specific promoter, MHCK7 or less active muscle specific promoter, MCK. To test the efficacy of the 4 constructs of AAVrh74.micro-dystrophin, we evaluated both functional and histological benefit 4 weeks post vector delivery. **Results:** The results of this study demonstrate that vector delivery of the AAVrh74.MHCK7.micro-dystrophin construct is the most advantageous in normalizing histologic and functional outcome measures in the mdx mouse model, among these constructs. Specific force output increased in the tibialis anterior muscle significantly compared to the other 3 constructs and there was no difference from wild-type levels. Additionally, muscle environment was normalized, which was demonstrated by reductions in centralized nucleation and normalized myofiber diameters. Finally, transgene expression through immunofluorescent staining and Western blot was significantly increased in comparison with the other constructs, indicating the functional and histological advantages of the AAVrh74.MHCK7.micro-dystrophin construct. **Conclusions:** The findings in this preclinical study provided proof-of-principle for safety and efficacy of systemic delivery of AAVrh74.MHCK7.micro-dystrophin in a dose-escalation study in the mdx mouse model for DMD.

180. AAV2.7m8 is a Powerful Viral Vector for Inner Ear Gene Therapy

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Background: Adeno-associated viruses (AAVs) are commonly used for inner ear gene delivery. While conventional AAVs are capable of transducing inner hair cells (IHC) in the cochlea to varying degrees, outer hair cells (OHC) and supporting cells are transduced less efficiently. In this study, we examined the transduction patterns of two synthetic AAVs (AAV2.7m8 and AAV8BP2) in the neonatal mouse inner ear. **Methods:** Neonatal (P0-P5) CBA/J mice were used in this study. Synthetic AAV-GFPs were injected into mouse inner ear using the posterior semicircular canal approach. Immunohistochemistry was used to assess the infection efficiency. Auditory function was assessed by auditory brainstem responses (ABR). **Results:** AAV2.7m8 transduced both IHCs and OHCs with very high efficiency. AAV8BP2 transduced the IHCs with high efficiency, but the transduction efficiency of OHCs was lower. AAV2.7m8 also transduced a subset of supporting cells (inner pillar cells and inner phalangeal cells) with high efficiency. Mice that underwent AAV2.7m8 injections had similar ABR thresholds compared to non-injected controls. **Conclusions**:

AAV2.7m8 transduced both IHCs and OHCs with high efficacy. In addition, it transduced inner pillar cells and inner phalangeal cells with high efficacy. Our results suggest that AAV2.7m8 is an excellent viral vector for inner ear gene delivery.

181. Modulation of Glycosylation Status of Adeno-Associated Virus (AAV) Vectors Improves its Hepatic and Ocular Gene Transfer In Vivo

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AAV mediated gene transfer has been successfully translated for gene therapy of hemophilia B and Leber congenital amaurosis. For this mode of therapy to be widely embraced in the presence of multiple transduction and immune challenges in humans, it is crucial to develop high transduction and permeating vectors to enhance gene delivery into the liver or the eye. We reasoned that a thorough study and modification of the capsid regions that trigger vector recognition and loss of function is likely to improve gene transfer. Given the paucity of data on post translational modifications in AAV2 capsids other than ubiquitination, we hypothesized that a study of glycosylation motifs in AAV2 capsid is likely to be rewarding as its role in several viruses such as HIV and Influenza towards improving their infectivity or their adaptation to host immunity is known. Towards this, we first characterized the glycosylation status of AAV2 capsid by MALDI-TOF analysis which revealed the presence of a low level of glycosylation. This was further confirmed by enzymatic de-glycosylation and immunoblotting analysis. We then estimated the regulation of AAV transduction and its packaging in conditions of altered cellular glycosylation. We observed that the transduction potential of scAAV2-EGFP vectors increased by 74% in HeLa cells with glycosylation inhibitors, but decreased (up to 1-fold) its vector yield in AAV-293 packaging cells under similar conditions. These studies presented preliminary evidence for the involvement of glycosylation during AAV2 life cycle. In the next set of studies, we postulated that modulation of glycosylation sites within or around specific epitope regions in the capsid will lead to the generation of improved vectors for gene delivery. The VP1 region of AAV2 capsid was further analysed to predict the T-cell and B-cell immunogenic epitopes as well sites of potential glycosylation. Twenty-four glycosylation sites were selected and further mutagenesis was performed with an aim to create or disrupt a potential glycosylation site within the target sites. AAV2-EGFP vectors containing the wild-type (WT) and each one of the 24 glycoengineered mutants were then evaluated for their transduction efficacy in multiple cell lines (HeLa, Huh7, ARPE19) *in vitro*. We observed a 1.3 to 2.5-fold increase in EGFP expression with at least three glycoengineered mutant vectors in all the three cell types. Further, we analysed the *in vivo*therapeutic potential of the two best glycoengineered vectors by hepatic gene therapy in a murine model of haemophilia B (B6.129P2-*F9*^{tm1Dws}/J) or by ocular gene transfer in C57BL6/J mice. During hepatic gene transfer of the novel vectors encoding human coagulation factor (F) IX in haemophilia B mice, we observed a 2-fold increase in FIX levels with the glycoengineered mutant vectors, 4-12 weeks after gene therapy. Our experiments also demonstrated no significant difference in T and B cell activation between WT-AAV2 treated and glycoengineered mutant treated hemophilia B mice. These findings suggest that it should be possible to obtain a similar level of therapeutic hFIX expression with a significantly lower dose of AAV2 glycoengineered vector than AAV2-WT vectors.Subsequently, retinal gene transfer of the top three glycoengineered vectors through intravitreal administration,demonstrated an enhanced EGFP expression (~2 to 4.4-fold at 4-8 weeks) for two of the glycoengineered vectors. Further, these vectors also demonstrated increased permeation across retina at significantly lower doses (3x10e8 vector genomes). Taken together, our studies demonstrate the role of glycosylation during AAV mediated gene transfer and highlights the improved translational potential of the glycoengineered AAV2 vectors for hepatic and ocular gene therapy.

182. Decoding AAV through In Silico Design and Multi-Parametric Screening

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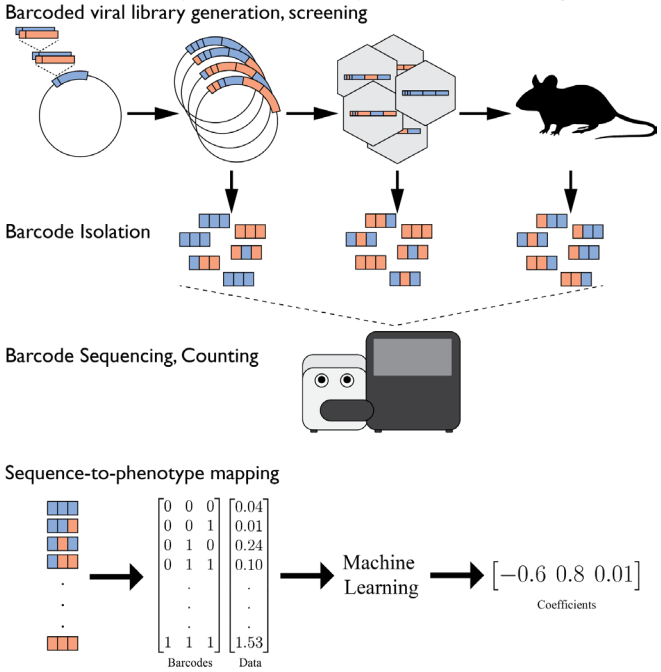
The Adeno-associated virus (AAV) capsid is a primary driver of the safety and efficacy of an AAV-based gene therapy drug. Biomining and mutational studies of the AAV capsid have highlighted that minor variation of the capsid's amino acid sequence can impact translationally relevant phenotypes such as manufacturability, tissue targeting, and host response. Researchers have leveraged this property of the AAV capsid to discover new and improved vectors through innovative approaches such as directed evolution and more recently through barcode-based sequencing of mutational libraries of AAV capsids. However, these studies have remained largely descriptive, in part due to the many challenges inherent to linking capsid sequence diversity with phenotypic diversity. Among these challenges is the limitation that the AAV capsid's structure imposes on the sequence space that a researcher can explore; capsid mutants within these libraries must fold and assemble correctly in order to be informative to many therapeutically relevant questions. Here, we aimed to overcome several of these technical constraints to build (a) DNA/RNA barcoded libraries of functionally assembling AAV capsids (b) libraries which are diverse with respect to several viral functions (e.g. viral assembly, tissue targeting, transcriptional activity), and (c) quantitative methods to characterize each capsid within a library with respect to each of these functions. To accomplish this, we revisited our earlier work using ancestral sequence reconstruction to build libraries of functionally diverse AAV capsids. This entailed developing a new combinatorial assembly method called CombiAAV to generate these libraries as well as to associate each mutant with a single, unique DNA/RNA barcode compatible with Illumina sequencing. As a proof of concept, a library of 2048 different AAV capsids plus eight well-characterized AAV controls were evaluated across a variety of phenotypes through a barcode-based sequencing approach which we call AAVSeq. Within the library we observed significant functional differences with respect to capsid

assembly and packaging, gene delivery to murine tissues (including liver, heart, skeletal muscle, and hippocampus), and the transcriptional activity of the library within those tissues. Moreover, the combinatorial nature of the library enabled discovery of novel sequence determinants governing the observed functional differences with respect to vector yield and tissue tropism. By leveraging the suite of methods and tools presented here, researchers can build large functional datasets and uncover novel capsid mutations which affect these capsid functions. We believe that these techniques will further inform capsid selection for clinical use and may provide a pathway toward rational design of AAV.



183. Computational Design of AAV Capsids Using Machine Learning Models Trained on Deep Mutational Libraries

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The complexity of AAV biology and the difficulty of predicting the functional effects of mutations have hindered efforts to engineer improved capsids for therapeutic applications. In many contexts, deep learning models have bypassed the need for detailed mechanistic understanding, instead learning patterns directly from data that cannot be detected by human intelligence but that are highly predictive of functional outcome. Can this approach be applied to AAV? Our previous work showed that experimental single site saturation mutagenesis data can be used to design highly mutagenized variants that produce capsids viable for packaging of DNA genomes and delivery to target tissues. Here, we ask whether adding limited random mutagenesis data to the corpus used to train deep learning models can provide additional lift over (i) our previous design strategy, and (ii) logistic regression models that have access to the same data. Post-hoc analysis of our existing set of 80,000 designed variants suggests the deep learning models increase performance, lifting the ROC curve. To prospectively test these approaches, we built convolutional and recurrent neural networks (NNs) together with baseline logistic regression models. We scored sequences from a large sweep of the design space, and selected sequences for synthesis and evaluation. Furthermore, we developed a local search strategy to follow model gradients and find sequences with even higher scores. In total, we experimentally tested 244,000 capsid variants for assembly and genome packaging. We identified viable sequences with 29 differences from wild type within a single contiguous 28 amino acid stretch of the AAV2 capsid protein. Our local search significantly improved the proportion of viable sequence variants found. This work highlights the potential of deep learning approaches that leverage limited amounts of data to search a broad space of putative AAV capsid sequences. These emerging technologies will diversify the set of functional AAV capsid designs that are available, enabling the creation of synthetic AAV capsids that are highly distinct in sequence from natural variants, and are enhanced for delivery, safety and manufacturability.

184. Determination of AAV Genome Content and Capsid Content by SEC

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AAV genome content can be determined by either polymerase chain reaction (PCR) or by optical density (OD). The latter method has a higher throughput and can also determine the capsid content and assess empty/full ratio[1]. However, the OD method lacks specificity and it is only applicable to relatively pure AAV samples. Alternatively, Size exclusion chromatography (SEC) separates analytes based on their size, which allows AAV to be separated from other components in the solution that may interfere with AAV UV absorbance, including excipients and in-process impurities. In addition, since the elution time and profile of AAV in SEC depends on genome size, the method is specific for each product. Here we explored the native UV absorbance of AAV at different wavelengths and developed two SEC methods to quantify AAV titer. Quantification of AAV titer by UV absorbance is complicated by the presence of both capsid protein and genome DNA. The UV absorbance coefficient of AAV varies with different ratios of empty to full capsids. In our previous study[1], the OD method was demonstrated to be capable of measuring both AAV DNA and protein by measuring UV absorbance directly at two wavelengths, with results that are in good correlation with PCR and AUC values. Here we found that SEC can be used to determine genome and capsid contents through UV absorbance peak areas at 260 nm and 280 nm. Similar to the OD method, the genome and capsid contents are absolute depending on the UV coefficients of the capsid protein and genome DNA only. The quantification of AAV content is direct without a calibration curve. The method, referred to as SEC-titer, utilizes detection similar to a spectrophotometer, with the advantage that there is no matrix or light scattering interference, and sensitivity is much improved. The method also can be extended to quantify any therapeutic proteins including antibody products absolutely without a

calibration curve. Quantification of AAV capsid content is also possible using UV absorbance at 214 nm, where capsid protein UV absorbance dominated. The small portion of UV absorbance contributed by genome DNA can be corrected by estimating the empty/full capsid ratio from A260/A280 and its correlation with DNA UV absorbance at 214 nm. A standard solution with only empty capsids is used to generate a calibration curve. The concentration of the empty capsid standard was determined by a conventional protein A280 method, which in general is considered to be an accurate method within 10% error to the true values[2]. The relative difference in capsid contents by OD and this method (referred as SEC-A214) is less than 5%, assessed over a wide capsid protein concentration range. Both SEC quantitation methods are very sensitive with Limits of Quantitation ≥ 1 x 10^{10} capsid/mL, allowing quantification of low AAV concentration samples. Reference: 1. Keith Webber, Brian Howie, Kevin O'Brien and Zhuchun Wu, "The Application of Spectrophotometry for the Estimation of Genome Copies and Full versus Empty Ratios of Adeno-associated Particles", poster presented to ASGCT 21st Annual Meeting (2018). 2. Pace, C.N. et al., "How to Measure and Predict the Molar Absorption Coefficient of a Protein," Protein Sci. 4, 2411-2423 (1995).

185. Characterization of a New AAV Vector Targeting Expression of Transthyretin to the Correct Tissues after Systemic Delivery in Mice

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Alzheimer's Disease (AD) is the most common neurodegenerative disorder and the leading cause of dementia and mortality in the industrialized world. Many risk factors have been associated with AD, like mutations in APP and presenilins, mitochondrial dysfunction, carrying the *APOE* ε4 allele, Down syndrome, among others. Presently there are no drugs commercially available that can reverse the disease and it is urgent to develop new therapies that can impact disease progression. The plasma- and CSF- circulating protein transthyretin (TTR) may modulate disease presentation or progression in humans as it has been associated with the prevention of Aβ aggregation, and it has also been described as a biomarker with AD patients exhibiting lower levels of TTR in the CSF when compared to normal individuals in the same age group. We proposed to develop an AAV-based gene therapy approach to express TTR somatically in a mouse model and assess the impact that overexpression of TTR has in disease progression by using AAV9 to express the gene in both liver and choroid plexus. We have developed a new AAV vector carrying a mouse TTR promoter driving expression of human TTR. Systemic infusion of 10^{12} gc AAV9-TTRp-TTR in C57BL/6J mice resulted in TTR expression in serum and CSF, at levels comparable to those in humans. When we used the same vector and dose in the AD mouse model APP^{NL-G-F}, we detected low levels of human TTR in the plasma of female mice and no protein in the CSF, whereas APP^{NL-G-F} males showed TTR levels comparable to C57BL/6J male mice. Interestingly, no differences in vector genome copy numbers were observed in either liver or choroid plexus of transgenic mice compared to wild-type littermates gender-matched. Therefore, we hypothesize the mouse TTR promoter used in the current AAV-TTRp vector might be subjected to hormonal regulation in females, similar to what has been documented for the endogenous *Ttr* gene. To our knowledge this is the first demonstration of an AAV vector that replicates the normal TTR tissue expression profile after systemic delivery, and thus an outstanding platform for therapeutics development and address biologically relevant questions about the role of TTR in AD.

186. Intrathecal Lumbar Administration of AAV9 in Nonhuman Primates is Safe and Effective in Targeting Cells of the Central Nervous System

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Naturally occurring and recently derived serotypes of adeno-associated viral vectors are used for CNS gene therapy because they can cross the blood brain barrier. The first description of an AAV serotype with these properties was AAV9. AAV9 is used by multiple academic and commercial groups as a research tool and a gene therapeutic including the AveXis product, AVXS-101. Intravenous delivery of AVXS-101, an AAV9 therapeutic for the treatment of the pediatric motor neuron disease spinal muscular atrophy, is currently under review for approval in the US, Europe and Japan. Data suggest that intrathecal administration is an important delivery route for CNS therapy development. Here we show the scale up of CSF delivery from mice to non-human primates with three different AAV9 vectors: scAAV9 CB GFP, AVXS-201 and AAV9-SOD1. AVXS-201 and AAV9-SOD1 are AveXis products in development for the treatment of the neurodevelopmental disorder Rett Syndrome and Superoxide Dismutase mediated Amyotrophic Lateral Sclerosis (ALS), respectively. Intrathecal infusion of AAV9 vectors via lumbar puncture followed by Trendelenburg tilting of subjects was safe and well tolerated in juvenile cynomolgus macaques through 18 months post injection. Distribution of vector genomes, transgene mRNA expression, RNAi target knockdown and protein expression were shown in the brain, spinal cord, and peripheral tissues including the heart, liver and skeletal muscle.

187. Physical Positioning Dramatically Improves Brain Transduction after Intrathecal Infusion of AAV9

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Effective treatment of the cerebral cortex is important for gene therapy of neurological disorders such as Alzheimer's disease, Lewy body dementia, frontotemporal dementia, Huntington's disease, and others.

The human cortex is extensively folded, with a total surface area of approximately five square feet, and methods to broadly transduce the cortex by direct intraparenchymal injection do not currently exist. Intrathecal administration of adeno-associated virus 9 (AAV9) to the cerebrospinal fluid (CSF) drives gene expression throughout the nervous system, but gene transfer to the cortex is inconsistent in rodents and monkeys. Following intrathecal AAV9 infusion in rats, we observed gene expression in brain regions where CSF appears to settle under gravity. We hypothesized that inverting rats with feet 30 degrees above the head, as well as rotating rats continuously between upright and inverted positions, for 2 hours after intrathecal AAV9 infusion would enhance gene delivery to the brain. Both inversion and rotation significantly increased the number of transduced neurons by more than 15-fold compared to rats that recovered in an upright position. In addition to entorhinal, prefrontal, frontal, parietal, and limbic cortices, transduction of hippocampus and of basal forebrain were also enhanced by more than 15-fold. 95% of transduced cells were neurons. This simple and effective method for broad gene transfer to cerebral cortex represents a potentially important advance in gene therapy for cortical disorders such as Alzheimer's disease.

188. AAV Vectors and Zinc: A Possible Role in Gene Therapy of Human Liver Cancer

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We have reported that of the 12 essential metal ions, Zn^{2+} is the most efficient in increasing the transduction efficiency of AAV2 vectors in HeLa cells. For example, at a final concentration of 30 μg/ml, an ~8-fold increase was observed (*Mol. Ther*., 26: 5S1, 2018). However, at a final concentration exceeding 30 μg/ml, Zn^{2+} also induced significant cytotoxicity, an observation consistent with a previously published report (*Exp. Biol. Med*., 235: 741-750, 2010). In subsequent studies, similar results were obtained with HEK293 cells. However, when these studies were extended to include a human hepatocellular carcinoma (HCC) cell line, Huh7, we also observed an ~7-fold increase in the transgene expression, but in the presence of only 15 μg/ml of Zn^{2+}, and concentrations exceeding 20 μg/ml of Zn^{2+} were cytotoxic to Huh7 cells. Further mechanistic studies to gain a better understating of these phenomena revealed that the increase in transduction occurred due to higher levels of extracellular levels of Zn^{2+}. Interestingly, hallmark decreases in intracellular Zn^{2+} levels have been reported in HCC patients dating back to the 1970s. Numerous population studies have reported a significant decrease (55-75%) in Zn^{2+} levels in HCC tissues, compared with healthy liver tissues (*Cancer Bio. & Ther*., 15: 353-360, 2014). It has also been reported that the decrease in Zn^{2+} levels in HCC tissues is due to under-expression of an essential metal ion transporter, SLC39A14 (ZIP14), and that ZIP14 plays a major role in the transport of Zn^{2+} in liver cells (*J. Gastrointest. Cancer*, 43: 249-257, 2012). We reasoned that targeting ZIP14-deficient HCC tumors would be ideal for Zn^{2+} infusions due to their tendency to prevent zinc uptake, and therefore, increase the level of environmental Zn^{2+}. To test this hypothesis, Huh7 cells were either mock-transfected, or transfected with a recombinant plasmid containing a CMV promoter-driven ZIP14-EGFP fusion protein, and were transduced with scAAV2-mCherry vectors under identical conditions. The results showed that ZIP14 over-expressing, but not mock-treated Huh7 cells, evaded Zn^{2+}-induced cytotoxicity at a final concentration of 20 μg/ml. We have now generated recombinant AAV vectors containing the ZIP14 gene, and we propose to develop a co-administration strategy with AAV vectors carrying a therapeutic gene such that subsequent Zn^{2+} infusions directly into ZIP14-deficient HCC tumors, especially in view of a negative correlation between ZIP14 expression and patient survival times (*J. Trace Elem. Med. Biol*., 49: 35-42, 2018) indicating intracellular zinc imbalances may play a role in cancer survival as well. These concerns notwithstanding, AAV vector-mediated delivery and expression of ZIP14 gene would be expected to augment not only Zn^{2+} uptake, but also Zn^{2+}-mediated increased expression of a therapeutic gene in human liver cancer tissues.

189. Identification of AAV-TT Residues Determining CNS Gene Transfer Efficiency

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AAV True-Type (AAV-TT) is a novel gene therapy vector, which contains 14 amino acids evolutionarily conserved in natural AAV2 human tissue isolates. AAV-TT displays potent neurotropism in vivo, and in a murine disease model of the lysosomal storage disease mucopolysaccharidosis IIIC AAV-TT displays superior efficacy compared to AAV9, currently the preferred choice for treatments of the CNS. Both serotypes restore whole brain HGSNAT enzyme activity, yet only AAV-TT corrects the behavioural phenotype.

One discernible difference between AAV-TT and AAV2 is the mutation of two key residues at the HSPG binding site. It is known that removal or attenuation of the HSPG binding site in AAV2 improves vector spread within the brain after local injection. We show here that while removal of HSPG binding from AAV2 improves vector spread and transduction in the brains of adult rats, levels are still inferior to those observed with AAV-TT. We further show that re-introduction of the HSPG binding site into AAV-TT does not reduce transduction and spread to levels seen with AAV2. These results indicate that some or all of the remaining 12 mutations also contribute to the improved transduction properties of AAV-TT.

To investigate which of the amino acids support the enhanced brain transduction and spread of AAV-TT, we conducted biodistribution studies after intrastriatal injection of multiple AAV-TT variants in adult rats. Amino acids were grouped based on putative function and reverted to those present in AAV2; some single amino acids were selected for reversion based on their position in the viral capsid. These AAV-TT alterations resulted in varying effects on transduction in the brain. Most notably, one amino acid at the base of the threefold spike, when mutated back to its original state, resulted in a significant reduction of viral spread. Reversion of another single amino acid,

with a side chain pointing toward the capsid interior resulted in comparable transduction to AAV-TT, but with a more favourable in vitro immunogenicity profile.

To summarise, we have engineered a novel gene therapy capsid that utilises key residues found in natural variants of AAV2 to produce a unique gene therapy vector for the treatment of diseases that affect the central nervous system. These mutations are being investigated in detail and the data generated have the potential to further improve the performance of AAV-TT, and potentially other serotypes as well.

190. Characterization of Hepatic and Retinal Cell MicroRNAome During AAV Infection Reveals Their Diverse Impact on Viral Transduction and Cellular Physiology

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Background: Adeno-associated virus (AAV) based vectors have shown excellent safety and efficacy profile for the treatment of inherited retinal diseases (Achromatopsia, Choroideremia and Leber congenital amaurosis) and liver diseases (Haemophilia A & B); however challenges to its widespread use remain. AAV enters the host cells through receptor mediated endocytosis, then escape from endosomes and traffick towards nucleus to uncoat and deliver the encapsidated transgene. Each of these steps are known to act as a barrier restricting AAV transduction. Understanding the host factors impacting viral transduction at early stages of AAV infection with high throughput technologies may help us to derive plausible therapeutic strategies to circumvent and enhance the AAV delivery system. **Methods:**We utilized Illumina NextSeq500 technology to profile the small RNA changes post 12 hours of AAV2 and AAV3 infection in Huh-7, a liver origin cell line and ARPE-19, a retinal origin cell line with 2 biological replicates under each condition. Differentially expressed miRs(DE-miRs) were mapped to specific pathways using InnateDb and MiRNet tools. Impact of selected pathways were validated; cell cycle analysis by propidium iodide staining-flow cytometry method, quantification of ER stress markers by qPCR; and assessment of lipids by NileRed staining-confocal microscopy. Synthetic mimic and inhibitors assays were performed to elucidate the role of a dysregulated miR-4488 in AAV transduction.**Results:**Six percentage of miRNAs were dysregulated between mock and AAV infected samples. Pathway characterization of experimentally validated miR target genes showed "Cancer, Viral carcinogenesis, FoxO signalling pathway, cell cycle, bladder cancer" as common significant hits and 'protein processing in endoplasmic reticulum' and 'sphingolipid signalling' as unique hits in Huh-7 and ARPE-19 respectively. Pathway validation showed AAV infected Huh-7 cells were arrested at G0/G1 check point (4%), increased expression of ER stress markersBiP (1.6 fold) and CHOP (2.2 fold) and IRE1A (1.53 fold),and unaltered lipid droplet levels. Whereas, ARPE-19 cells were delayed in S-phase (2%) with downregulation of ER stress markersATF4 (-8.26), ATF6 (-8.24), BIP (-2.56), IRE1A (-4.97), PERK (-7.03) and moderate increase in the percentage of lipid droplets. Irrespective of serotypes and hosts used, we found miR-4488 as commonly dysregulated(-2.24 fold in Huh-7 and -3.32 fold in ARPE-19). Increasing miR-4488 levels showed a positive regulation of AAV transduction in Huh-7 cells (126-128%), whereas inhibiting the function of miR-4488 showed increased transduction in ARPE-19 cells (142-158%). **Discussion:**Overall our findingssuggest that recombinant AAV mediated changes to microRNA expression is altered during AAV infection and can target specific biological pathways. We have further demonstrated that miR-altered biological pathways such as cell-cycle regulation, endoplasmic reticulum stress and lipid signalling are impacted upon AAV2 or AAV3 transduction. We have also identified for the first time that miR-4488 is commonly dysregulated during AAV infection of these cells, independent of the serotype employed. Furthermore, our studies have shown that modulation of miR-4488 levels can improve AAV transduction. **Conclusion:**A comprehensive study of the specific biological functions of the many small RNAs described in our work and their interaction with multiple AAV serotypes is likely to be rewarding to improve their hepatic or retinal gene transfer efficiency.

191. Adeno-Associated Virus Serotypes Screening in Non-Human Primates for Hemophilia A Genome Editing Treatment

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Adeno-associated virus (AAV) can be engineered to safely deliver DNA to target cells *in vivo*, and is being widely used in emerging gene therapies. Several AAV serotypes have been isolated in recent years, however, the landscape for their optimal use for therapeutic purposes has not fully investigated. Here we report the results of a comprehensive study comparing liver infection efficiencies of several AAV serotypes (AAV5, AAV8, AAV100M and Anc80) in Non-Human Primates (NHP) *in vivo*, delivering CRISPR/Cas9 genome editing vectors for Hemophilia A treatment (See abstract # 2019-A-523-ASGCT for details and proof of concept in mice). In this study, sixteen adult male cynomolgus macaques (*Macaca fascicularis*) received intravenous injections of two viruses, AAV-Staphylococcus aureus Cas9 (SaCas9)-guideRNA and AAV-B domain-deleted human FVIII (BDD-F8) Donor, in several doses ($3x10^{10}$- $5.5x10^{12}$ vg/kg). Liver infectivity of both viruses was determined up to 18 weeks later by droplet-digital PCR (ddPCR), showing higher infectivity efficiencies for AAV8 and 100M serotypes compared to AAV5 and Anc80 serotypes. Liver SaCas9 mRNA expression was determined by RNAscope *in situ* hybridization (ISH) method, showing higher saCas9 levels following infections with AAV8 and 100M serotypes, supporting the infectivity findings. Safety studies, including clinical observations, food consumption monitoring, body weight and temperature, liver enzyme and gross pathology evaluation showed no toxicity effects following administration of the tested serotypes. Anti-SaCas9 immunogenicity was evaluated prior and post viral administration and no increase of anti-SaCas9 antibodies post-dosing was detected. Taken together, AAV-based *in vivo* delivery to non-human primate livers was the most efficient using AAV8 or 100M serotypes with no safety concerns. Further studies are ongoing to determine optimal dose as well as genome editing efficiency.

192. Cre-Dependent Reporter Mice Improve the Sensitivity of AAV Tropism Screens: Implications for AAV as a Genome Editing Platform

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Conventional methods to discern adeno-associated virus (AAV) vector tropisms are based on the high, stable expression of a reporter gene in the AAV transgene cassette. As a consequence, conventionally described AAV tropisms omit cell types that exhibit transient or low transgene expression. This creates a blind spot for AAV vectors that deliver genome editing machinery because only minimal transgene expression is required for activity. To fill in this blind spot, we present an AAV tropism screening method that captures both stable, high transgene expression and transient expression. Using AAV8, we demonstrate the superiority of the approach in a side-by-side comparison with traditional screening and find unexpected sites of transduction. From these results, we predict cell-specific off-target events upon AAV-delivery of CRISPR/Cas9 components. We anticipate that this system, which captures transient and persistent gene expression from AAV vectors *in vivo*, will be foundational to future applications of AAV.

193. AAV3-miRNA Vectors for the Potential Gene Therapy of Human Liver Cancer

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Liver cancer is predicted to be the sixth most commonly diagnosed cancer, and the fourth leading cause of cancer death worldwide reported in 2018, 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. AAV8 vectors expressing miRNA-26a (*Cell*, 137: 1005-1017, 2009) and miRNA-122 (*J Clin Invest*, 122: 2871-2883, 2012) have been used to achieve strong inhibition of growth of mouse liver tumors. Since we have 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), in the present studies, we wished to evaluate the efficacy of AAV3-miRNA-26a and AAV3-miRNA-122 vectors. To this end, a human hepatocellular carcinoma (HCC) cell line, Huh7, was transduced with various multiplicities of infection (MOIs) of scAAV3-miRNA-26a or scAAV3-miRNA-122 vectors, both also co-expressing a Gaussia luciferase (Gluc) reporter gene. A modest yet significant (~12-13%; $p<0.05$) level of dose-dependent growth inhibition of Huh7 cells was observed at the highest MOI ($1x10^5$ vgs/cell) with each vector. When Huh7 cells were co-transduced with both vectors, the extent of growth inhibition was additive (~26%). Similar results were obtained with Huh7 cells stably transfected with a Firefly luciferase (Fluc) expression plasmid. Studies are currently underway to evaluate the efficacy of capsid-optimized next generation of scAAV3-miRNA-26a and scAAV3-miRNA-122 vectors alone, or in combination, in targeting Huh-Fluc cells-derived human liver tumors in a mouse xenograft model *in vivo*. These studies should yield information on whether in contrast to mouse liver tumors, the miRNA-26a and/or miRNA-122-mediated suppression is an effective gene therapy strategy to target human liver cancer.

194. AAV Gene Therapy Vectors for Remote Modulation of Peripheral Nerves Targeting the Pancreas

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Type 2 diabetes mellitus (T2DM), which affects greater than 300 million people worldwide, is marked by an inability to produce appropriate amounts of insulin in response to high blood glucose. Long-term complications of T2DM include blindness, heart disease, stroke and lower limb amputation. One novel approach to treat T2DM is to control production of glucose regulatory hormones by delivery of neuromodulatory agents directly to the peripheral nerves that target endocrine cells of the pancreas. The development of this strategy relies on a better understanding of the anatomy of pancreatic innervation and how stimulation and inhibition of these nerves affects pancreatic production of insulin, glucagon, somatostatin and pancreatic polypeptide.One means of remotely controlling activity in pancreatic nerves to regulate pancreatic hormone production and ultimately glucose homeostasis is through the use of magnetogenetics, a technique for manipulating neuronal activity using magnetic fields. We fused a ferritin binding nanobody to the transient receptor potential V1/TRPV1/vanilloid receptor-1/capsaicin receptor (NbFT-TRPV1). The NbFT-TRPV1 receptor recruits iron binding ferritin complexes to the membrane. Magnetic stimulation of neurons expressing NBFT-TRPV1 leads to increased intracellular calcium *in vitro*. Here we focus on three approaches to efficiently express neuromodulatory NbFT-TRPV1 in pancreatic nerves: 1) optimizing the regulatory elements within AAV constructs, 2) comparing different capsid serotypes for AAV-mediated delivery to pancreatic nerves and 3) downregulating expression of therapeutic protein in off-target organs.We compared mCherry expression in Neuro2A and non-neuronal cell types using three promoters: the prototypic neuron-specific promoter for human synapsin (hSyn), the small, synthetic JeT promoter, and the promoter for neuron-specific enolase (NSE) a marker for neuroendocrine cells. We found that the NSE promoter results the highest level of neuron-specific protein expression while the JeT promoter leads to highest expression overall and is ideal for inducible systems such as Cre-activation. Because the NBFT-TRPV1 receptor is nearly 3kb, we conserved space within the AAV construct by testing different promoter/enhancer/ polyadenylation signal combinations. Our second strategy to deliver AAV to pancreatic nerves involved testing different

AAV capsid serotypes. We directly compared the ability of AAV serotypes -6, -8 and -9 to accumulate in the celiac ganglia innervating the pancreas and we are currently testing additional capsid moieties for delivery of AAV to peripheral nerves of the pancreas. Finally, we incorporated micro RNA antisense target sequences into the 3' untranslated region of our expression vectors in order to downregulate expression of neuromodulatory constructs in off-target organs such as the liver. These studies have shaped the design of the AAV vectors we are testing for maintaining glucose homeostasis through remote modulation of peripheral nerves targeting the pancreas.

195. Integrase-Deficient Lentiviral Vector for Efficient CRISPR/Cas9-Mediated Gene and Epigenome-Editing Applications

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The CRISPR/Cas9 systems have revolutionized the field of genome editing by providing unprecedented control over gene sequences and gene expression in many species, including humans. Lentiviral vectors (LVs) are one of the primary delivery platforms for the CRISPR/Cas9 system due to their ability to accommodate large DNA payloads and sustain robust expression in a wide range of dividing and non-dividing cells. However, long-term expression of LV-delivered Cas9/guide RNA may lead to undesirable off-target effects characterized by non-specific RNA-DNA interactions and off-target DNA cleavages. Integrase-deficient lentiviral vectors (IDLVs) present an attractive means for delivery of CRISPR/Cas9 components because: (1) they are capable of transducing a broad range of cells and tissues, (2) have superior packaging capacity compared to other vectors (e.g., adeno-associated viral vectors), and (3) they are expressed transiently and demonstrate very weak integration capability. Here, we aimed to establish IDLVs as a means for safe and efficient delivery of CRISPR/Cas9. To this end, we developed an all-in-one vector cassette with increased production efficacy and demonstrated that CRISPR/Cas9 delivered by the improved IDLV vectors can mediate rapid and robust gene editing in human embryonic kidney (HEK293T) cells and post-mitotic brain neurons in vivo, via transient expression and with higher gene-targeting specificity than the corresponding integrase-competent vectors (Ortinski et al, 2017; Mol.Therapy Methods & Clin. Dev.). As to further validate the novel system in the clinically-relevant setting, we most recently developed an all-in-one IDLV vector for the delivery of gRNA-dCas9- fused with the catalytic domain of DNA-methyltransferase3A (DNMT3A) enzyme. To facilitate the drug discovery pipeline, we applied the system into human induced pluripotent stem cells (hiPSC)-derived 'aged' dopaminergic neurons from a PD-patient with the *SNCA* triplication. The experiment resulted in fine-tuned downregulation of *SNCA*-mRNA and protein levels mediated by targeted DNA-methylation at intron 1, thus providing a strong foundation for advancing the IDLV- platform towards various therapeutic applications involving genome and epigenome editings prospectives. Key Word: Integrase-Deficient Lentiviral Vectors, transient delivery, CRISPR/Cas9 systems, off-target effects, hiPSC-derived neurons, *SNCA*, expression regulation, aging, epigenome editing, Parkinson's disease

196. Stability Evaluation of rAAV Serotype 1, 2, 8 ,9 and Wild-Type AAV2 Under Various Conditions

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Background: The different recombinant adeno-associated virus (rAAV) serotypes display different tissue tropisms *in vivo*. Because of this, rAAVs have emerged as a versatile delivery vehicle for genetic treatment. Currently, there are several rAAV-based drugs for gene therapy that have already been approved. In order to further increase the possibility of introducing new rAAV-based treatments on the market, it is necessary to investigate how rAAV shedding from patients could influence biodiversity. However, there is not enough data available concerning the stability of the different rAAVs that are released from the patients under treatment into the ecosystem. In this study, to research the environmental effect on the stability of rAAVs, they were exposed to ultraviolet ray (UV), 0.1M Sodium hydroxide (NaOH), 0.06% Sodium hypochlorite (NaClO), tap water, and 70% ethanol (EtOH). Furthermore, to compare the stability between recombinant and wild type AAV, wtAAV2 was also analyzed. **Methods:** rAAVs (ssAAV(1, 2, 8, 9)-CMV-ZsGreen1) were produced by the triple-transfection of HEK293EB cells in serum-free medium. For rAAV1, rAAV8, and rAAV9, the cultured supernatant was harvested 5 days after transfection, filtrated through a 0.45-μm filter, and finally purified by AAVpro® Concentrator (TaKaRa Bio, Japan). rAAV2 was purified by AAVpro® Purification Kit (All Serotypes, TaKaRa, Japan) from the cell lysate. Next, 1.12 x 10^9 v.g. of each rAAV and wtAAV2 were exposed to UV for 40 min, 0.1M NaOH for 15 min, 0.06% NaClO for 15min, 70% EtOH for 10 days, and 0.22μm filtrated tap water for 10 days. The treated samples were transduced to HeLaRC cells (a clone of HeLa cells that harbors *rep-cap* gene) with adenovirus type 5, and their biological activity was analyzed by fluorescent microscopy and $TCID_{50}$.

Results and Conclusion: The activity of all rAAVs was weakened by exposure to UV, NaOH, and NaClO for 15 or 40 min. rAAV1, 8, and 9 were not inactivated at all by tap water nor 70% EtOH even after exposure for several days, but rAAV2 and wtAAV2 were inactivated to some extent. Furthermore, there was no difference observed between the stability of wtAAV2 and of rAAV2. We consider that these results can be an important contribution to the data necessary for the study of the impact of rAAV on biodiversity.

Adenovirus Vectors and Other DNA Virus Vectors

197. Development of Adenoviral Vectors for the Treatment of Duchenne Muscular Dystrophy with One Single Vector

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Advances in gene editing enable the correction of mutations causing Duchenne muscular dystrophy (DMD) by developing personalized CRISPR/Cas9 treatments for the different mutations underlying the disease. Recent studies showed efficient in vivo genome editing following AAV delivery of a DMD specific CRISPR/Cas9 machinery. Nevertheless viral delivery of all required CRISPR/Cas9 components including multiple guide RNA (gRNA) expression units within one single vector has not been fully exploited yet. Gene deleted high-capacity adenoviral vectors (HCAdVs) can transport up to 35 kb of foreign DNA and allow e.g. to deliver the complete CRISPR/Cas9 machinery including several gRNAs within a single vector. Exemplarily we produced a CRISPR-HCAdV containing two gRNAs specific for intronic sequences flanking DMD exon 51. We used this DMD specific CRISPR-HCAdV to transduce immortalized dystrophic skeletal myoblasts carrying a Δexon 48-50 mutation leading to frameshift and premature stop codon in DMD exon 51 and absence of full length protein. Upon transduction locus specific PCR and sequencing confirmed efficient locus specific deletion of DMD exon 51 on genomic level and seamless splicing of DMD Exon 47 to exon 52 on mRNA level. Reconstitution of DMD expression after treatment of dystrophic muscle cells was shown using immunofluorescent DMD staining. Moreover DMD protein quantification using in cell western analysis showed that treatment lead to DMD levels of up to 50% of wild type myoblasts. As Adenovirus (AdV) serotype 5, which is commonly used as gene therapy vector, has limitations for in vivo applications, we used a library of 19 different reporter gene expressing AdV serotypes and tested their efficiency to transduce various human and rodent skeletal and cardiac muscle cells. In rodent cells no other AdV could compete against AdV5. In contrast human cells were easier to transduce with AdV37, 35 and 21 compared to AdV5. These are promising candidates for the conversion into highly efficient vectors for the treatment of muscular diseases including DMD. We believe that in addition, or as an alternative to AAV, HCAdVs could be efficient delivery vehicles for gene therapy in the context of DMD and other muscular disorders.

198. A Hypoxia-Responsive Oncolytic Adenovirus Expressing Secretable TRAIL for Cancer Gene Therapy

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Here, we hypothesized that overexpression of proapoptotic therapeutic gene in glioblastoma by oncolytic adenovirus may overcome apoptosis resistance of glioblastoma while enhancing viral distribution through induction of apoptosis. In this regards, we constructed a hypoxia-responsive and cancer-specific modified human telomerase reverse transcriptase (HmTERT) promoter to drive replication of an oncolytic adenovirus (HmTERT-Ad), aiming to overcome hypoxia-mediated downregulation of viral replication. As our primary objective was inducing apoptosis in glioblastoma, we have also generated HmTERT-Ad expressing secretable trimeric tumour necrosis factor-related apoptosis-inducing ligand (HmTERT-Ad-TRAIL). HmTERT promoter-regulated oncolytic adenoviruses showed cancer-specific and superior cell killing effect in contrast to a cognate control oncolytic adenovirus replicating under the control of the endogenous adenovirus promoter or conventional human TERT promoters. The cancer cell-killing effects of HmTERT-Ad and HmTERT-Ad-TRAIL were both enhanced under hypoxic conditions, owing to hypoxia-responsiveness of the promoter. HmTERT-Ad-TRAIL showed more potent antitumor efficacy than HmTERT-Ad in subcutaneous and orthotopic glioblastoma models that are resistant to conventional TRAIL therapy. The potent antitumor effect of HmTERT-Ad-TRAIL was mediated by superior induction of apoptosis and more extensive virus distribution in the tumor tissues. Collectively, our findings show that HmTERT-Ad-TRAIL can promote dispersion of an oncolytic adenovirus through robust induction of apoptosis in a highly TRAIL-resistant glioblastoma.

199. HIV Tat-Induced HIV Gene Expression Precedes the Proliferation and Dedifferentiation of Renal Cells in HIV-Tg26 Mice

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Background: The HIV-Tat protein is a powerful activator of HIV transcription, but can also be released by infected cells and induce the dysregulation and proliferation of cultured human podocytes not infected with HIV-1. However, it is unclear whether HIV-Tat can induce proliferation and de-differentiation of podocytes and activation of the ERK pathway thereby enhance renal injury of effect of HIV-1 gene in vivo. **Objective:** To determine whether HIV-Tat can induce the renal expression of HIV-1 gene and the proliferation and de-differentiation of glomerular epithelial cells, and activation of ERK pathway thereby effect of renal injury in HIV-Transgenic (HIV-Tg26) mice. **Methods/Design:** rAd-Tat and LacZ control vectors (2 x 10^9) were expressed in renal glomeruli of WT and HIV-Tg 26 young mice without pre-existing renal disease. Mice were sacrificed at seven days and thirty five days. The renal expression of HIV-genes, nephrin, synaptopodin, WT-1, Cyclin D-1, FGF-2, VEGF and signaling pathways that modulate the growth of glomerular epithelial cells was assessed by RT-PCR, immunohiostochemistry and Western blots. **Results/Discussion:** At seven days, rAd-Tat induced the renal expression of HIV envelope gene in HIV-Tg26. This change was associated with activation of the ERK pathway and up regulation of FGF-2 and VEGF that preceded the proliferation and de-differentiation

(down regulation of nephrin, synaptopodin and WT-1) of renal glomerular epithelial cells. rAd-Tat reduced activate caspase-3 and apoptosis of renal cells at seven days. It also preceded the down regulation of Cyclin D1, WT-1, nephrin, synaptopodin and the proliferation of renal glomerular epithelial cells at 35 days. rAd-Tat induces the expression of HIV-1 gene (env) in the kidney of HIV-Tg26 mice. This change was associated with activation of the ERK pathway and up regulation of FGF-2 and VEGF that preceded the development of proliferative changes in renal epithelial cells, and de-differentiation of podocytes in HIV-Tg26 mice.

200. Adenovirus-Mediated Expression of Relaxin for Treatment of Scar Remodeling

In-WooK Kim[1], Bok Ki Jung[2], Won Jai Lee[3], Eunhye Kang[3], Yong Oock Kim[3], Dong Kyun Rah[3], In Sik Yun[2], Chae-Ok Yun[1]

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Scars occurring as the result of wound healing are able to elicit functional disability. Although a large number of studies have investigated the mechanism of scar development to both minimize and alter scar formation, treatments for scars have not yet been fully realized. Relaxin is a peptide hormone that functions as transforming growth factor β1 antagonist. As transforming growth factor β1 is associated in extracellular matrix regulation and fibrosis, we hypothesized that relaxin gene delivery may improve scar remodeling. To determine the effects of relaxin on scar reduction, we investigated the scar remodeling process by injecting relaxin-expressing adenoviruses using a pig scar model. In specific, scars with full thickness were generated on the backs of Yorkshire pigs. At 50 days after the treatment, relaxin-expressing adenovirus-treated groups exhibited decreased surface areas of scars, normalization in color of scars, and increase in pliability of scars compared to those treated with vehicle control. Collectively, our findings demonstrate that relaxin-expressing adenovirus improves the surface area, color, and pliability of scars.

201. Relaxin Expression by Oncolytic Adenovirus Chemo Sensitizes and Enhances Therapeutic Efficacy in Highly Desmoplastic Tumors

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In advanced stages of pancreatic cancer, the only treatment option available is chemotherapy, which often combines gemcitabine with other chemotherapeutics. However, these chemotherapeutics are highly toxic and lack therapeutic efficacy. Specifically, clinical beneficial response to gemcitabine are observed in approximately 25% of cases, but even this limited therapeutic efficacy rapidly declines owing to long-term tolerance, resulting in a median overall survival of 6 months. Here, we examined the ability of combined treatment with gemcitabine and relaxin-expressing oncolytic adenovirus (oAd), which degrades extracellular matrix (ECM), to efficiently treat chemoresistant pancreatic cancer. Our findings show that gemcitabine alone induced low level of cancer cell death in 3 different pancreatic cancer cell lines. The anticancer effect of gemcitabine was greatly enhanced through combination therapy with oAd; a subtherapeutic dose of gemcitabine (0.01~0.05 uM) resulted in potent anticancer effects through robust induction of apoptosis. Importantly, oAd combined with gemcitabine attenuated the expression of major ECM components in both tumor spheroids and xenograft tumors compared with gemcitabine or oAd treatment alone, resulting in potent induction of apoptosis, gemcitabine-mediated cytotoxicity, and an oncolytic effect through degradation of tumor ECM. Our results demonstrate that oAd can selectively degrade aberrant ECM to sensitize desmoplastic pancreatic tumor to both oncolytic virotherapy and conventional chemotherapy.

202. Considerations for Biosafety Testing of Cell and Gene Therapies

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Regulatory authorities such as the US Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) impose stringent limits on the amount of microbial contaminates and impurities present during the manufacturing of biological medicines and vaccines, and present in cell and gene therapy products. These regulations ensure sterile products and thus patient safety. To establish that the testing procedures are accurate, regulatory authorities require proof of testing before clinical trials can be approved or a batch of commercial biopharmaceuticals or vaccines be released. Consequently, all components of the manufacturing process must undergo extensive safety testing to demonstrate identity, stability, and purity. This talk will review general approaches to biosafety testing, with specific focus related to cell and gene therapies.

203. Development of Helper-Dependent Adenovirus for Gene Therapy in the Inner Ear

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The most common type of hearing loss is sensorineural, due to loss of hair cells and spiral ganglion neurons, the two inner ear cell types essential for transducing auditory information. Gene therapy strategies to restore inner ear function by reestablishing functional sensory cells may require the expression of large or multiple transgenes with promoters or cis-acting regulatory elements that impart coordinated expression with cell-type specificity. Such approaches are only achievable using a viral vector that has sufficient capacity to

accommodate multiple coding sequences and necessary regulatory elements. Helper-dependent adenovirus (HdAd) has great potential for the development of such strategies due to its large capacity for genetic cargo. Further, HdAd is completely devoid of viral genes, eliminating the potential for ototoxicity that is associated with first generation adenoviral vectors. Despite its promise, technology for the use of HdAd for inner ear gene therapy has thus far not been developed. Therefore, we have generated reporter vectors for the characterization of HdAd as a platform technology for use in the inner ear in vivo. Vectors were delivered to the adult murine inner ear by injection through the round window membrane, a clinically viable route of delivery for therapeutic strategies that require the preservation of existing cochlear function. Using this surgical technique, transduction was observed in various cell types throughout each turn of the cochlea. Expression of the reporter gene was stable at 28 days post-injection, with minimal damage to hair cells compared to cochlea injected with a first generation adenoviral vector. Studies are ongoing to further characterize the transduction pattern, safety, and stability of HdAd in the inner ear.

204. Precise Genetic Modification of Canine Adenovirus Type 2 (CAV2) Genome by CRISPR/CAS9

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Genetically modified oncolytic adenoviruses are an outstanding and common vehicle for efficient cancer gene therapy. They account for 23.8% of nearly 1700 clinical trials that were conducted using both viral and non-viral vectors. However, several concerns, such as normal cell/organ toxicity, lack of suitable cell surface receptors to allow viral entry to the desired cancer cell, and activation of both innate and adaptive immune systems in patients restrict the successful clinical application of adenoviral-mediated cancer gene therapy. As a result, successful virotherapy will require efficient transductional and transcriptional targeting to enhance therapeutic efficacy by ensuring targeted and long-term adenoviral replication or therapeutic transgene expression. Targeted modification of several viral components, such as viral capsid, fiber knob, and the insertion of transgenes for expression, are prerequisites for conducting the necessary transductional and transcriptional targeting of adenovirus. However, the conventional approach to modify the adenoviral genome is not only complex but also time consuming and expensive. It is solely dependent on the presence of unique restriction enzyme sites that may or may not be present in the target site. Clustered regularly interspaced short palindromic repeat (CRISPR) along with an RNA-guided nuclease called Cas9 (CRISPR/Cas9) is one of the most powerful tools that has been adopted for precise genome editing in various cells and organisms. However, whether the CRISPR/Cas9 system can precisely and efficiently make genetic modification as well as gene replacements in adenoviral genomes remains essentially unknown. Here, we propose to utilize in vitro CRISPR/CAS9 mediated editing of the canine adenovirus type 2 (CAV2) genome to promote targeted modification in the viral genome. To demonstrate the feasibility of this goal, we have conducted CRISPR/Cas9 mediated insertional mutagenesis and successfully inserted the RFP (red fluorescent protein) reporter construct into the CAV-2 genome. Initial results provided high efficiency and accuracy for in vitro CRISPR mediated editing of the large CAV2 genome. Furthermore, we are also in the process of utilizing the CRISPR/Cas9 system to conduct wild type gene replacement with our desired ligand into the CAV-2 genome to construct tumor-targeted vectors. Thus, we hope that our work will provide a significantly improved and efficient method for targeted editing of adenoviruses to generate oncolytic adenoviruses in the shortest possible time.

Gene Targeting & Gene Correction

205. Enhancing Genome Editing with Engineered CRISPR Nucleases

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The rapid pace of genome editing technology development has led to important advances in biomedical research, while also enabling clinical applications to treat human disease. Despite the vast potential of these technologies, several natural characteristics of CRISPR-Cas9 and Cas12a enzymes, evolved over millennia to combat pathogens, are suboptimal for modern-day implementations. Three such properties include on-target activity (the ability to edit the intended site), genome-wide specificity (the ability to edit precisely and avoid off-targets), and targeting range (the ability to target accurately and frequently within a desired sequence). Thus, the intrinsic properties of CRISPR nucleases must be optimized to enable their safe and effective implementation in therapeutic settings. To impart more desirable properties into Cas9 and Cas12 nucleases, we have developed and utilized protein engineering strategies that independently enhance each of these properties. We are pursuing high-throughput approaches to engineer and characterize hundreds of variants simultaneously, enabling a more thorough understanding of protein-DNA and protein-protein interactions that govern these properties. Using this information, we have engineered CRISPR nuclease variants with improved on-target activities, genome-wide specificities, and targeting ranges, and demonstrate the superiority of these variants for applications including genome, epigenome, and base editing. Collectively, we have improved the on-target activities of several CRISPR nucleases by more than 2-fold, reduced their off-target propensities to undetectable levels, and have increased their targeting ranges by 4-to-8 fold. Our results provide insight into

important properties of Cas9 and Cas12a nucleases, and offer optimized technologies to the research and therapeutic communities for the study and treatment of genetic diseases.

206. Heterotopic Activation of Enhancers as a General Targeted Strategy to Increase Human Gene Expression

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Enhancers serve an essential function in regulating spatiotemporal gene expression but are typically active in only certain cell types. Leveraging enhancer activities in heterotopic cell types (i.e., cells in which an enhancer is not normally active) would increase options for variably increasing the expression of specific target genes. We have developed a general strategy that leverages synthetic CRISPR-Cas-based gene regulators to enable the robust activation of enhancers in cell types in which they are normally inactive. We have used this approach to increase the expression of a series of different endogenous human genes across multiple cell types. In addition, we show that this method can be used to selectively direct the enhancer activity of the locus control region (LCR) to different gene promoters in the beta-globin gene cluster, enabling differential regulation of epsilon-, gamma-, and beta-globin genes in cells in which these genes are normally not expressed. The general strategy for heterotopic enhancer activation we have developed provides a powerful method for modulating human gene expression and also has important implications for understanding how enhancers function in their normal cell-type specific contexts.

207. A Complete Strategy for Characterizing On- and Off-Target CRISPR/Cas9 Editing Events via Target Enrichment and High-Resolution NGS Analysis

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Genome editing with the CRISPR/Cas9 system is moving towards therapeutic applications, which drives an increased need for in-depth characterization of both on- and off-target genome editing events. Here we present a workflow and useful guidelines for prediction, validation, quantification, and analysis of on- and off-target editing events. First, we provide comparison of several commonly-used off-target prediction tools for *in silico* selection of target sites throughout the genome. Then, using previously-published, unbiased methods for experimental off-target validation, we are able to compare these algorithm-based selections to bona fide sites exhibiting editing mediated by more than twelve guide RNAs. The unbiased detection methods are carried out with experimental and bioinformatics advancements to improve the efficiency of empirical off-target validation. A multiplexed, amplicon-based enrichment method (rhAmpSeq™) for next-generation sequencing is then employed for strict quantification of editing events at validated as well as predicted off-target sites. The rhAmpSeq technology enables interrogation of >1000 genomic loci in a single reaction using RNase H2-cleavable primers which facilitate almost complete suppression of non-target location and primer dimer amplification. Moreover, amplicon coverage is uniform and reproducible, removing the need for primer re-balancing. The relationship between coverage depth and statistical confidence in quantification of editing events was characterized. Finally, we developed and rigorously validated a data analysis package to confidently detect Cas9 edits via synthetic reads that contain diverse editing events and varied genomic target complexities.

208. Strategies for Multi-Gene Editing and Reduction of Translocations with CRISPR-CPF1 in T Cells for the Development of Improved Cell Therapies

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Gene editing using RNA-guided nuclease technology has gained widespread attention for its potential application to current cell therapies. The CRISPR-Cpf1 system (also known as Cas12a) is complementary to Cas9 with several distinct differences. Cpf1 uses a single ~40 nucleotide crRNA and can target T- and C- rich PAMs with the WT and engineered PAM variants. The expanded targeting space, when compared to the purine rich PAMs of Cas9, makes it an attractive addition to enable broader targeting opportunities. Unlike SpCas9, Cpf1 makes a staggered cut in the DNA leaving behind a 4-5 nucleotide 5'-overhang, which could result in different editing outcomes. We screened multiple loci of therapeutic interest, including TRAC, B2M, and CIITA, in primary human CD4+ and CD8+ T cells with Cpf1 and its engineered RR and RVR PAM variants. We obtained active hits for all variants when these different enzymes were delivered as ribonucleoproteins (RNPs). We conducted specificity studies using GUIDE-seq, Digenome-seq, and in silico analysis followed by targeted next-generation sequencing and verified that there was no off-target for these top hits for any RNP at a sensitivity level of ~0.1% editing. The results were consistent with Cpf1 being a highly specific enzyme. With these top hits, we were able to consistently achieve robust single (>95% KO) and multiplexed (>80% triple KO) gene disruption. To examine the genomic consequences of multiplexed editing, we applied a set of detection technologies including targeted and genome-wide methods to quantitate editing efficiency and genomic rearrangements within and between target loci. Simultaneous targeting of multiple loci resulted in readily detectable translocations between loci. We have developed and characterized multiple strategies with which to reduce rearrangement frequencies. We observed that multi-gene editing with a CRISPR-Cas9/CRISPR-Cpf1 combination reduced translocation frequencies compared to multiplexing with only CRISPR-Cas9. Taken together, for the development of T cell-based medicines, these data suggest

that CRISPR-Cpf1 is both robust, specific, and capable of reducing genomic rearrangements when making multiple gene edits compared to the CRISPR-Cas9 system alone.

209. CRISPR-READI: Efficient Generation of Knock-In Mice with CRISPR RNP Electroporation and AAV Donor Infection

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Genetically modified animal strains are invaluable assets for modeling human disease and assessing the safety and efficacy of gene therapies. In particular, knock-in mice harboring large sequence insertions or substitutions are essential for a variety of applications including endogenous gene tagging, conditional gene knockout, site-specific transgene insertion, and gene replacement. However, existing methods used to generate such animals remain laborious, costly, or suboptimal in efficiency. To address these shortcomings, we developed a novel approach, designated as CRISPR-READI (CRISPR RNP Electroporation and AAV Donor Infection), which combines adeno-associated virus (AAV) donor delivery with Cas9/sgRNA RNP electroporation to engineer large site-specific modifications in the mouse genome with high efficiency and throughput. We first identified AAV1 as an efficient, naturally occurring serotype for robust transduction of mouse zygotes. When paired with RNP electroporation, recombinant AAV donors mediate precise sequence modification by homology-directed repair (HDR) in up to 77% of treated zygotes, exceeding the 46% editing frequency previously achieved with short oligo donors. Using CRISPR-READI, we successfully inserted a 774 bp fluorescent reporter, a 2.1 kb inducible CreERT2 driver, and a 3.3 kb expression cassette into targeted endogenous loci with *ex vivo* knock-in efficiencies ranging from 33% to 69%. We also applied CRISPR-READI to produce viable transgenic mice, resulting in 18% of live pups harboring the correctly targeted fluorescent tag. Currently, we are assessing the ability of CRISPR-READI to generate live animals with multi-kilobase insertions and verifying germline transmission of our targeted gene knock-ins. Our findings show that CRISPR-READI is applicable to a variety of widely used gene targeting schemes, enabling rapid mouse line generation for gene expression studies and disease modeling with great economic and technical ease. Altogether, CRISPR-READI provides a simple, efficient, and high-throughput alternative to microinjection and ESC-based methods for sophisticated mouse genome engineering.

210. Inducible Staphylococcus Aureus Cas9 Mediated Hypermutation

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The ability of catalytically inactive *S. pyogenes* Cas9 (SpCas9) proteins to precisely target specific genomic loci, while also delivering additional functional domains has recently been leveraged to yield numerous molecular tools. For example, the commonly used SpCas9 has been repurposed to allow somatic hypermutation at endogenous target sequences by using catalytically inactive versions of Cas9 (referred to as dCas9) to recruit variants of activation-induced cytidine deaminase (AID). Using such programmable hypermutators, sequence diversity can be introduced at defined genomic loci in a mammalian cell context, allowing for the evolution of proteins and study of gene function, which may facilitate novel gene therapy approaches involving evolved envelopes, nucleases and base editors. In order to expand the number of loci and breadth of experiments that may be conducted with this strategy, we developed an inducible hypermutator using a catalytically dead *Staphylococcus aureus* Cas9 (dSaCas9). The use of an inducible approach may allow multiple rounds of evolution to be conducted, while minimizing toxicity. To do this, lentiviral vectors were used to stably deliver sequences encoding dSaCas9, a hyperactive AID variant fused with a MS2 coat protein (MCP) domain, as well as gRNAs containing MS2 aptamer sequences that recruit MCP-AID. As a proof of principle, we investigated changes at four regions of the EGFP coding sequence in HEK293 cells. Loss of EGFP expression was observed in doxycycline treated cells, indicating mutagenesis activity. The EGFP sequences of unsorted cell populations were then deep sequenced. Utilizing four guide sequences we observed the expected increase in the mutation rates of guanine and cytosine bases. Increases of substitution frequency of approximately 20-fold were observed within a window of 600 base pairs. The highest rates of mutation were observed within the guide sequence. The use of SaCas9 in this work allows for additional experiments to be designed, as SaCas9 operates orthogonally to SpCas9 and is substantially smaller. For example, such Cas9 orthologues could be used in parallel to evolve functionality in a mammalian context. Additionally, as SaCas9 has a different PAM sequence requirement than SpCas9 it expands the range of loci that can potentially be targeted by programmable hypermutators.

211. AAV-Mediated Delivery of Single Base Editors Using Intein Trans-Splicing

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Since their inception, CRISPR-Cas9 editing systems have gained widespread adoption for introducing targeted modifications in genomic DNA of living cells. However, their reliance on creating double

stranded breaks (DSBs) in DNA to achieve targeted modifications limits their therapeutic potential due to the unpredictability of the mutations created during repair of the DSB. Recently developed single base editors (SBEs) consisting of a deaminase domain fused to a Cas9 nickase overcome these limitations by introducing C>T or A>G targeted mutations without creating a DSB. SBEs have been employed for a wide variety of therapeutic strategies, such as correcting disease-causing single nucleotide polymorphisms or controlling gene splicing by selectively mutating splice donor and splice acceptor sites. While these tools show great promise for the treatment of human disease, the large size of the constructs that encode them has made in-vivo delivery a challenge. One effective delivery vehicle for *in vivo* gene therapy are adeno-associated virus (AAV) vectors. AAV are non-integrating viral vectors capable of infecting a wide range of cell types including heart cells, muscle cells, and neurons. However, one important shortcoming of AAV is its limited packaging capacity, which prevents the packaging of large open reading frames. To overcome the packaging limitation of AAV and enable in vivo delivery of SBEs, we have split the open reading frame of an adenine base editor (ABE) into two separate vectors through the use of *Rhodothermus marinus* inteins, which can be packaged and delivered as two separate AAV particles. Upon translation, the two intein peptides dimerize, and simultaneously excise themselves to reconstitute the full length ABE. We have tested this split-base editor architecture *in-vitro* using multiple cell lines and confirmed the introduction of precise A>G mutations at genomic target sites through both Sanger and high-throughput sequencing. Additionally, we were able to achieve programmable exon skipping using the split ABE for several gene targets as confirmed by RT-PCR and RNA-seq. Finally, we demonstrated that split base editors packaged into AAV can effectively infect cells and induce targeted exon skipping. We anticipate that *in vivo* delivery of SBEs will enable a wide range of future studies to expand their therapeutic relevance. We envision this system will gain widespread use as a new, minimally disruptive, and permanent treatment modality for a variety of genetic diseases.

212. A Novel Light-Inducible CRISPR/dCas9 System for Controlling Gene Expression

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Gene expression both directly influences and is altered by cellular activity, and rapid gene expression fluctuations are critical to normal cellular physiology and disrupted in many diseased states. Development of technology capable of mimicking these fine-tuned alterations has proven difficult, and currently available approaches suffer from significant drawbacks such as unintended interactions with endogenous proteins and leaky baseline effects. Through the fusion of the light regulated Flavin Kelch-repeat F-box1 (FKF1) and GIGANTEA (GI) elements with deactivated Cas9 (dCas9) and a transcription activator, we created a new system to target specific genes for upregulation in the presence of blue waveform (470nm) light. When this FKF1 Light Induced CRISPR Construct system (FLICC) is compared to an existing light-activated CRISPR effector system (LACE), FLICC confers several specific advantages. For example, previous reports suggest that FLICC will require shorter exposure times to harsh blue light than LACE to activate. Under the control of blue waveform light, FLICC successfully upregulated both endogenous gene targets at the mRNA level and a luciferase reporter at the protein level, allowing tight temporal control of gene expression. FLICC also demonstrates less leaky baseline gene upregulation relative to LACE at targeted loci. FLICC provides the tight temporal control of gene expression required to mimic fluctuations in gene expression at the time scale on which they occur, while offering specific benefits over currently available technologies. Future directions include using this system to dissect gene regulatory contributions to neuronal function in the context of experience-dependent plasticity and memory formation.

213. A Single Vector Approach in the Self-Inactivating KamiCas9 System with a Viral-Mediated Retrograde Transport

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Huntington's disease (HD) is a fatal neurodegenerative disorder caused by CAG expansion in the *huntingtin* (*HTT*) gene. Genome editing with the recently discovered CRISPR/Cas9 system represents an exciting alternative to tackle dominantly inherited genetic disorders such as HD. In a previous study, we developed a KamiCas9 self-inactivating editing system to achieve transient expression of the Cas9 protein and high editing efficiency using a double viral vector approach. Mutant huntingtin (HTT) was efficiently inactivated in mouse models of HD, leading to an improvement in key markers of the disease. Sequencing of potential off-targets with the constitutive Cas9 system in differentiated human iPSC revealed a very low incidence with only one site above background level. This off-target frequency was significantly reduced with the KamiCas9 system. In the present study, we improve this system in term of editing and delivery. At first, we combine the KamiCas9 in a single vector to avoid editing of the Cas9 gene during viral vector production in HEK-293T cells using the inhibitor proteins encoded by Listeria monocytogenes prophages (acrIIA4). Furthermore, to maximize viral-mediated gene transfer and retrograde transport, we took advantage of the neuronal circuitry using an improved version of HiRet lentiviral vector. This FuG/B2 envelope efficiently transduces neuronal and glial cells around the injection site and lead to high retrograde transport. This new generation of the KamiCas9 self-inactivating system will facilitate pre-clinical validation of gene editing in the CNS.

214. CRISPR/Cas9 Genome Nuclease Editing Induces Megabase Scale Chromosomal Truncations

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CRISPR/Cas9 is a promising technology for genome-editing. Using the congenital erythropoietic porphyria disease model, we demonstrate that using Cas9 nuclease-induced double strand break DNA (DSB), homology-directed repair is rare compared with the preferred non-homologous end-joining (NHEJ) repair pathway leading to in-target insertions/deletions (indels), and causing unwanted dysfunctional protein. Moreover, using FISH analysis with a subtelomeric probe at Chr10 p-arm and another one at Chr10 q-arm, we describe for the first time unexpected alarming chromosomal megabase-scale terminal truncations (up to 10% of transfected cells) resulting from only one Cas9 nuclease-induced DSB in cell lines (HEK293T and K562). High-resolution Array-CGH confirms on several clones the complete loss of chromosomal arm extremity exactly starting at CRISPR/Cas9 nuclease DSB. In addition, one clone has an unexpected 30 megabases duplication upstream DSB. This truncation is critical not only for our gene of interest (*UROS*) activity but also for the elimination of multiple downstream genes and telomere. We extend our data from cell lines to primary cells with higher impact for future CRISPR clinical application. We demonstrate that this chromosomal truncation can also occur in primary fibroblast cells risk if *TP53* is invalidated. Our findings reveal the global damaging effects of DSB on human genome. Further studies will be necessary to evaluate if chromosomal truncation risk is DSB locus dependent. We show that single nickase could be an efficient and a safer approach to edit genome: it drastically prevents in- and off-target indels and dramatically reduces chromosomal truncations. These results demonstrate that single nickase but not nuclease approach should be preferred to model disease, but more importantly for safe management of future CRISPR/Cas9-mediated gene therapies.

215. Efficient In Vitro and In Vivo Self-Repression of Spcas9 Gene and Reduction of Off-Target Mutations Using a Molecular Hara-Kiri Method

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The CRISPR/Cas9 system is currently a major revolution in the field of biology. Because of its simplicity compared to other endonucleases, this system is being experimented in diverse fields. However, a major disadvantage is the toxicity linked to sustained Cas9 expression. In the present study, we present an approach to effectively suppress the expression of the Streptococcus pyogenes Cas9 (SpCas9) gene. This approach that we call the molecular Hara-Kiri method, involves two sgRNAs targeting two sequences in the SpCas9 gene. The SpCas9 enzyme binds to the Protospacer Adjacent Motifs following the two sequences targeted by the sgRNAs and induces two Double Strand Breaks (DSBs) in its own gene (Hara-Kiri). The sequence located between the DSBs is then deleted. Most of the time, the SpCas9 gene is repaired by Non Homologus End Joining without INDELs. By adequately selecting the targeted sequences, the junction of the SpCas9 gene residues generates a TAA type stop codon within this truncated gene to effectively suppress its expression. This results in a dramatic decrease of the SpCas9 protein *in vitro* and *in vivo*. *In vitro* repression in 293T and *in vivo* in the muscles of the 52hDMD / mdx model mouse was made by two different methods. The first method, called immediate repression, was tested in 293T cells *in vitro* by transfecting a pBSU6 plasmid coding for two gRNAs (i.e., gRNAs 1-50 and 5-54 targeting exons 50 and 54 of the DMD gene) and the pX458 coding for SpCas9. The plasmid $pBSU6_{1\text{-}2SpCas9}$ coding for two gRNAs targeting the SpCas9 gene was also transfected simultaneously. The second method was a delayed repression, the plasmid $pBSU6_{1\text{-}2SpCas9}$ was transfected either 12h, 24h or 48h after the first transfection. The results obtained showed an efficient repression of the SpCas9 gene by the formation of a truncated gene with a stop codon of TAA at the junction point of the residues of SpCas9 gene in the samples transfected with pBSU61-2SpCas9. They also showed a dramatic decrease in Cas9 protein in the samples treated with the sgRNAs targeting SpCas9 compared to untreated samples. The results finally showed that the delayed repression allowed the editing of the DMD gene resulting in the formation of the hybrid exon 50-54 while avoiding the sustained SpCas9 expression. Similar results were also obtained *in vivo* in the 52hDMD/mdx model mouse, which contains a human DMD gene with an exon 52 deletion resulting in a reading frame shift. Two AAV9 were injected intra-venously, i.e., $AAV9_{SpCas9}$ coding for SpCas9, $AAV9_{1\text{-}50/5\text{-}54}$ coding two sgRNAs targeting DMD exons 50 and 54. Twenty-four later, the $AAV9_{1\text{-}2SpCas9}$ coding for the two sgRNAs targeting the SpCas9 gene was injected into the same mouse. The mice were sacrificed 4 weeks later. The formation of a truncated SpCas9 gene with a TAA stop codon was detected in the heart and skeletal muscles. A large decrease in the Cas9 protein was observed in the muscles of the mouse treated with $AAV9_{1\text{-}2SpCas9}$ compared to the untreated mouse. However, the formation of the DMD hybrid exon 50-54 was only slightly reduced. A study of off-target mutations with the Guide-Seq method indicated an important reduction of off-target mutations in 293T cells, which received the $pBSU6_{1\text{-}2SpCas9}$ (Hara-Kiri treatment). Our results demonstrated that the Hara-Kiri treatment could eventually reduces the risks of off-target mutations in therapeutic application of the CRISPR/Cas9 technology.

216. Recombinant R2 Retrotransposon for Targeted Integration of Large Genetic Cassettes into the Human Genome

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Maintaining stable and uniform transgene expression following delivery, particularly of large genetic constructs and in dividing cells, is a major challenge in gene therapy. Homologous recombination mediated insertion is promising but is limited by efficiency and insert size. DNA transposons can integrate large constructs, but the insertion sites are difficult to control or predict. A second class of transposons termed retrotransposons also has the ability to integrate large constructs, and some possess the unique capability to integrate this cargo site-specifically. We have developed to our knowledge the first retrotransposon-based system for the use of site-specific integration in mammalian cells using a human-codon optimized R2 from the silk worm *B. mori*. Our aim was to achieve *in vitro* expression of transgenes at a defined locus using a two-vector system involving of a donor plasmid and a retrotransposase construct. The transgene donor is flanked by targeting sequences from R2, all of which is transcribed into an RNA that is bound and reverse transcribed by the retrotransposase. We show that the system can successfully integrate DNA cassettes of >10 kb in both Human Embryonic Kidney (HEK) 293 cells and primary T cells at the defined target locus of R2, ribosomal DNA (rDNA). Junctions between the R2 construct and human genome at the target site were sequenced to confirm site-specific integration, and a full length integrant was successfully amplified. Additionally, we see a 5-fold increase the fraction of transgene-expressing cells in the presence of human-codon optimized R2 (5% GFP+) as compared to when only the donor is present (1% GFP+). This increase was further confirmed by a HEK293 colony counting assay of drug resistant colonies that survived 2 weeks under selection. We were also able to integrate and probe for expression of a Chimeric Antigen Receptor (CAR) in HEK293s as a proof of concept (1.8% CAR+). In sum, recombinant retrotransposons offer the potential for site-selective integration of large genetic constructs into the human genome, with broad applications for gene therapy.

217. Precise Therapeutic Gene Correction by a Simple Nuclease-Induced Double-Stranded Break

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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 methods 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. These limitations call for the development of other strategies that will enable precise correction of pathogenic mutations. Here, we show 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 demonstrate efficient repair of microduplications in patient-derived cell lines for two diseases: Limb-Girdle Muscular Dystrophy 2G (LGMD2G) and Hermansky-Pudlak Syndrome Type 1 (HPS1). Clonal analysis of *Streptococcus pyogenes* Cas9 (SpyCas9) nuclease-treated LGMD2G iPSCs revealed that ~80% contained at least one wild-type allele and that this correction restored *TCAP* expression in LGMD2G iPSC-derived myotubes. Efficient genotypic correction was also observed upon SpyCas9 treatment of an HPS1 patient-derived B-lymphoblastoid cell line (B-LCL). Inhibition of PARP-1 (poly (ADP-ribose) polymerase) suppresses the nuclease-mediated collapse of the microduplication to the wild-type sequence, confirming that precise correction is mediated by the MMEJ (microhomology-mediated end joining) pathway (Figure 1).

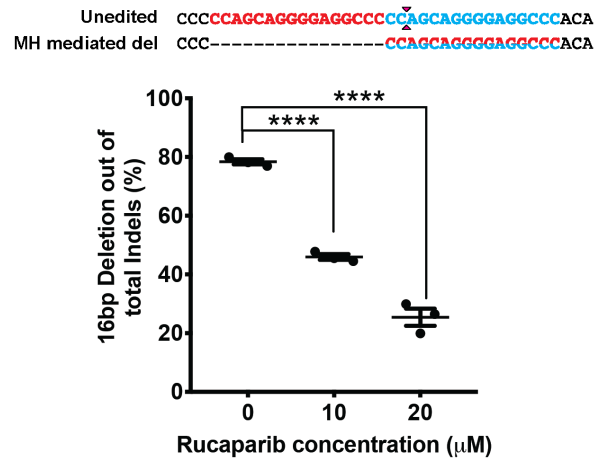

Figure 1. (Top) Schematic of *HPS1* 16bp microduplication (Unedited) (repeats shown in red and blue text). SpyCas9 induced DSB is denoted by magenta carets. The expected sequence upon MMEJ mediated collapse of the repeat is denoted by red/blue text (MH mediated del). (Bottom) Percentage of wild-type reverted alleles (16bp deletion) among all other edited alleles (indels) observed in patient-derived HPS1 B-lymphoblastoid cells treated with SpyCas9 in the presence of 0, 10 and 20 μM rucaparib based on UMI-based Illumina deep sequencing. Each value corresponds to the mean ±s.e.m. from 3 biological replicates. "****" indicates P value <0.0001 calculated using unpaired two tailed t-test.

Analysis of editing by SpyCas9 and *Lachnospiraceae bacterium ND2006* Cas12a (LbaCas12a) at non-pathogenic microduplications within the genome that range in length from 4 bp to 36 bp indicates that this correction strategy is broadly applicable to a wide range of microduplication lengths and can be initiated by a variety of nucleases. The simplicity, reliability and efficacy of this MMEJ-based therapeutic strategy should permit the development of nuclease-based gene correction therapies for a variety of diseases that are associated with microduplications.

218. Gene Delivery to Non-Human Primate Embryos Using AAV Vectors

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Delivering CRISPR reagents to mammalian zygotes for gene editing has greatly expedited the generation of genetically modified animals. However, the delivery of nucleic acid or proteins mostly relies on laborious and time-consuming procedures including microinjection, as well as specialized equipment and techniques that collectively incur substantial expenses and turnaround. Furthermore, such mechanical procedures face species-specific difficulties regarding practicality. For example, the bovine and porcine zygotes appear opaque due to lipid droplets, making the pronucleus not readily discernable for microinjection. Previously, we and others developed a simple method for gene delivery to mouse pre-implantation embryos using AAV vectors. This technique allowed us to conveniently deliver the Cre recombinase or CRISPR components to mouse zygotes by incubating with AAV vectors expressing the related genes, which enabled highly efficient genome editing and resulted in the generation of genetically modified mice (Yoon and Wang et al. Nature Communications 2018). Encouraged by the results obtained in rodent, we tested this approach in non-human primate (NHP). We found that cynomolgus monkey zygotes are also permissive to rAAV infection. Through a series of optimization steps, highly efficient gene editing (up to 100% in most embryos) can be achieved without detectable off-targeting or rAAV genome integration, consistent with our mouse study. We attempted to utilize this method to create NHPs modeling Canavan disease, an inherited lethal pediatric leukodystrophy caused by *ASPA* gene mutations, which would provide a valuable pre-clinical large animal model to test gene therapy for this currently untreatable disease. We obtained three aborted fetuses carrying *ASPA* gene editing, and two live births negative for editing. Possible reasons include, but not limited to, the small sample size and impact of *ASPA* gene editing on NHP embryo development. Together, our rAAV-mediated approach offers unparalleled ease and efficiency of gene delivery to pre-implantation embryos, and is potentially suitable to a broad range of mammalian species. In addition to gene editing and animal modeling, this method can also facilitate gene function studies in early-stage embryos. *Co-first authors; #Co-corresponding authors.

219. Carboxylated Branched Poly(Beta-Amino Ester) Nanoparticles Enable Non-Viral CRISPR/Cas9 Gene Editing via Intracellular Ribonucleoprotein Delivery

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Introduction: Direct intracellular delivery of CRISPR/Cas9 ribonucleoproteins (RNPs) reduces the probability of off-target editing by reducing RNP persistence time. Intracellular protein delivery is challenging, however, as naked proteins are generally membrane impermeable. In this study, we modified poly(beta-amino ester) s (PBAEs) with amino acid-like carboxylate ligands and examined their ability to enable intracellular protein delivery. We hypothesized that the carboxylate end-caps facilitate protein encapsulation through hydrogen bonding and salt bridges, resulting in a versatile protein delivery platform.

Methods: Carboxylate ligands were synthesized via acrylation of amino acid derivatives to yield a series of acrylated amino acids with varying numbers of carbons between the carboxyl and amide groups (ligands are referred to by the number of carbons). PBAEs were synthesized via a Michael addition reaction and end-capped with carboxylate ligands. Protein-encapsulated nanocomplexes were formed by allowing polymer and proteins to self-assemble in sodium acetate solution. **Results:** Nanoparticles encapsulating FITC-labeled bovine serum albumin were used to assess nanoparticle uptake in CT-2A murine brain cancer cells and human mesenchymal stem cells (MSCs); C5 and C7 polymers achieved the highest levels of uptake. Intracellular delivery of the ribosome-inactivating protein saporin demonstrated functional cell killing in several cell lines. Nanoparticles delivering FITC-labeled human IgG antibodies revealed FITC-IgG fluorescence distributed throughout the cytosol 5 hours after delivery using confocal microscopy imaging.

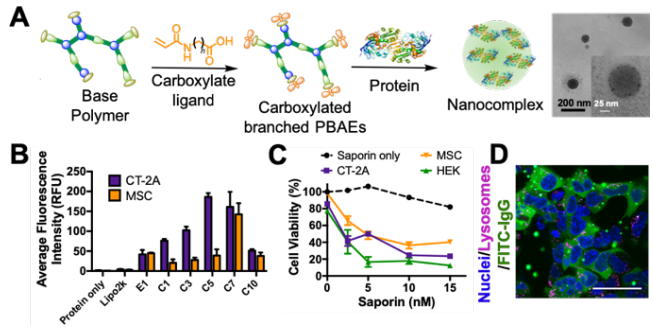

Figure 1.*(A)Assembly of carboxylated branched PBAEs with proteins. (B) Nanoparticle uptake using FITC-BSA reveals biphasic response. (C) Polymer C5 encapsulating saporin enables robust cell killing. (D) Distributed cytosolic fluorescence visible 5 hours after FITC-IgG delivery; scale bar = 50 μm.* CRISPR RNPs were encapsulated into nanoparticles approximately 180 nm in diameter. Over 50% knockout of GFP was observed in HEK-293T cells and GL261 murine brain cancer cells following a single administration of nanoparticles delivering 35 nM RNPs. To assess efficiency of gene deletion edits, we designed a reporter system whereby excision of a 630 bp stop cassette turns on

red-enhanced nanolantern (ReNL) fluorescence. Delivery of CRISPR RNPs targeting the stop cassette resulted in 23% editing in GL261 cells and nearly 45% editing in B16-F10 murine melanoma cells.

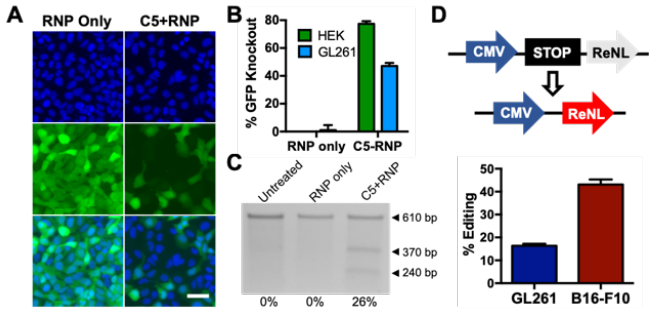

Figure 2.*(A) Fluorescence micrographs show GFP-null cells after Cas9 RNP delivery; scale bar = 50 μm. GFP knockout was quantified using (B) flow cytometry and (C) Surveyor® endonuclease assay. (D) CRISPR-mediated deletion of transcription stop cassette turned on ReNL expression.* **Conclusions:** Carboxylated branched PBAEs enabled high levels of cytosolic protein delivery and CRISPR gene knockout up to 80%. These polymers are a versatile protein delivery platform and a promising tool for gene editing applications.

Neurologic Diseases

220. AVXS-101 Gene Replacement Therapy for Spinal Muscular Atrophy: A Comparative Study with a Prospective Natural History Cohort

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Background: Spinal muscular atrophy type 1 (SMA1) is the leading genetic cause of infant mortality for which therapies, including onasemnogene abeparvovec (AVXS-101) gene-replacement therapy, are emerging. **Objectives:** This study compared the effectiveness of AVXS-101 in SMA1 infants with a prospective natural history cohort and a cohort of healthy infants. **Approach:** Between December 2014 and December 2015, 12 SMA1 infants received the proposed therapeutic dose of AVXS-101 (NCT02122952). Where possible, the following outcomes were compared with a natural history cohort of SMA1 infants (n=16) and healthy infants (n=27) enrolled between December 2012 and September 2014 in the NeuroNEXT (NN101) study (NCT01736553): event-free survival (composite endpoint of time to death or permanent ventilation), Children's Hospital of Philadelphia - Infant Test of Neuromuscular Disease (CHOP-INTEND score), motor milestone achievement, electrophysiology assessment (compound muscle action potential [CMAP]), and adverse events. **Results:** Baseline characteristics of SMA1 infants in the AVXS-101 and NN101 studies were similar with respect to age at first study visit or dosing (3.4 *vs* 4.0 months, respectively) and genetic profile (homozygous *SMN1* exon 7 deletions and 2 *SMN2* copies). The proportion of AVXS-101-treated infants who survived by 24 months of age was 100% compared with 37.5% in the NN101 study. The average baseline CHOP-INTEND score for NN101 SMA1 infants was 20.3, worsening to 5.3 by 24 months of age; average baseline score in AVXS-101-treated infants was 28.2, improving to 56.5 by 24 months of age. Eleven infants (92%) receiving AVXS-101 sat unassisted, and two stood and walked independently. Improvements in CMAP peak area were observed in AVXS-101-treated infants at 6 and 24 months of age (1.1 and 3.2 mV/s, respectively). During long-term follow-up, two patients gained the ability to stand with support. **Conclusions:** AVXS-101 significantly increases probability of survival, rapidly improves motor function, and enables motor milestone achievement in SMA1 infants.

221. Primary Mouse RPE as a Cellular Model of Oxidative Stress to Evaluate the Antioxidant Therapy

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The elevated oxidative stress and associated retinal pigment epithelium (RPE) damage are hallmark to incidence of age-related macular degeneration (AMD). Primary mouse RPE and human immortal APPE-19 cells were cultured under oxidative stress induced by blue light exposure at 4000 lux for 1 h. The blue light could induce oxidative stress damage in both cells. Western blot analysis showed that oxidative stress markers, hypoxia-inducible factor 1-alpha and 4-hydroxynonenal, were significantly upregulated. Fluorescent probes (MitoSox and live dead assay) indicated an increased level in ROS-oxidized products and a reduced level of cell viability in both types of cells. Compared to ARPE-19 cells, the mouse primary RPE cells exhibited a lower viability and were more sensitive to oxidative stress. After light exposure, we carried out the treatment using our previously studied glycol chitosan coated nanoceria as an antioxidant (namely glycol chitosan coated ceria nanoparticles (GCCNP)). The treatment significantly reduced blue light-induced oxidative stress in ARPE-19 and mouse primary RPE cultures. We showed that GCCNP significantly reduced oxidative stress and cell damage as revealed by Western blot, Mitosox, and live/dead assay. The cellular model we reported here provided a relatively easy and fast approach to obtain oxidative damage phenotypes in vitro for studying and evaluating the treatment using antioxidative nanomedicine.

223. CNS Anti-Phospho-Tau Gene Therapy for Chronic Traumatic Encephalopathy

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Chronic traumatic encephalopathy (CTE) is a devastating, debilitating neurodegenerative disorder of soldiers, contact sports athletes, and trauma victims, resulting from concussive traumatic brain injury (TBI) to the central nervous system (CNS). CTE is a CNS tauopathy, generated by the accumulation of hyperphosphorylated forms of the microtubule-binding protein Tau (pTau), resulting in neurofibrillary tangles and the progressive loss of neurons. There are no therapies to effectively treat CTE. We hypothesized that direct CNS administration of an adeno-associated virus (AAV) vector coding for an anti-pTau antibody would generate sufficient levels of anti-pTau antibodies in the CNS to suppress pTau accumulation. To evaluate this hypothesis, we developed a mouse model of CTE inducing the accumulation of pTau in the CNS by repeat traumatic brain injury using a closed cortical impact procedure and 2X/daily impacts at 3 m/s and 2.2 mm depth. C57Bl/6 mice (n=5 sacrificed at each of 6 wk, 3 and 6 months post impact) receiving a total of 10 impacts over 5 consecutive days developed high levels of CNS pTau for at least 6 months ($p<0.002$ compared to untreated). To assess the optimal antibody for treatment of CTE, the variable region of 2 different anti-Tau antibodies were cloned into AAVrh.10 gene transfer vectors (AAVrh.10PHF1, AAVrh.10IPN). Three wk after the impacts, the animals were randomly assigned to cohort groups (n=10 mice/group), and 10^{11} genome copies (gc; $5x10^{10}$ gc in 2 µl/hemisphere) of the anti-pTau vectors or control vector (AAVrh.10-Null) were administered directly to the hippocampus. Mice were euthanized 6 wk post-vector administration, the brains extracted and pTau levels were assessed by protein quantification and immunohistochemistry. Protein quantification data of pTau levels demonstrated that vector expressed antibodies PHF1 and IPN significantly reduced the pTau levels after impacts compared to untreated group (impacts, no AAV; $p<0.03$, $p<0.02$, respectively). As a further demonstration of the efficacy of this therapy, a detailed analysis of the mouse CNS cortical and hippocampal regions was assessed using immunohistochemical detection of pTau and ImageJ quantification. Mice treated with AAVrh.10PHF1 demonstrated marked suppression of the accumulation of pTau in the CNS in both the outer cortex and hippocampus regions (PHF1 *vs* untreated group in cortex, $p<0.0001$; or in hippocampus, $p<0.0002$). In summary, using doses that can be safely scaled to humans, direct CNS delivery of AAVrh.10 expression vectors coding for anti-pTau antibodies to the CNS of mice with repeated traumatic brain injury significantly reduced pTau levels across the CNS. This strategy provides new modality in the prevention of the CTE consequences of TBI.

224. CRISPR-Cas9 Gene Editing Strategies of ATXN2 for the Treatment of Spinocerebellar Ataxia Type 2

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Spinocerebellar ataxia type 2 (SCA2) is an autosomal dominant neurodegenerative disease characterized by progressive loss of coordinated movement. SCA2 is caused by a CAG repeat expansion within exon 1 of *ATXN2,* encoding mutant ATXN2 with an expanded polyglutamine (polyQ) tract. Cerebellar Purkinje cells and other neurons of the central nervous system (CNS) are affected in SCA2, with neuronal dysfunction and atrophy caused by a toxic gain-of-function mechanism. There are currently no effective treatments for SCA2. We and others have shown that RNA interference (RNAi) and antisense oligonucleotide (ASO)-mediated gene silencing strategies provide therapeutic benefit in SCA1 and SCA2 mouse models, respectively. However, these gene silencing strategies have their own unique limitations. To overcome these, we propose targeted gene editing of *ATXN2* at the DNA level with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein 9 (Cas9). We hypothesize that CRISPR-Cas9 editing of *ATXN2* will reduce toxic, mutant ATXN2 expression and provide therapeutic benefit in a SCA2 mouse model. For this, we are investigating two editing strategies: an indel mutation strategy with one sgRNA (1-sgRNA) and a deletion strategy to remove the CAG repeat with two sgRNAs (2-sgRNAs). With co-expression of the endonuclease, *Streptococcus pyogenes* Cas9 (SpCas9), we screened for candidate single-guide RNAs (sgRNAs) that target SpCas9 to *ATXN2* in HEK293 cells. Evaluation of genomic editing of *ATXN2* by PCR amplification and Sanger sequencing showed 1-sgRNA resulted in indel formation at the target site and 2-sgRNAs resulted in deletion of the CAG repeat. Both strategies reduced ATXN2 protein levels by western blot analysis. For *in vivo* application, we packaged AAV1 with an expression cassette encoding a neuronal-specific promoter driving expression of SpCas9, and, AAV1 with a Pol III promoter driving expression of either 1-sgRNA or 2-sgRNAs. We also included an eGFP reporter to visualize transduction biodistribution. Stereotaxic injection of AAV1 into the deep cerebellar nuclei (DCN) of the cerebellum of SCA2 mice resulted in widespread transduction of Purkinje cells and other cells of the cerebellum, and western blot of cell lysates showed reduced mutant ATXN2 protein levels with both 1-sgRNA and 2-sgRNA strategies. Our results indicate the feasibility of AAV delivery of CRISPR-Cas9 into the cerebellum of SCA2 mice to reduce mutant ATXN2 and suggest the potential for translating the therapy into humans.

225. Novel AAV Capsids Demonstrate Strong Retinal Expression in Non-Human Primates after Intravitreal Administration

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The eye represents a unique opportunity for the use of gene therapy due to its immune privileged status, relatively small size and the availability of non-invasive imaging to monitor safety and efficacy. Historically, AAV administration to the eye has been performed by subretinal injection between the neural retina and underlying retinal pigmented epithelium (RPE). While this method provides the benefit of positioning the vector directly next to its cellular target, it requires a retinal detachment, possibly damaging the retina, as well as an operating room and trained retinal surgeon, likely limiting the broad applicability of any therapy. Ideally, a therapy that is administered directly into the vitreous of the eye would provide a safer and likely more applicable approach. While testing of natural serotypes for their ability to reach the outer retina have failed after intravitreal injection, we have used both shuffled and rationally designed vectors to identify several promising AAV candidates. Here, we describe the identification of a novel AAV capsid that provides robust expression in both the inner and outer retina after intravitreal administration in both mice and non-human primates (NHPs). Using an EGFP reporter, we demonstrate broad expression in photoreceptors, bipolar cells and RPE in wild type mice dosed at 2.5E+10 vg/eye that shows a time dependent increase in expression. Administration of this novel AAV to NHP retinal explant cultures demonstrated similar or higher expression compared to naturally occurring AAV, including expression in the photoreceptors. To evaluate the potential for clinical translation, intravitreal administration to NHPs at 1.5E+12 vg/eye showed strong foveal expression as well as wide-spread expression in the peripheral retina within four weeks post-treatment. NHP retinal expression was highest in the photoreceptor cell layer, found in both rod and cone cells, bipolar, ganglion and RPE cells. Due to its broad cellular tropism, this engineered AAV capsid is clinically translatable to treat a wide range of retinal dystrophies, including both inherited and acquired forms. Importantly, this novel AAV can be administered using a relatively safe method that can be administered in an out-patient retinal clinic, allowing broader availability of any new therapy to previously under-treated communities.

226. XIST-Mediated Trisomy Silencing Enhances Neuron Formation in a Human Cell Model of Down Syndrome

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Although Down Syndrome (DS) is the leading genetic cause of intellectual disability in children, the developmental pathogenesis remains largely unknown, and better strategies are needed to investigate this. We previously showed that one copy of chromosome 21 can be epigenetically silenced in DS iPSCs by insertion of an *XIST* transgene, which produces a non-coding RNA that normally silences one X chromosome in female cells. *XIST* was shown to induce heterochromatin and silence transcription across chromosome 21 in pluripotent stem cells, the natural developmental context of *XIST* function. Prior literature indicated that initiation of chromosome silencing is only possible within 48 hours of mES cell differentiation, however it would be highly advantageous experimentally if trisomy silencing could be initiated in differentiated cells, and this is critical for any therapeutic potential of *XIST*. Here we use RNAseq and molecular cytology to investigate the effectiveness of *XIST* for trisomy silencing in cells undergoing *in vitro* neural differentiation and examine the potential cell phenotypic effects of chromosomal silencing. Induction of *XIST* from the onset of differentiation resulted in comprehensive silencing of chromosome 21 genes, providing a powerful approach to examine effects of trisomy on neurogenesis. To determine whether human neural stem cells can initiate *XIST*-mediated silencing, we induced *XIST* at several times during neural differentiation. We demonstrate for the first time that differentiated normal human cells can initiate chromosome silencing in response to *XIST* expression. While this process only takes a few days in pluripotent cells, we show that it takes 2-3 weeks in differentiated cells. Importantly, single-cell RNAseq revealed that cells which express *XIST* preferentially differentiate into neurons, providing evidence of phenotypic improvement with trisomy silencing, which is seen even when *XIST* is initiated weeks into differentiation. We are currently investigating whether silenced neurons show other non-chromosome 21 transcriptional changes suggestive of potentially improved function. These studies have important implications for the understanding of *XIST* biology and DS neurobiology, and also further open the possibility of a *chromosomal* therapy for DS using a single gene: *XIST*.

227. Using MAPseq to Assess Serotonergic Hyperinnervation in Non-Motor Circuits in the Parkinsonian Rat

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It is becoming increasingly clear that non-motor symptoms (NMS) in Parkinson's disease (PD) comprise a significant portion of debilitating features of disease, and such symptoms are often more debilitating than the motor symptoms themselves. Despite its initial effectiveness, chronic L-DOPA administration leads to motor side effects such as L-DOPA-induced dyskinesias (LID) in up to 90% of patients and NMS in as many as 60% of patients. Reports have revealed extensive modifications to serotonergic (5-HT) innervation of the striatum as dopamine (DA) cells are lost, and that these 5-HT terminals are crucial in the formation of LID. However, it is unclear what changes occur in other areas of the brain. To address this we utilized an AAV reporter virus to label the dorsal raphe (DR) 5-HT innervation in the hemiparkinsonian rat. 5-HT innervation from the DR was dramatically enhanced in areas such as the amygdala and prefrontal cortex, suggesting that changes in 5-HT circuitry may be responsible for NMS. In order to better understand the role of this circuitry in

NMS we generated a projection-specific viral vector approach whereby specific DR 5-HT terminals are targeted using novel AAV vectors with retrograde transport capacity, and paired with viral vectors expressing regulatory elements specifically in the 5-HT soma. However, in order to achieve single circuitry precision we need to better understand individual projections. Thus, in ongoing experimentation we are using Multiplexed Analysis of Projections by Sequencing (MAPseq) in combination with AAV, DNA barcoding, and Next Generation Sequencing in order to fully map each individual 5-HT projection from the dorsal raphe. This approach will systematically determine how neuroadaptation in 5-HT innervation and function modulate DA neurotransmission in the parkinsonian brain, facilitating therapeutic targeting of such maladaptive changes to optimize NMF treatment and therefore quality of life for PD patients.

228. Transfer of Therapeutic miRNAs within Extracellular Vesicles Secreted from Huntington's Disease iPSC-Derived Neurons

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Huntington's disease (HD) is a neurodegenerative disorder caused by an autosomal dominant mutation in the huntingtin gene (HTT), which leads to mutant protein aggregation, toxicity and neuronal cell death. microRNA (miRNA)-based gene therapies have emerged as a potential approach to lower the disease-causing protein in HD. We have developed an engineered microRNA targeting human huntingtin (miHTT), delivered via adeno-associated serotype 5 (AAV5) virus. AAV5-miHTT have demonstrated an efficient lowering *in vitro* and *in vivo* in the brain in different HD animal models. For adequate efficacy in HD, brain-wide biodistribution (striatum, cortex) of the therapeutic miRNA is of crucial importance. Recently, extracellular vesicles (EVs), and in particular exosomes and microvesicles, have been identified as carriers of RNA species. Therefore, understanding how therapeutic miRNAs are transferred between neuronal cells might be relevant for delivery, translational studies and biomarker discovery in gene therapies for brain disorders. The aim of this study was to investigate the presence of miHTT molecules within EVs secreted from AAV5-miHTT-treated neuronal cultures originating from an HD patient. For this purpose, we developed an *in vitro* model based on the differentiation of induced pluripotent stem cells (iPSCs) from HD patients to mature neuronal cells. iPSC-derived neuronal cultures were transduced with AAV5-miHTT and the cultured medium was collected for the precipitation of EVs. We observed a dose-dependent transduction of neuronal cells and a dose-dependent expression of miHTT in the cells measured by TaqMan qPCR. At 5 days and 12 days after transduction we detected mature miHTT molecules within EVs enriched for exosomes and microvesicles secreted from AAV-transduced iPSC-derived neurons. EV-associated miHTT levels strongly correlated with AAV dose and miHTT expression in neuronal cells. Moreover, isolated EVs were taken up by other naïve neuronal cells resulting in a concentration-dependent transfer of miHTT molecules to recipient cells. This study indicates a contribution of EVs to the transfer of therapeutic molecules for miRNA-based gene therapies for HD. EV-containing therapeutic miRNAs can thus be regarded as novel mediators of cell-cell communication and as potential source of biomarkers in neurodegenerative disorders.

229. Therapeutic Efficacy of Gene Therapy for Tuberous Sclerosis Type 2 by Delivery of AAV Encoding a Condensed form of Tuberin (ctuberin) in a Mouse Model

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Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder caused by second hit somatic mutations in tumor suppressor genes, *TSC1* or *TSC2,* encoding hamartin or tuberin, respectively. These proteins act as a complex which inhibits mTOR-mediated cell growth and proliferation. Loss of either protein leads to overgrowth of cells in many organs, most commonly affecting the brain, kidneys, skin, heart and lung. Gene therapy using adeno-associated virus (AAV) is a promising strategy to correct this disease by providing a replacement cDNA for the mutated allele of *TSC2*. Unfortunately, the size of tuberin cDNA exceeds the packaging capacity of AAV. Here, we engineered a condensed form of tuberin (ctuberin) comprised of discreet functional domains of the parent protein, which can be encoded in an AAV vector. We found that ctuberin was functional and non-toxic in culture assays. To test its therapeutic efficacy we used a stochastic mouse model of TSC brain lesions in which loss of *Tsc2* is achieved in multiple cell types in the brain by injection of an AAV1-Cre recombinase vector into the cerebral ventricles of homozygous *Tsc2*-floxed mice around the time of birth (P0 - P3). This leads to a shortened life span (median survival 60 days) and brain pathologic findings, including subependymal lesions, consistent with TSC. Remarkably, when these mice were then injected intravenously (IV) on day 21 with an AAV9 vector encoding ctuberin, most survived for more than 280 days (ongoing) in an apparently healthy condition. This study demonstrates the feasibility of treating life-threatening TSC2 lesions with IV injection of an AAV vector encoding ctuberin. This gene therapy approach has the advantages that therapy can potentially be achieved from a single application, as compared to repeated treatment with drugs with side effects, that an "extra copy" of the tuberin gene-equivalent may protect other cells from consequences of subsequent mutations in the normal allele.

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230. Multi-Modal Gene Therapy for Neuropathic Pain

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Background and Aims: Neuropathic pain is chronic pain state syndrome and has complex underlying pathophysiology. Current neuropathic pain treatment regimens employ anticonvulsants, antidepressants, and opioids. These single target analgesic treatments

have limited analgesic efficacy and show low patients' responsiveness. Simultaneous targeting of multiple pathologic elements in neuropathic pain is required to develop effective neuropathic pain medicine. KLS-2031 consists of two AAV vectors expressing three transgenes (AAV-hGAD65 and AAV-hGDNF/hIL-10) to control the major pathological mechanisms of neuropathic pain, including nerve injury, neuroinflammation, and neuronal hyperactivity. In this study, we evaluated KLS-2031 as a novel therapeutics for neuropathic pain. **Methods:** The model system utilized in this study was a rat spared nerve injury (SNI) model. KLS-2031 was administered one time into a lumbar vertebra 4 (L4) intervertebral foramen using micro-catheter for targeted delivery of the drug to peri-dorsal root ganglion (DRG) space. Pain-related behavior was measured with von Frey filament and Hargreaves test. The effects of KLS-2031 on nerve injury, stress, and neuronal inflammation were assessed by the expression of activated caspase3, activating transcription factor 3 (ATF3), and ionized calcium binding adaptor molecule 1 (Iba1). The effects of KLS-2031 on sensitization were evaluated by electrophysiology study such as the current clamp method for DRG neuronal excitability, the voltage clamp method for synaptic plasticity in spinal cord, and the in vivo extracellular recording technique for network activity of wide dynamic range (WDR) neuron in the spinal cord. **Results:** KLS-2031 showed significant analgesic effect, i.e., mechanical allodynia and thermal hyperalgesia in the SNI model. KLS-2031, the three gene combination vectors, significantly reduced mechanical allodynia in the SNI model compared to the single or two gene combination usage. Furthermore, KLS-2031 showed to be more potent on reducing the expression of ATF3, activated caspase 3 and Iba1 than GDNF or IL-10 alone respectively. When the long-term analgesic effect was evaluated with the von Frey test, the effect was maintained for up to ten weeks in both female and male rats. Pathological features of neuropathic pain, such as the hyperexcitability of DRG neurons, impairment of primary afferent synaptic plasticity in the spinal cord, and the hyperactive activity of spinal WDR neurons were restored to the normal level when the SNI model received TF injection of KLS-2031. **Conclusions:** KLS-2031 is being developed as combinational gene therapy to treat multiple aspects of pathophysiology involved in neuropathic pain. Gene combination approach turned out to be more potent than single gene approach in analgesic effect and restoring pathophysiology of neuropathic pain. Especially, KLS-2031 effectively normalized the peripheral and central sensitization of pain with a single administration. Based on these result, KLS-2031 could be a promising therapeutic option for refractory neuropathic pain and be a disease-modifying agent.

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

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GM3 synthase (GM3S) deficiency is a rare monogenic neurological disorder common within Old Order Amish communities due to an *ST3GAL5* c.862C>T founder variant segregating with a population-specific carrier frequency of ~4%. GM3S mediates synthesis of GM3, which serves as the common precursor for all cerebral gangliosides. GM3S deficiency abolishes ganglioside biosynthesis. *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. Gene replacement to restore cerebral GM3S protein could rescue this severe neurodevelopmental phenotype and serve as a paradigm for treating similar neurogenetic disorders. Here, we report preliminary results using recombinant adeno-associated virus (rAAV) to deliver human *ST3GAL5* to mice. Previous studies indicate that one mRNA variant (hST3GAL5-1a-2) is the most abundant isoform in human tissues, including brain. However, hST3GAL5-1a-2 can produce at least three protein products that initiate from different start codons. Therefore, we generated multiple h*ST3GAL5* constructs that differ in their initiating start codons, codon optimization, and the presence of the full length 5'-UTR. We first examined their expression efficiency in HeLa cells, which normally have no detectable endogenous *ST3GAL5* expression. As expected, all constructs expressed the desired GM3S isoform and the 5'-UTR reduced gene expression. Assuming that high cerebral expression of GM3S is necessary to optimize neurological rescue, we further pursued constructs without the 5'-UTR; these restored GM3 production in patient-derived fibroblasts, indicating their strong therapeutic potential. We are currently validating patient iPSC-derived neurons to further explore this effect. Based on these *in vitro* data, we packaged h*ST3GAL5* constructs into AAV-PHP.eB capsids that efficiently target murine neurons and astrocytes. To examine the safety and efficacy of transgene expression, we injected AAV vectors via facial vein into neonatal wild type mice and observed no dose-related toxicity. AAV vector delivery and GM3S protein over-expression in the brain were quantified by Droplet Digital PCR (ddPCR) and western blot, respectively. Encouraging results support further development of rAAV-based h*ST3GAL5* replacement vectors for application to *St3gal5-/-* and *St3gal5-B4galnt1* double knockout mice. This represents first gene therapy approach to GM3S deficiency, and has the potential to translate directly into clinical application. *Co-corresponding authors

232. Developing Gene Therapy for Treating Intractable Epilepsy in Dravet Syndrome

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Dravet Syndrome, also known as Severe Myoclonic Epilepsy of Infancy (SMEI), is a genetic disorder characterized by sudden unexpected death in epilepsy (SUDEP), febrile seizures, and autism-like behaviors. Approximately one in 15,700 individuals is diagnosed with this disorder, typically between the ages of one and three. Most patients become resistant to anti-convulsant drugs and therefore there is no long-lasting, effective treatment available for these patients. Eighty

percent of Dravet Syndrome patients have loss-of-function mutations in the SCN1A gene, impairing the function of a transmembrane protein, the voltage-gated sodium channel alpha subunit 1 (Nav1.1) that mediates action potentials in neurons. We hypothesize that selectively expressing an adeno-associated virus (AAV) vector encoding a sodium channel subunit in inhibitory interneurons of the CNS will rescue or mitigate the Dravet Syndrome phenotype in *Scn1a* heterozygous (*Scn1a*$^{+/-}$) mice, a model of Dravet Syndrome. This mouse model has reduced expression of Nav1.1 which impairs the action potential initiation and propagation in neurons. This mutant mouse line also displays characteristics similar to those of Dravet Syndrome patients including SUDEP, spontaneous generalized tonic-myoclonic seizures, heat-induced seizures, and autistic features. Treatment of *Scn1a*$^{+/-}$ mouse pups with the therapeutic vector via injection into the cerebral spinal fluid at postnatal day two resulted in a wide distribution of the transgene in the brain and expression of the sodium channel subunits in GABAergic neurons. Treatment also increased the average lifespan and ameliorated several autism-like behaviors. Our findings indicate that AAV-based gene therapy has therapeutic benefits in a mouse model of Dravet Syndrome. This research is supported by the Canadian Institutes of Health Research, Dravet Canada, and the Dravet Syndrome Foundation, USA.

233. Development of Adeno-Associated Virus (AAV9)-Mediated Gene Delivery to Treat a Mouse Model of Cockayne Syndrome

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Cockayne syndrome (CS) is a rare, autosomal recessive disease characterized by neurodegeneration and premature aging. CS is caused by mutations in various genes involved in DNA repair mechanisms. There is no disease modifying therapy currently available for these patients. One subtype of CS is caused by mutations in the *ERCC5* gene that encodes the Xeroderma Pigmentosum group G (XPG) endonuclease protein. The hybrid C57BL6/FVB F1 Xpg$^{-/-}$ mouse model developed by Drs. Hoeijmakers and Vermeij replicates the CS phenotype well. Our evaluations in Xpg$^{-/-}$ mice as compared to age and sex matched healthy controls (n = 10 per cohort) have revealed lower birthweights, decreased growth, reduced response to incline in negative geotaxis tests at 1 week of age, hindlimb clasping by week 3 (a sign of neurodegeneration), kyphosis development at 9 weeks, tremor development at 10 weeks, and ataxia at 12 weeks. Whole body ActiTrack analyses revealed significant decreases in distance travelled, number of rearings to cross a horizontal plane, and increased time at rest by 3 weeks of age which all became even more dramatic by 12 weeks of age. Xpg$^{-/-}$ mice display growth impairment by 10 weeks of age and reach a moribund state between 14-17 weeks of age. We developed an adeno-associated virus (AAV) mediated *ERCC5* gene replacement strategy to determine whether this approach can prevent development or reduce severity of the CS phenotype in these mice. AAV9-CMV-*ERCC5* was intravenously administered to Xpg$^{-/-}$ mouse 1 day old neonates (n ≥ 8 per cohort) at one of the following doses ($5x10^{12}$, $1x10^{13}$, $3x10^{13}$, or $3x10^{14}$ vg/kg) via the temporal vein. Although most outcome measures were not significantly improved in the two lowest dose treatment groups, tissue biodistribution and gene expression analyses did revel approximately 1.5 to 2 fold increased expression in all tissues assessed. Both low dose treatment groups also showed on average 3 to 4 weeks of increased survival as compared to untreated controls and brain histological analyses demonstrate normalization of nucleus sizes in treated mice which suggests some improvement in the underlying DNA repair disorder. Studies to fully evaluate the two higher treatment cohorts are underway. Currently, (at 15 weeks of age) no mice from the higher dose treatment cohorts have displayed any signs of tremors, kyphosis, or ataxia, and none have reached a moribund state. ActiTrack analyses have revealed normalization of distance travelled, rearings, and resting times in both of the high dose treatment cohorts at 12 weeks of age suggesting significant improvements in whole body activity ability. Evaluations will continue to 24 weeks of age at which time full necropsies will be performed for further analyses of vector genome biodistribution, *ERCC5* transcription, histological evaluations, and other protein studies. In summary, our data our promising thus far and support further development of gene replacement strategies to treat Cockayne Syndrome.

234. The Adeno-Associated Viral Anc80 Vector Efficiently Transduces Hair Cells in Cynomolgus Macaques (M. Fascicularis): Development of a Non-Human Primate (NHP) Model for Cochlear Gene Therapy

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Gene therapy is a promising approach to address both genetic and acquired hearing loss, and Anc80-mediated delivery of healthy genes of interest has successfully recovered cochlear function in rodent models of genetic deafness. An intermediary model system that is anatomically, developmentally, and immunogenically more similar to humans is important for translating these findings to the clinic. Previous work demonstrated efficient transduction of NHP inner hair cells with Anc80L65 (Andres-Mateos et al., 2018). Here, we extend those findings to demonstrate the dose-response of transduction efficiency and preliminary safety of Anc80L65 in 6 pre-pubescent monkeys with relatively low levels of pre-existing neutralizing antibodies (nAbs) against the vector capsid. Monkey pairs were assigned at random to receive one of three doses of Anc80L65 vector encoding a green-fluorescent protein (GFP). Following a single intracochlear injection and a 3-wk survival, serum and CSF were collected, and cochleae were harvested. Using hair-cell and anti-GFP immunolabels, we quantified hair-cell survival and the efficiency of GFP expression in surviving hair cells at 9 logarithmically-spaced frequency positions spanning

the length of the cochlear spiral. Transduction efficiency was dose-dependent and graded along the length of the cochlea. The minimal hair-cell loss observed in both injected and uninjected control ears was not dose-dependent. Serum nAb levels were similar to pre-injection levels for two of the three doses tested, as determined by relative inhibition of transduction in an *in vitro* assay, and CSF nAb levels were similar to pre-injection serum nAb levels. These data, demonstrating efficient transgene expression and preliminary safety in NHPs, support our approach of intracochlear injections of Anc80L65 as a promising strategy to evaluate in human clinical trials.

235. Impact of the Age of Administration of AAV9 Murine and Human Bicistronic Hexosaminidase Vectors in Sandhoff Mice on Correction of GM2 Gangliosidosis

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GM2 Gangliosidoses are severe neurodegenerative diseases caused by a Hexosaminidase A (HexA) enzyme deficiency leading to the neurotoxic accumulation of GM2 gangliosides (GM2). The HexA enzyme is comprised of an α- and β- subunit, and complexes with the GM2 activator protein to breakdown GM2. Mutations in the *HEXA*, *HEXB*, or *GM2AP*cause deficiencies in the α-, β- subunit, or GM2AP, giving rise to Tay Sachs Disease (TSD), Sandhoff Disease (SD), or the AB-variant disease, respectively. The infantile form of GM2 Gangliosidosis are fatal by 4 years old, and there are no current treatments. Here, we evaluated the impact of the age of administration of gene therapy. AAV9 vectors containing either a human bicistronic (h*B-A*), a murine bicistronic (m*b-a*), or a murine hexb only (m*hexb*) transgene were administered intravenously to SD mice at 0 weeks of age (at 2.5E11vg/mouse or ~2.5E14vg/kg) or 2, 4 or 6 weeks of age (at 1E12vg/mouse, 1.88vg/mouse, and 2.5E12vg/mouse (respectively) or ~1.25E14vg/kg). Half of the mice in each cohort were euthanized at 16 weeks, equivalent to the humane endpoint for untreated controls. The rest were observed until their humane endpoint. Mice treated at 0, 2, 4, and 6 weeks with the h*B-A* vector survived to a median age of 59, 68.4, 43.6, and 43.8 weeks and mice treated with the m*b-a* vector survived to median age of 42.2, 76.8, 65.8, and 68.14 weeks, respectively. Mice treated with the m*hexb* vector at 0 and 6 weeks are currently surviving to a median age of 65.72 and 70.71 weeks respectively, with one mouse from the 0 week *mhexb* group still alive past 95 weeks of age. All the survival curves are significant vs vehicle controls ($p<0.001$). A significant increase in behavioural performance was observed at 16 weeks in all treatment groups across all ages of administration ($p<0.01$). Preliminary analyses showed slight increases of midbrain HexA activity across all treatment groups and ages of administration, significant decreases in GM2 storage in the h*B-A* and m*b-a* groups at all ages of administration ($p<0.001$), and constant h*B-A* biodistribution across the midbrain at all ages of administration. Anti-hB-A antibodies arose in mice receiving hB-A at 6 weeks of age ($p<0.01$). A cross-species antibody response was expected; the human bicistronic vector may not elicit an immune response in patients. Study limitations include the existence of an alternative sialidase pathway for GM2 catabolism in mice, the differential affinity of the subunits, the immature blood-brain barrier in mice at birth, and the possible interaction of the human HEXA enzyme with the murine GM2AP, each of which may have enhanced efficacy. Nevertheless, this preliminary data demonstrates that a bicistronic hexosaminidase AAV9 vector may be a viable option for the treatment of SD and TSD in humans.

236. Liver-Targeting Gene Editing Achieves Significant Neurological Benefits in MPS I Mice

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Gene therapy is promising for treating lysosomal diseases due to its potential for a permanent, single-dose treatment. Currently, there lacks treatment protocols providing sustained therapeutic benefits with minimized risks for patients with lysosomal diseases. To this end, we designed a PS-813 gene editing system to integrate a promoterless alpha-L-iduronidase (IDUA) cDNA sequence into the albumin locus. To evaluate this strategy, 2 AAV8 vectors delivering the PS-813 system were injected into neonatal MPS I mice at different doses. Plasma IDUA activities reached 750 fold of wildtype levels in treated MPS I mice (n=13). IDUA activities in the heart, liver and spleen increased to 2.7-fold, 19-fold and 4.6-fold of wildtype levels, respectively. More importantly, IDUA activity in the brain increased to 75% of wildtype levels. Further, glycosaminoglycan levels in these tissues including the brain reduced to normal levels. At 10 months post-dosing, the fear conditioning showed that treated MPS I mice had better memory and learning ability, while the pole test showed that treated MPS I mice had better neuromotor function. Kaplan-Meier survival analysis showed that the survival of treated MPS I mice was significantly better than controls. This gene editing strategy will enable us to use lower dose of AAV vectors for treating lysosomal diseases, which brings minimized risk, ease of vector production and less expense.

237. Codon-Optimized, Reduced-Size ATP7A Plus Subcutaneous Copper Histidinate for CSF-Directed AAV Gene Therapy in Two Menkes Disease Mouse Models

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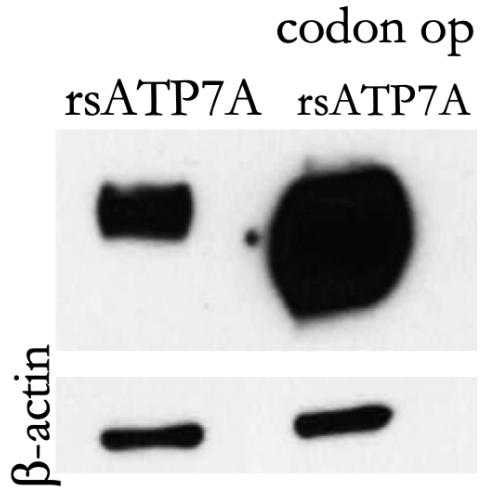
Menkes disease is a X-linked recessive neurodegenerative disorder caused by mutations in the highly conserved copper transporter, *ATP7A*. Untreated affected children typically die by three years of age from copper deficiency, notably in the brain. The *mottled brindled* (*mo-br*) mouse is a naturally occurring mammalian model with an in-frame deletion in the mouse ortholog (*atp7a*), that accurately recapitulates the disease phenotype. Animals exhibit depigmented

coats, curly vibrissae, failure to thrive, ataxia, and premature death around 2 weeks of age, stemming from copper deficiency in the blood and in the developing mouse brain. We recently showed that CSF delivery of recombinant AAV9 carrying a reduced-size version of the human ATP7A complementary DNA (rsATP7A), in combination with subcutaneous clinical grade Copper Histidinate, improved median survival (300 days) and enabled >50% long term rescue of the mo-br mice on a C57BL/6 background. Neither treatment alone rescued the mutant mice. In an effort to futher refine this AAV gene therapy approach, we generated a codon-optimized version of rsATP7A to increase the expression level of the transporter in the brain. Expression in cultured HEK293T cells documented 20-fold higher levels of co-rsATP7A in comparison to the non-codon optimized rsATP7A (Figure). We are currently evaluating AAV9-co-rsATP7A doses ranging from 5e+8 vg to 1.6e+10 vg in combination with variable subcutaneous doses of clinical grade CuHis in the *mo-br* mutation on two different genetic backgrounds (C57BL/6, and CBA/C3H). Preliminary evidence indicates approximately five-fold increased expression in mouse brain in vivo. Utilization of co-rsATP7A may enhance safety and efficacy in a future first-in-human pilot gene therapy clinical trial for this illness.



238. CRISPR/Cas9-Mediated Allele-Specific Disruption of a Mutant Human Rhodopsin Gene in a Transgenic Mouse Line Modeling Autosomal Dominant Retinitis Pigmentosa

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Retinitis pigmentosa (RP) is a group of inherited retinal diseases characterized by progressive death of photoreceptors. Mutations in the rhodopsin (RHO) gene are the most common cause of autosomal dominant RP (adRP), with no treatment available. The CRISPR/Cas9 genome editing technology has been widely used for gene disruption, which provides a new avenue for developing treatment of *RHO*-associated adRP. The threonine-17 to methionine (T17M) mutation caused by a single base-pair change (C to T) is a class II human rhodopsin mutation. In the present study, we tested if the CRISPR/Cas9 approach could specifically disrupt the T17M human allele in a transgenic mouse line and rescue the disease phenotype. Another transgenic mouse line carrying the proline-347 to serine (P347S) human rhodopsin mutant allele was used to control for off-target events of genome editing, as it does not have the mutation accounting for the T17M change. A small guide RNA (sgRNA) was designed to target the mutant T17M allele, with PAM sequence located right adjacent to the the single-base mutation. Two Cas9 nucleases, SpCas9 and eSpCas9 which reportedly has a higher specificity were used and compared. The Cas9 and the sgRNA expression cassettes were packaged into type 8 adeno-associated viral vectors (AAV8) respectively. Each T17M or P347S mouse, aged at post-natal day 14, received subretinal administration of vectors carrying Cas9 and the sgRNA against the mutant T17M allele (sgRNA-T17M) in one eye, and vectors carrying Cas9 and the sgRNA against EGFP in the control fellow eye. Electroretinography (ERG) was used to examine retinal function of these mice when they were 3-month-old. An initial high dose of the SpCas9 vector (2.5 e+09 vg/eye) together with the sgRNA-T17M vector (2.5 e+09 vg/eye) resulted in markedly higher ERG amplitudes in the therapeutic eyes than in the control eyes in the T17M mice, suggesting an efficient genome editing at the mutation site. However, higher ERG response was also observed in eyes of P347S mice receiving the same treatment, implying that the editing may also occur at the WT allele. Sanger sequencing of PCR amplicons spanning the mutant locus confirmed the on-target and the off-target events. When a lower dose of the SpCas9 vector (1.0 e+09 vg/eye) was used, a better ERG response of therapeutic eyes than control eyes was observed only in the T17M mice, suggesting a reduced off-target effect at the WT allele. However, Sanger-sequencing results indicated that the off-target effect was not completely abolished. Treatment of the eSpCas9 vector (7.5 e+09 vg/eye) together with the sgRNA-T17M (2.5 e+09 vg/eye) resulted in a slight but statistically significant ERG improvement in T17M mice but not in P347S mice. Sanger sequencing only detected on-target editing events while no off-target events were observed. Our study demonstrated the feasibility of using CRISPR/Cas9 genome editing to disrupt a human mutant allele for treatment of *RHO*-associated adRP caused by a single-base mutation. Our current work is focused on increasing the editing efficiency while minimizing the off-target effect.

239. Evaluating Gene Editing for Rhodopsin Linked Autosomal Dominant Retinitis Pigmentosa

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Rhodopsin is the visual pigment that mediates vision under dim light. Mutations in the rhodopsin gene are the most common cause of the blinding disease autosomal dominant retinitis pigmentosa, (adRP), with P23H being the most prevalent mutation found in the US. Disruption of the diseased RHO mutant while preserving the wild-type (WT) functional allele is a potential therapy for this disease. Using a

cellular model of adRP, HEK-293 cells containing an integrated copy of the hP23H Rho locus, we validated a gene editing construct designed to specifically inactivate the P23H RHO mutant, without affecting the WT allele. DNA sequencing results confirmed that the hP23H locus was specifically targeted, leaving the wt Rho locus unaffected. The insertion/deletion (in/del) frequency of the hP23H Rho locus was 15%, while no insertions or deletions were detected at the wt hRho locus. Digital droplet PCR (ddPCR) analysis was also used to analyze the in/del frequency at the hP23H locus in these cells, these data demonstrated an in/del frequency of 11%. Next we designed a gene editing approach that allows for broader target sequence specificity by relaxation of the DNA recognition site. This strategy allows for all *Rho* sequence variants to be targeted in a mutation independent manner. HEK-293 cells containing an inserted copy of either the mouse or human Rhodopsin locus were transfected with a pan-Rho gene editing construct. A nuclease assay confirmed the disruption of both the mouse and human wt Rho locus. Data suggest that this construct could be paired with a gene editing resistant wtRho replacement sequence for a Rho ablation/replacement strategy for the treatment of all Rho causative adRP mutations. Our gene editing constructs are designed to eliminate the size restriction typically imposed by AAV, and allow use of a single AAV gene therapy vector. As a prelude to evaluating the gene editing constructs in the mouse retina, an AAV hP23H targeting vector was generated and used to infect P23H expressing cells; ddPCR analysis confirmed disruption of the hP23H locus. This result supports the further ongoing evaluation of this AAV gene editing vector in a P23H mouse model of adRP.

240. Multiple Novel Engineered AAV Capsids Demonstrate Enhanced Brain and Spinal Cord Gene Transfer after Systemic Administration in Adult Mice

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Adeno-associated viral (AAV) gene therapy vectors are a promising new class of treatment for diseases of the adult central nervous system (CNS). One of the major challenges of AAV gene therapy for the CNS is broad delivery of the transgene of interest to target cells within the brain and spinal cord parenchyma. Genes delivered with AAV vectors have been shown to achieve safe and long-term therapeutic expression in the CNS, but the limited capacity of natural AAV serotypes to cross the blood-brain barrier in adult mammals has restricted their use for targeting the CNS via intravenous (IV) dosing. To identify and characterize novel AAV vectors that provide CNS transduction following IV dosing, we screened a barcoded engineered AAV library following systemic administration in adult mice. Multiple AAV variants with >100-fold increases in CNS gene delivery relative to their parental capsid were identified, as measured by quantifying DNA and RNA barcodes. Functional comparisons of two of these novel AAVs - VoyC01 and VoyC02- to a previously described highly BBB-penetrant VOY101 capsid demonstrated enhancement of CNS biodistribution and transgene expression, with low peripheral exposure following IV injection in mice. Of note, one of the novel capsids, VoyC01 significantly outperformed VOY101. At a cellular level, these vectors successfully transduced both neuronal and astrocytic cell types in the brain. In summary, these barcode analyses and early results from systematic evaluations of a novel AAV vector repertoire demonstrated enhanced CNS gene transfer after systemic dosing over a previously described highly BBB-penetrant capsid. Furthermore, this characterization of capsids showing improved CNS transduction after a single IV injection validates continued efforts to engineer and identify novel BBB-penetrant capsids for application to gene therapy for CNS diseases.

241. Novel Insights into the Mechanobiology of Dystrophin Informs Design of Transgenes for DMD

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Mutations in the dystrophin gene result in the two allelic diseases Duchenne and Becker Muscular Dystrophy (DMD and BMD). Patients with DMD usually cease ambulation by age 13 and a majority have deletional frameshift with resultant deficiency of dystrophin. 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. The cloning capacity of vectors based on adeno-associated viruses (AAV) is limited to less than half the length of the dystrophin coding sequence. Relative to vectors with larger cloning capacity, AAV vectors exhibit reduced immunogenicity and efficient systemic delivery to cardiac and skeletal muscle, prompting intense interest in optimization of "AAV-sized" transgenes for DMD. To gain further insight into structure-function relationships for proteins that might substitute for dystrophin we have integrated two approaches as reported in other ASGCT abstracts (Greer et al, and Morales, et al., 2019). In the first approach 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 tested them in animal models for DMD by systemic delivery of AAV-encapsidated transgenes. In the course of this research we made a startling discovery with immediate implications for both the mechanobiology of dystrophin and for translational research in gene therapy. The findings suggest that dystrophin is mechanically loaded in such a way that "BMD dystrophins", including those of supraphysiological length as a result of gene duplication, have a common structural flaw: a weakest link at the deletion/duplication junction. We have formulated this as a testable hypothesis anchored in the structural biology of the crystallographically studied members of the supergene family. We have reverse-engineered a promising solution to the design problem and report on our progress in blinded, rigorous tests using the most sensitive structural assays developed to date, re-defining the integrative mechanobiology of dystrophin. Our findings shed new light on hitherto unexplained clinical observations that date back over 25 years, and offer the prospects for an AAV-based treatment that will cure rather than merely "Beckerize" DMD

242. Cerebellar Transduction of Astrocytes as Gene Therapy Strategy for Megaloencephalic Leukoencephalopathy with Subcortical Cysts (MLC)

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Efficient cerebellar transduction is important for the treatment of diseases affecting motor function and ataxias among other neurological disorders, but it has not been sufficiently explored so far. With the aim to achieve global and robust gene expression in the cerebellum, we tested different delivery routes of administering viral vectors, like direct intraparenchymal injection, lumbar intrathecal administration or subarachnoid injection to the cerebrospinal fluid (CSF). For that purpose, we used an AAVrh10 vector driving the expression of GFP under the regulation of the GFAP promoter, specific for glial cells. We found that subarachnoid injection in the CSF was the most efficient route in transducing Bergmann's glia, severely affected in Megaloencephalic leukoencephalopathy with subcortical cysts (MLC), a neurological disorder characterized by macrocephaly, deterioration in motor functions, cerebellar ataxia and mental decline. It is a type of vacuolating myelinopathy and its diagnosis is through the identification of swollen brain with diffuse white-matter abnormalities and subcortical cysts, mainly in temporal regions. AAVrh10 coding for MLC1 under the control of the GFAP promoter was injected it in the subarachnoid space of the cerebellum of MLC KO and wild type animals at 2 months of age, before the onset of the disease, as a preventive approach; at 5 months, once the disease has been already stablished; or at 15 months, when it has progressed to a more severe pathology, as a therapeutic strategy. Western-blot, quantitative RT-PCR and immunohistochemistry showed MLC1 expression in the cerebellum and extremely reduced myelin vacuolation, a hallmark of the disease, in treated 8- and 18-month old animals. In addition, GlialCAM, an Ig-like adhesion molecule, and the chloride channel CLC2, both implicated in MLC, restored its cellular localization in Bergmann glia after AAV-mediated MLC expression. These results may have implications for gene therapy to treat MLC patients as well as for other diseases affecting the cerebellum.

243. AAV-HLA-G Prevents Allogeneic Corneal Transplant Rejection and Xenogeneic Graft Survival

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Allogeneic corneal transplantation is a treatment for many genetic and acquired anterior eye diseases including corneal dystrophies, herpes, keratoconus, vascularization, and ocular surface trauma. Corneal transplantation to treat vision loss is the most common form of tissue transplantation worldwide with more than 50,000 surgeries occurring annually in the United States alone. In low risk patients, transplant rejection after 2 years is considerable at 15%. However, most patients undergoing corneal transplantation are considered high risk with an alarming 50-70% rejection rate after only 2 years. Furthermore, many patients with severe corneal disease are not candidates for the procedure. Current treatments, in part, rely on topical and systemic corticosteroids that exhibit limited success and serious adverse side effects including, ironically, vision loss. The second major concern in corneal transplantation is that the need surpasses the availability of transplant-grade corneal tissue, especially in developing countries. This reality, along with the increasing prevalence of high-risk procedures necessitates an alternative healthy tissue source for human corneal transplantation. Initially, AAV-HLA-G vectors administered post-corneal trauma were validated for inhibition of corneal vascularization, immune cell infiltration, and resulted in decreased fibrosis compared to corneas treated with AAV vector control. Then, AAV-HLA-G ex vivo transduction prevented allogeneic cornea transplant rejection in rabbits while control treated corneal explants were rejected 2 weeks following transplantation. HLA-G abundance in the graft and peripheral host tissue correlated to the therapeutic effect. In xenotransplantation studies HLA-G gene delivery to donor pig corneas delayed the onset of vascularization and increased the overall time to graft rejection in a non-human primate. Again, HLA-G staining was evident in donor porcine and primate corneal tissue. The collective data suggest that AAV-HLA-G ex vivo corneal graft transduction has the potential to prevent corneal graft rejection in xenotransplantation thereby addressing deficits in corneal tissue availability.

244. CRISPR-Cas9-Mediated Pre-Transcriptional Silencing of Mutant ATXN3 Decreases Motor Impairments of an In Vivo Machado-Joseph Disease Model

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Machado-Joseph disease (MJD) is a hereditary neurodegenerative disorder, caused by mutation of the *ATXN3* gene. The pathogenic

mutation consists in an abnormal expansion of CAG repeats that codify a region of polyglutamine repeats. When expanded, the gene product - ataxin-3 (atxn3) - aggregates and causes cell demise in select regions of the nervous system. MJD remains an incurable disease, but silencing *ATXN3* is recognized as a promising therapeutic approach, considering that studies employing RNA interference were able to counter disease-related phenotypes in animal models of MJD. Reports have however highlighted that mRNA transcripts of mutant *ATXN3* are toxic as well, prompting investigation into methods that counter *ATXN3* expression at a pre-transcriptional level. CRISPR systems of gene editing employ nucleases - usually the bacterial Cas9 - guided by RNA molecules in order to produce modifications in precise regions of the DNA, but CRISPR-Cas9 can also be modified to target other proteins to particular genetic loci, including transcriptional inhibitors such as the Krüppel associated box domain (KRAB). The aim of the current study was to develop a silencing strategy that would target a fusion protein composed by inactive Cas9 and KRAB (dCas9-KRAB) to the human expanded *ATXN3* gene, in order to selectively repress mutant atxn3 protein expression and thereby produce phenotypic amelioration. Our results demonstrate that, when targeted to a particularly disease-relevant region of *ATXN3*, this pre-transcriptional silencing system is able to selectively reduce the expression of mutant atxn3 protein in cell cultures, while leading to no decrease in wild-type atxn3. Importantly, in a transgenic mouse model of MJD, lentiviral delivery of these molecular tools to the cerebellum decreases motor incoordination. Our results support the potential of CRISPR-based pre-transcriptional silencing as a putative approach to MJD treatment.

245. Base Editing Strategy Allows High Frequency Insertion of the Protective A673T Mutation in APP Gene for the Development of an Alzheimer's Disease Therapy

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The Alzheimer's disease (AD) represents 70 % of the cases of dementia, which amount to 47.5 million cases in the world at present, and 75.6 million in 2030 according to the World Health Organization. Amyloid precursor protein (APP) is usually cut by the alpha-secretase, however an abnormal cut by beta-secretase leads to the accumulation of beta-amyloid peptides, which form plaques in Alzheimer patient brain. Numerous APP gene mutations favour the accumulation of plaques. However, it was discovered that a variant of the APP gene (A673T) in Icelanders reduces by 40 % beta-secretase cutting. We hypothesized that the insertion of this mutation in the patient genome is an effective and sustainable treatment to slow down the progression of familial (FAD) and sporadic forms of Alzheimer disease. The objective of our project was in a first time to show the protective effect of A673T in a FAD APP gene and determine against which mutation the treatment was the most effective. Secondly, we wanted to achieve a permanent correction by base editing to insert the A673T mutation and obtain evidence of the reduced formation of amyloid plaque. Plasmids containing one mutation responsible for a FAD were transfected in neuroblastoma SH-SY5Y and the supernatant was harvested 72 hours later. Another plasmid containing this mutation in addition of the A673T mutation was transfected in parallel. Every FAD mutation in exon 16 and 17 of APP were tested. The results were characterized and quantified by Meso Scale Discovery's Aβ kit. We succeeded to show an Aβ40 and Aβ42 peptides decrease with most of the FAD mutations with scores reaching 80% in some cases. Next we tried to create the A673T mutation with genome editing. APP was modified in HEK 293T cells and neuroblastoma SH-SY5Y using a nCas9-deaminase enzyme, which allows to change a cytosine into a thymine. Several deaminase variants were tested to compare the efficiency of conversion. The results were characterized and quantified by Deep Sequencing. We succeeded to modify the APP gene with a total conversion rate of 56.7%. Our approach aims to attest the protective effect of A673T against Alzheimer's familial and sporadic forms and the efficiency of base editing in the development of treatments against genetic diseases.

Cancer-Oncolytic Viruses

246. Pre-Clinical Assessment of Efficacy and Safety of Novel Oncolytic Adenovirus for Therapy of Disseminated Lung Cancer

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The majority of lung cancer patients do not respond to current immune-oncology drugs due to low PD1 and PD-L1 expression on immune and cancer cells. Oncolytic viruses are being actively explored as an alternate modality for cancer therapy due to their natural ability to kill infected cells through viral replication. In this study, we analyzed the anti-tumor efficacy and safety profile of AVID-317 oncolytic virus in pre-clinical mouse models of disseminated lung cancer. AVID-317 is a novel oncolytic adenovirus possessing a set of mutations in the virus capsid, which ablate virus interactions with cellular b3 integrins on macrophages and hepatic cells, resulting in virus de-targeting from the liver after intravenous administration. In vitro studies demonstrated that 70% of tested human non-small cell lung cancer (NSCLC)-derived cell lines (N=16) are sensitive to AVID-317 infection, and 85% of AVID-317-sensitive NSCLC lines express no or low PD-L1. Intravenous injection of control parental human adenovirus type 5 (HAdv5) at a dose of 1e11 v.p. per mouse into wild-type mice resulted in release of pro-inflammatory cytokines TNF-a, IL-1b, IL-6, IFN-g into the bloodstream at 6 hours post virus administration. Furthermore, this dose of HAdv5 was lethal due to severe hepatotoxicity within 48 h post virus injection. In contrast, intravenous administration of AVID-317 in the range of doses up to 1e12 v. p. per mouse resulted in no symptoms of acute toxicity and near background levels of TNF-a, IL-1b, IL-6, and IFN-g in the blood. Importantly, AVID-317 administration did not result in mortality. Next, we grafted PD-L1-low human adenocarcinoma A549-Luc-C8 cells into lungs of NCr nude mice to establish an orthotopic model of disseminated lung cancer. When tumor burden reached between 2xe6 to 8e6 RLU, mice were enrolled in three cohorts and treated with AVID-317 at doses of 5e10 or 1e11 per mouse or administered with saline only (control group; N=>9 in

each cohort). Mice were euthanized upon detection of 20% weight loss compared to baseline. The median survival in saline-treated group was 57 days post treatment. However, median survival has not be reached for AVID-317-treated groups for the duration of the study (80 days). Taken together, our study demonstrates feasibility of using AVID-317 oncolytic adenovirus for systemic therapy of disseminated lung cancer and its greatly improved safety profile observed upon intravenous virus delivery. The study was supported by AdCure Bio, LLC. N.C.D.P, H.W., and D.M.S are shareholders and officers of AdCure Bio, LLC.

247. Developing a New, Simian Foamy Virus - Based, Retroviral Platform for Oncolytic Virotherapy

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Simian Foamy Viruses (SFVs) are members of the ancient group of retroviruses - *Spumaretrovirinae*. They infect a variety of non-human primates with sporadic cases of zoonotic transmission to humans. SFVs are non-pathogenic and their replication is limited to dividing cells. Unlike Gammaretroviruses, SFVs can successfully infect cells with long doubling times due to their ability to remain intact at the centrosome for weeks. Furthermore, the large size of the SFV genomes facilitates a larger transgene carrying capacity than other retroviruses. These features make SFV an appealing platform for cancer gene therapy. Here, we describe an initial characterization of primate SFVs and engineered molecular infectious clones. Two strains of the chimpanzee Simian Foamy Virus (SFVcpz) tested in this study - PAN1 and PAN2 - were able to infect a broad range of human cancer cell lines and induce a strong cytopathic effect. We generated infectious molecular clones of PAN1 and a PAN1/PAN2 chimeric virus (PAN1/2). Infection of a panel of human cancer cell lines demonstrated that the PAN1/2 virus killed infected cells more efficiently than either of the parental strains. To facilitate virus titration, we engineered a BHK-21 cell line that expresses a reporter gene, mCherry, under the control of the SFVcpz U3 promoter, thus expressing mCherry in response to SFV infection. SFVcpzs slowly replicated in the indicator cell line with their titers reaching approximately 10^6 infectious units per milliliter on day 8 after infection at a low MOI. To test the ability of SFVcpz to carry foreign transgenes, we engineered a PAN1/2 clone encoding *gfp* in place of the *bel-2* gene (PAN1/2-GFP). This virus successfully infected various human cancer cell lines, although it replicated slower than the parental PAN1/2 virus. To verify whether SFV can propagate *in vivo* in a tumor, we generated a human glioblastoma U251 cell line expressing two reporter genes, mCherry and luciferase, under the control of the SFV U3 promoter. Subcutaneous implantation of the cells in CB-17 SCID mice resulted in tumor formation and the established tumors were injected with $2*10^6$ Infectious Units of PAN1/2 or PAN1/2-GFP. Both viruses efficiently propagated *in vivo,* demonstrated by serial luciferase imaging of the tumor bearing mice and flow cytometric analysis of the infected tumor cells. Our data show that SFVcpz can be utilized as a new replication-competent retroviral platform for cancer gene therapy.

248. Dual microRNA Engineered Oncolytic Coxsackievirus Virotherapy for Clinical Trial

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Recently, oncolytic viruses (OVs) have emerged as new modalities for cancer treatment depending on tumor cell lysis via preferential replication of OVs in tumor cells, followed by activation of the host's antitumor immunity. Our group previously reported that coxsackievirus B3 (CVB3) is a novel OV with a strong ability to lyse human non-small cell lung cancer. And two organ-specific(enriched in muscle and pancreas)miRNAs target sequences were constructed into the 3' untranslated region (3'UTR) of the CVB3 genome (CVB3-HP) resulting in the markedly reducedoccurrence of CVB3-induced pancreatitis and myocarditis. However, non-clinical acute toxicity testing of recombinant CVB3 in mice and monkeys showed mild hematological and histopathological abnormal findings in the highest dose group.To further suppress replication of virus in wide normal organs, target sequence complementary to miR-217 (enriched in pancreas) and miR-34a (enriched in normal organs) were inserted into both of 5'UTR and 3'UTR of CVB3 genome (CVB3-BHP). This novel virus elicited massive viral lysis in various tumor cell lines, but reduced replications and cytotoxicities in miR-217/miR-34a mimics-transfected cells. *In vivo* therapeutic studies using human tumor-bearing nude mice showed that CVB3-BHP preserved the same antitumor activities as wild-type CVB3 (CVB3-WT) without losing weight. Mice treated with CVB3-WT showed significantly increased serum levels of AST, ALT, LDH and Amylase, and histological findings of pancreatic injury. In contract, CVB3-BHP showed neither significantly increased serum levels of any enzymes nor pancreatic injury.Collectively, tissue-specific microRNA could be incorporated into 3' UTR and 5' UTR of CVB3 genome. Replication of the recombinant viruses was demonstrated in tumor but not in normal organs of infected tumor-bearing mice. Our current results suggested CVB3-BHPwould be a promising candidate for safer and effective oncolytic virus therapy.

249. A Novel Virus-Drug Combination to Enhance Oncolysis in Renal Cell Carcinoma

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Background: Oncolytic viruses (OVs) represent promising novel option for treatment of advanced cancers. There are few studies assessing the effects of oncolytic viruses in renal cell carcinoma. Oncolytic measles virus (MV) is a promising viral platform that previously been shown to induce cell fusion and cytotoxicity in a CD46-dependent manner. The goals of our study are to identify novel agents that used in combination with oncolytic MV will enhance the virus' cytotoxic effects in renal cell carcinoma (RCC) models. **Results:** To achieve this goal, we performed

in vitro assays combining the Edmonston strain of Measles virus expressing eGFP (MV-GFP), whose oncolytic activity in vitor and in vivo has been demonstrated, with several targeted novel agents. The agents chosen include Cabozantinib, (new generation multikinase inhibitor used in the treatment of renal cell carcinoma), EP321670, a Bromodomain inhibitor (associated with epigenetic regulation) and Triptolide (T-reported as apoptosis, ER stress, and oxidative stress inducer and an inhibitor of angiogenesis). 786-0 (VHL-mut), A498 (VHL mutant), Caki-1 (VHL-wt) and ACHN (VHL-wt) RCC cells were seeded and treated with oncolytic MVs, cabozantinib, EP321670 or triptolide. Cell viability as well as cytotoxicity were quantitated at 24, 48 and 72h. Evaluation of single agent activity showed that MV-GFP and triptolide were associated with the most significant growth inhibitory or cytotoxic effects, compared to cabozantinib or BRD4 inhibitors. Among the MV-drug combinations, we found that low dose triptolide was associated with the most significant augmentation of MV oncolysis in RCC cell lines, demonstrated by both viability cell count and by real time monitoring of cell growth by xCELLigence based assays. We found that the treatment sequence (virus infection followed by triptolide treatment) was an important determinant of the combined tumor cytotoxic effects. In addition to enhanced tumor cytotoxicity, we also observed that triptolide enhanced viral replication in RCC, which may explain in part the mechanisms of drug synergy. Finally, enhanced viral oncolysis induced by the MV-Triptolide combination was found to be associated with enhanced apoptosis (PARP cleavage), ER stress induction (pEIF2a, CHOP), and down regulation of survival and proliferation pathways (pAKT and pERK) between 24 and 36 hours post-treatment. **Conclusion:** We identified a novel strategy to enhance RCC viral oncolysis by combination of MV with triptolide, by mechanisms including enhanced the viral replication with subsequent increase in the cell death, ER stress pathways and down regulation of pAKT and pERK. Current efforts are focused on identifying in vivo antitumor effects and mechanisms of this combination.

250. Immunodominant and Cryptic Tumor Neoantigen Specific Immune Responses Activated by an Armed Oncolytic Virus Expressing a PD-L1 Inhibitor

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Tumor neoantigens are exclusively expressed in malignant cells representing an ideal target for tumor immunotherapy. Oncolytic virus selectively infects and lyses tumor cells releasing a full-array tumor antigens and danger factors. Combined with PD1 checkpoint blockade, oncolytic virotherapy showed to facilitate cross-presentation of tumor neoantigens boosting tumor-specific T cell immune responses. The present study developed a novel therapeutic vaccine that combines ddVV, GM-CSF, and a PD-L1 inhibitor into a single therapeutic agent by engineering ddVV to co-express GM-CSF and a PD-L1 inhibitor. The novel therapeutic vaccine has triple functions: ddVV-mediated selectively lysing of tumor cells releasing a full-array of tumor antigens and danger signals, GM-CSF-mediated immune enhancement of dendritic cell recruitment, activation, and function, and PD-L1 inhibitor-mediated interruption of PD-1/PD-L1 checkpoint signaling in immune effector cells followed by tumor-specific immunity boosted. As a result, the recombinant oncolytic virus enhanced tumor microenvironment dendritic cell maturation, promoted both dominant and cryptic neoantigen-specific T cell responses, eradicated primary tumor and prevented tumor recurrence more efficiently after local administration.

251. Development of an Oncolytic Adenovirus for Systemic Intravascular Administration for Treatment of Solid and Disseminated Tumors in Mouse Lung Adenocarcinoma Model

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Development of safe and effective oncolytic viruses for cancer therapy is a rapidly growing area of translational research that has great potential to improve outcomes of currently incurable malignancies. Using a systemic intravascular route for delivery is vital for the oncolytic vector to successfully eliminate tumors especially in the disseminated metastatic stage of the disease. However, pharmacokinetic studies of unmodified adenovirus vectors after intravascular delivery demonstrate that the majority of an administered virus dose is rapidly sequestered from the circulation by the liver and tissue resident macrophages, triggering systemic inflammation. Mechanisms that determine virus sequestration and immunological toxicity rely on molecular determinants located on each of the major virus capsid proteins, hexon, penton, and fiber. Using extensive mutagenesis we developed a novel oncolytic adenovirus vector Ad-3M, which contains several modifications in the surface capsid proteins. Modifications in the hexon highly variable region (HVR) 1 and 7 rendered the vector unable to infect hepatocytes and Kupffer cells. In addition, replacement of the RGD loop in the penton base protein to the laminin-derived mimetic peptide proved the vector to be incapable to activate severe inflammatory cascade in the spleen. CryoEM studies of the Ad-3M virion allowed for determination of the overall position of the shortened HVR1 of Ad-3M on the hexon tower. Localization of HVR1 required filtering the density to 5A resolution to help visualize density of this region. In contrast, structures of the wild type virus are completely missing coordinates for the long and highly flexible wild type HVR1. Additionally, molecular dynamics simulations of models of the laminin-derived peptide on a penton base suggest that this region is highly solvent accessible. Overall the cryoEM studies confirm the structural integrity of the Ad-3M virus particle and provide a starting point for modeling adenovirus-host interactions. Intravascular injection of unmodified human adenovirus 5 at a dose of 1e11 vp resulted in release of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α in the bloodstream at 6 h post injection. At 48 h post injection the mice succumbed due to severe hepatotoxicity of the wild type virus. In contrast, intravenous administration of Ad-3M in mice resulted in no hepatocyte transduction or toxicity, and demonstrated a greatly improved safety profile compared to the unmodified adenovirus. The improved safety profile of Ad-3M allowed us to administer high doses of the vector into mice bearing tumors derived from human non-small cell lung cancer cells. In a highly aggressive disseminated orthotopic lung model, Ad-3M treatment resulted in a complete elimination of

tumor in 30% of mice. In subcutaneous model the Ad-3M treatment resulted in complete elimination of tumors in 40% of mice and 40% of mice had partial response. Collectively, our study provides evidence that capsid modifications render adenovirus nontoxic and safe for intravascular administration.

252. Translational Research on Synergy of PD-1 Checkpoint Blockade and Oncolytic Measles Virotherapy

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Immune checkpoint blockade (ICB) has revolutionized immuno-oncology. However, the majority of cancers remain refractory to ICB alone. Preclinical and clinical data indicate that oncolytic virotherapy can break resistance to ICB. In this study, we aimed to pinpoint the underlying mechanisms in preclinical models by performing correlative research to identify biomarkers of response. We found unique signatures in tumor-infiltrating lymphocytes, cytokine/chemokine profiling, transcriptome analysis, and T cell receptor sequencing after combination treatment of anti-PD-1 and oncolytic measles virus. For clinical validation, these markers will now be implemented in the translational research program accompanying a planned Phase I/II trial of oncolytic measles virus and anti-PD-1 antibody.

253. Intravenous Application of Mesothelin-Targeted Oncolytic Adenovirus to Pancreatic Cancer

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Pancreatic cancer is a highly malignant and lethal disease. It 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 efficacy upon systemic application has been quite limited to date. Unlike loco-regional therapy, systemic application of cancer therapy mandates better tumor distribution and transduction. When adenovirus vectors are injected intravenously, most of the virus goes to the liver. As a consequence of large sequestration of adenovirus by liver, the tumor transduction rate is low and the *in vivo* efficacy of systemic therapy is limited. Therefore, the improvement of cancer selective transduction and vector distribution to avoid liver sequestration would overcome the obstacles for systemic delivery and enable efficient systemic treatment of cancer with OAd. To improve the tumor transduction, we have generated the pancreatic cancer-targeted OAd by screening of Ad-fiber 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 anti-tumor effect against MSLN-positive subcutaneous tumor model in both intratumoral (i.t.) and intravenous (i.v.) injection. Importantly, when we assessed viral distribution after i.v. injection, the liver sequestration of MSLN-targeted OAd was lower than untargeted OAd (Ad5 WT virus) at 48 hrs after injection. By day 7, the viral copy number of MSLN-targeted OAd in the tumor was significantly higher than Ad5 WT virus. These results suggest that systemic injection of the tumor targeted-OAd showed significantly lower liver sequestration and better tumor accumulation. Additionally, antitumor effect of MSLN-targeted OAd was assessed in patient-derived xenograft (PDX) model. After intravenous administration, only the MSLN-targeted OAd showed significant antitumor effect compared to the untreated group ($p<0.05$), while the growth of Ad5 WT virus injected group was same as untreated group.Next, to further reduce liver sequestration and increases antitumor efficacy, we developed an improved vector which is a double-modified vector with both hexon- and fiber-modification. The MSLN-targeted OAd was additionally modified by substitution of Ad5 hexon-hypervariable region 7 (HVR7) with Ad26 hexon-HVR7. We assessed liver distribution of the double-modified vector after i.v. injection. When the virus copy number was compared after 48 hrs, double-modification resulted in significantly decreased liver sequestration. These data suggest that hexon-modification allowed to escape from the liver. The detailed mechanism of this finding is currently being investigated and focuses on the viral uptake by hepatocytes and Kupffer cells. In this study, MSLN-targeted OAd showed remarkable anti-tumor effect in both systemic and intratumoral injections. We believe that the fiber and hexon double-modified vector might be a promising vector to reduce hepatotoxicity and enhance antitumor effect for systemic treatment of pancreatic cancer.

254. Optimization of Mengovirus Targeting for Oncolytic Infectious Nucleic Acid Based Virotherapy

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Formulating oncolytic viruses as infectious nucleic acid can significantly reduce treatment costs and is a potential mechanism for avoiding neutralization during repeat dosing. We have demonstrated the feasibility of formulating oncolytic viruses as infectious RNA (iRNA) with Coxsackievirus A21 (CVA21), a member of the Picornaviridae family. However, CVA21 replication is restricted to human cells limiting preclinical evaluation of iRNA to immunodeficient xenograft models or transgenic animals. Mengovirus, a well-studied zoonotic picornaviral pathogen, has a very broad species tropism, including mice and primates, allowing the impact of the immune system on iRNA-based therapy to be evaluated in genetically unmodified immunocompetent tumor-bearing animals. Intratumoral (IT) administration of in vitro-derived RNA transcripts encoding an attenuated Mengovirus (MC24) genome to immunocompetent Balb/C mice bearing syngeneic mouse plasmacytomas results in tumor regression, but causes lethal toxicity. We previously showed that inserting microRNA response elements targeted by neuronal and cardiac specific microRNAs into the 5' and 3' UTRs (MC24NC), respectively, inhibits viral replication in cells expressing the cognate microRNAs and ameliorates MC24 toxicity in

vivo. However, the oncolytic infection established by IT administration of iRNA encoding the MC24NC genome was insufficient to prolong survival. In vitro analyses determined that the specific infectivity of the in vitro-derived RNA transcripts was reduced compared to the unmodified genome and was attributed to the 3' UTR insert. Therefore, the goal of this study was to generate a safe, detargeted Mengovirus genome without reducing the specific infectivity of the iRNA. We generated a comprehensive panel of detargeted Mengoviruses using three different strategies; i) attenuation by eliminating known virulence factors including the poly C tract in the 5' UTR (MC0) and a stem loop in the 3' UTR (MC24ΔSL); ii) combined attenuation with microRNA response element insertion (C-MC24ΔSL, MC24ΔSL-C); and iii) exchange of the internal ribosomal entry site (IRES) or complete 5' non-coding region with those of picornaviruses (HRV2, FMDV) that do not replicate in neuronal tissues (MC24HRV2; MC24FMDV; MG-FMDV). Attenuation proved the most efficacious strategy in vitro with MC0 and MC24ΔSL iRNAs exhibiting specific infectivities similar to unmodified MC24 iRNA in transfected H1-HeLa cells. Furthermore, the rescued viruses were as cytotoxic to MPC-11 murine multiple myeloma cells as unmodified MC24. Interestingly, although IRES or complete 5' UTR switching resulted in reduced specific infectivity of the iRNA in producer BHK cells, the virus progeny were similarly cytotoxic to MPC-11 cells as unmodified MC24 and may be a differential mechanism for targeting MC24 virus formulations. Animal studies to test in vivo efficacy and safety of intratumorally administered iRNA encoding each of the detargeted constructs in the presence and absence of immune modulators is currently underway.

255. Neural Stem Cell Delivered Conditionally Replication-Competent Oncolytic Adenovirus (CRAd-SPARC-pk7) for the Treatment of Ovarian and Brain Cancers

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The 5-year survival rates for ovarian and brain cancers are significantly lower than those for other cancer types, at 47.4% and 33.2% respectively. Replication-competent oncolytic virotherapy is a promising approach for patients with recurrent disease, given that oncolytic viruses selectively replicate in tumors and induce cancer cell death irrespective of radio- or chemo-resistance. In addition, secondary immune responses are expected to be induced upon exposure of tumor antigens following the lysis of cancer cells. Although clinical trials have demonstrated the safety of oncolytic virotherapy, its efficacy has been limited by numerous obstacles, including poor viral penetration in tumors and poor viral spread through tumor-associated stroma and the tumor microenvironment. Previous studies showed that neural stem cells (NSCs) have inherent tropism to tumors, making them an ideal delivery vehicle. The NSC-delivered adenovirus, CRAd-Surivin-pk7, is protected from rapid immune-mediated clearance and neutralization, resulting in more effective distribution to tumors compared to free virus. This result was observed in immunodeficient and immunocompetent murine models of peritoneal ovarian metastases and orthotopic glioma. However, the therapeutic efficacy of the virus is limited by the *survivin* promoter, which is highly expressed only in a subpopulation of tumor cells. In contrast, CRAd-SPARC-pk3/5 replicates under the SPARC promoter, which is overexpressed in both tumor and tumor-associated stromal cells. In addition, CRAd-SPARC-pk3/5 contains enhancer elements that facilitate viral replication in response to hypoxia and inflammation, conditions commonly present in tumor microenvironments. Therefore, by targeting both the tumor and the tumor microenvironment, CRAd-SPARC-pk3/5 is expected to be more significantly more efficacious, compared to CRAd-Surivin-pk7. To compare the relative potencies of the same virus replicating under two different promoters, a fiber knob modification was performed on CRAd-SPARC-pk3/5 to create CRAd-SPARC-pk7. The viral uptake and lysis kinetics of NSCs carrying CRAd-SPARC-pk7 were optimized. CRAd-SPARC-pk7 was first examined *in vitro* using multiple murine and human glioma and ovarian tumor cell lines. Results demonstrated robust infection and significant tumor killing. *In vivo* efficacy studies are in progress to compare NSC.CRAd-Survivin-pk7 vs. NSC.CRAd-SPARC-pk7 in immunodeficient and immunocompetent murine orthotopic tumor models. Thus far, we have observed that the NSC delivery of CRAd-SPARC-pk7 increases viral distribution and spread in tumors, tumor-associated stroma, and the tumor microenvironment. Ongoing experiments are expected to demonstrate improved anti-tumor efficacy of CRAd-SPARC-pk7, resulting in prolonged long-term survival. The addition of immune checkpoint inhibitors is also being evaluated for potential therapeutic enhancement. Thus, in the long term, we aim to incorporate NSC-delivered CRAd-SPARC-pk7 as an adjunct to standard regimens for the treatment of brain and ovarian cancers. We acknowledge the financial support of City of Hope, the Anthony F. & Susan M.Markel Foundation, the Ben and Catherine Ivy Foundation, the Rosaline and Arthur Gilbert Foundation, the California Institute of Regenerative Medicine, and the Alvarez Family Charitable Foundation for making this research possible.

256. Abstract Withdrawn

257. Decorin Expression from Oncolytic Adenovirus Potentiate Antitumor Efficacy via Mitochondrial Apoptosis in Cancer Cells

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Oncolytic adenoviruses (Ads) have the ability to target, replicate in, and lyse cancer cells. However, due to a modest cytolysis activity of oncolytic Ads as antitumor agent, the development of an improved therapy for cancer patients is necessary. In order to achieve this challenge, insertions of powerful therapeutic gene into oncolytic Ad have been explored. Here, we have investigated the detailed mechanism of apoptotic tumor cell death induced by decorin (DCN) expression from adenoviruses (Ad). DCN-expressing replication-incompetent Ads and oncolytic Ads induced more potent cancer cell killing effect compared to their cognate control Ads lacking DCN through more robust induction of apoptotic cell death. Improvement in apoptosis was due to DCN expression improving the stability of p53, increasing

p53 expression level, and ultimately inducing mitochondrial apoptosis. Similarly, DCN-expressing oncolytic Ad induced a greater antitumor effect in a xenograft tumor model compared with control Ad. Similar to our in vitro findings, significantly higher levels of apoptosis in tumor tissues were observed from mice treated with DCN-expressing Ads compared to those treated with control Ads. Collectively, our findings provide new insight into how DCN expression via viral vector induces apoptotic tumor cell death.

258. Oncolytic Vaccinia Virus and Anti-PD1-Based Combination Therapy Restores AntitumorImmunity in Tumor Microenvironment

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Tumor microenvironments (TME) consist of various types of regulatory cells, and also limit the therapeutic efficacy of most of the drugs. To overcome these hurdles, immunotherapy-based combination therapies are being developed to modify the immunosuppressive nature of TME and block immune checkpoints on immune cells of tumors. In this study, we used cancer-favour oncolytic vaccinia virus (CVV) and α-PD1 to treat mouse coloncancer. Weekly-based intratumoral injection of CVV and intraperitoneal injection of α-PD1 were tested in subcutaneous CT26 tumor models in Balb/c mice. Tumor volume, survival curve and hemotoxylin and eosin staining, and immunohistochemistry-based analysis indicated benefit of co-treatment received groups, especially simultaneous treatment of CVV and α-PD1.The infiltration of CD8+ T-cells also correlated with these results. Furthermore, PD1 and PD-L1 mRNA expression from these groups confirmed the therapeutic efficacy of this combination therapy. CVV and α-PD1-based combination therapy enhanced tumor infiltrating lymphocytes and also tumor cells killing.

259. One-Step Monolith Purification of Oncolytic Influenza Virus Produced in Vero Cells

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Chromatography is a useful purification method for large biomolecules and virus manufacturing and it is easily scalable to large production volumes. Convective Interaction Media (CIM) monolithic columns constitute of large flow-through channels and consequently have high surface accessibility of binding sites. Preferences of CIM monolithic columns are flow independent performance, resulting in fast separation, concentration, purification, impurities removal, and analytics of biopharmaceuticals. The aim of the study was to develop Influenza virus purification platform, which can be used for several virus strains. The main objective was to develop a process with as little as possible of intermediate steps, especially omitting Tangential Flow Filtration (TFF) or other sample pre-treatments with high host-cell DNA and protein removal, as well as to achieve high binding capacity of the Influenza virus per mL of monolithic support.

260. Novel Oncolytic Virus Armed with Cancer Suicide Gene and Normal Vasculogenic Gene for Improved Cancer Specificity

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We developed a new oncolytic vaccinia virus (NOV) with the dual advantages of cancer selectivity and normal vessel construction by adopting TRAIL and Angiopoietin. To demonstrate the cancer-specific and oncolytic potency of NOV, different panels of cancer cell lines (CRC, HCC, CC, PanC etc.) were tested. NOV were injected into tumor mice models and tumor growth and gene expressions was examined after treatment. Also, the early apoptotic cell population, which represents apoptosis, and its relation to oncolytic, was examined. NOV showed higher toxicity than the WT virus with TK deficiency in all the examined various ranges of cancer cell line (hepatocellular carcinoma,human epithelial adenocarcinoma, human bone osteosarcoma epithelial cell lines, murine melanoma, pancreatic cancer cell, metastatic pancreatic cancer cell etc). NOV treated CT26 mouse colon cancer xenograft model showedattenuated tumor growth resulting from successful lysis of cancer cells by NOV. The percentage of early stage in apoptosis of NOV treated group was much higher than that of WT virus. In addition, normal vessel construction byNOV was along with its antitumor activity and immunity. From results, we concluded that the improved antitumor effects of our novel oncolytic virus (NOV) is contributed both by specific proliferation only in cancer cells inducing their apoptosis and by normal vessel construction.

261. Enhancement of Oncolytic Activity of Newcastle Disease virus Through Combination with Retinoic Acid Against Digestive System Malignancies

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Combination of an oncolytic virus with chemotherapy has a significant hope for the treatment of digestive system malignancies. In addition, the combination with chemotherapy allows lower doses to achieve the anti-cancer effect, thus resulting in lower undesirable toxicities. In the current investigation, we examined whether the combination of an Oncolytic Newcastle disease virus Iraqi attenuated strain (NDV) with Retinoic acid (RA) could result in higher anti-digestive system malignancies in vitro and whether RA and NDV have a synergistic effect on cancer cells of inducing apoptosis. The Esophagus carcinoma (SKG), hepatocellular carcinoma (HC) and colon carcinoma (CC) cell lines, were used as in vitro model. Viability was determined by MTT method after treating by NDV and RA alone or in combination. Apoptosis induction in SKG, HC and CC cancer cell lines under different treatments were detected using mitochondrial membrane potential assay. Cytopathic effect was examined to check the enhanced

replication in infected cells that treated with RA. Rat embryonic cells used as normal control cells for toxicity studies. Results, NDV and RA, showed anti-tumor activity and induce apoptosis in cancer cells, and when RA and NDV used in combination, there is an enhanced synergistic effect against tumor cell growth and more apoptosis induction. Cancer cells treated with combination therapy showed more severe cytopathic effect that indicates the synergistic effect between the treatment modalities. Combination therapy showed no cytotoxic effect on normal rat embryonic cells at all doses tested (table-1). In conclusion, We propose that this effective therapeutic synergy can help to reduce the dose of each drug, to improve efficacy, reduce adverse drug reactions, provides a novel therapy for Digestive system malignancies.

262. NIS-Expressing Oncolytic Adenovirus for Non-Invasive Imaging of Lymph Node Metastasis in Melanoma

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Melanoma represents an ideal target for oncolytic viral therapy. In addition, melanoma is particularly susceptible to immunotherapy and oncolytic virus therapy is known to evoke antitumor immunity. Our lab has designed a number of oncolytic adenoviral (OAd) vectors with different transgenes inserted into the adenoviral E3 region to drive tumor expression of various therapeutic and diagnostic proteins including immunotherapeutic agents and the sodium iodide symporter (NIS). The latter can be used for diagnostic cancer imaging and radiotherapy. Melanoma staging is dependent on identification of lymph node metastasis, currently identified through a surgical procedure known as the sentinel lymph node biopsy. Our objective is to develop a non-invasive approach for identification of lymph node metastasis in melanoma utilizing NIS-expressing oncolytic adenoviruses. Here we aim to identify the most efficient combination of viral modifications for maximal NIS expression in human melanoma cells. Modifications included tropism modification (Ad5 fiber and serotype chimeric Ad5/3), tissue specific promoter modifications (Cox2 and wild type replication), and deletion of the Adenoviral Death Protein (ADP). Four human melanoma cell lines (SH-4, A375, WM-115, HTB-66) were tested. We observed variability in the binding efficiency of OAd5 fiber and OAd5/3 fiber modified adenoviral vectors amongst the cell lines. OAd5 fiber vectors were more efficient at killing two cell lines (SH-4 and HTB-66) and the other two lines (A375 and WM-115) were killed more efficiently by OAd5/3 vectors. Immunohistochemistry studies showed increased NIS expression with deletion of ADP from the E3 region. We are currently optimizing a hamster melanoma metastasis model utilizing AbC1 hamster melanoma cells. This model will allow us to investigate the feasibility of subcutaneous delivery of OAd-NIS to lymph nodes which could then be imaged with standard techniques. This work lays the foundation for development of a novel implementation of OAds which has potential to lead to more efficient identification of early cancer metastasis.

Cancer-Targeted Gene & Cell Therapy

263. Neural Stem Cell Delivery of a Chimeric Orthopoxvirus in Combination with PD-1 Antibody for the Treatment of Metastatic Ovarian Cancer in Mice

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An estimated 13,980 women will die from ovarian cancer in 2019, making it the fifth most common cause of cancer-related deaths in women, and the number one cause of reproductive-related cancer deaths in the US. Current standard of care (SOC) treatment for late-stage ovarian cancer involves highly toxic intolerable chemotherapy with only a limited therapeutic benefit. Indeed, patients with stage III metastatic ovarian cancer have only a 34% 5-year survival rate following SOC treatment. Hence, alternative therapeutic approaches, such as immunotherapies and oncolytic virotherapies, are under investigation. Studies have shown increased efficacy of checkpoint inhibitors (e.g., PD-1/PD-L1 blockade therapies) after the administration of replication-competent oncolytic viruses, indicating that the combination of oncolytic virotherapies and blockade immunotherapies may result in improved clinical efficacy. One of the major obstacles to *in vivo* delivery of oncolytic viruses is rapid inactivation by the host immune system. To overcome this hurdle, we loaded an oncolytic virus into HB1.F3.CD21 neural stem cells (NSCs) that are tumor-tropic and have demonstrated safety in several first-in-human clinical brain tumor trials. NSCs can shield the virus from the immune system en route to targeting metastatic tumor sites. In this study, NSCs were modified to deliver CF33 chimeric orthopoxvirus with an attenuated thymidine kinase (Tk) gene, providing selectivity of viral replication only within tumor cells that are TK positive. *In vitro* studies of CF33-expressing NSCs (CF33-NSCs) co-cultured with murine (ID8) and human (OVCAR8 and SKOV3) ovarian cancer cell lines and non-cancerous mouse fibroblast cell lines at a ratio of 1:1000 demonstrated rapid infection and complete lysis of tumor cells after 4 days. We also observed increased expression of PD-L1 after 1 day. Thus, we hypothesized that CF33-NSCs would selectively infect tumor cells, causing lysis and increased expression of PD-L1. In addition, we anticipated that blocking the T cell PD-1 receptor with antibodies (Abs) would lead to further anti-tumor activity in the treated mice. For these *in vivo* experiments, we developed an immunocompetent, syngeneic ovarian cancer model by injecting female C56BLK/6 mice intraperitoneally with ID8 cells labeled with eGFP and firefly luciferase (ID8-eGFP-ffluc). Treatment consisted of 3 weekly intraperitoneal injections of PBS, free CF33, or CF33-NSCs starting 1 week post-tumor implantation. A single intraperitoneal administration of a PD-1 Ab was given 2 days before, after, or concurrently with the first PBS, CF33, or CF33-NSC injection. Weekly bioluminescence imaging (BLI) was conducted to monitor tumor growth/regression, as mice were followed for long-term survival. A subgroup of mice were euthanized 2 days after each weekly treatment and tumor and organ tissues harvested and prepared for IHC and FACS analysis. FACS analysis of tumor tissues indicated the highest increase in PD-L1 expression in mice treated with CF33-NSCs. IHC indicated a higher distribution of CF33 virus to

tumor sites in mice treated with CF33-NSCs vs. free CF33. The group of mice receiving concurrent combination therapy (CF33-NSCs/PD-1 Ab) exhibited the lowest BLI tumor signal and highest survival rate, as compared to groups receiving the PD-1 Ab before or after CF33 NSC administration. **Acknowledgements** The Anthony F. & Susan M. Markel Foundation, the Rosalinde and Arthur Gilbert Foundation, California Institute of Regenerative Medicine, the Alvarez Family Charitable Foundation, NIH/NCI RO1 CA197359 01.

264. The Sodium Iodide Symporter (NIS) is a Versatile Reporter Gene for Tracking Cell and Vector Fate in Living Animals

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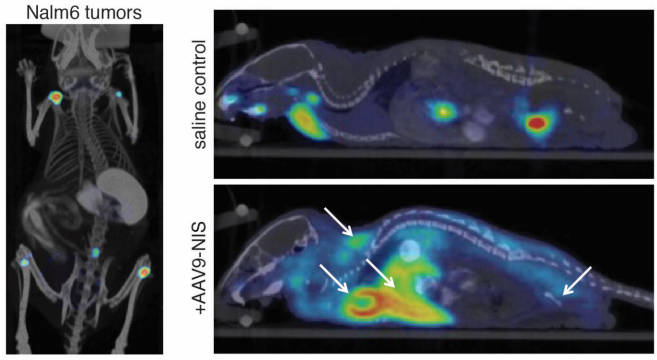
The success of cell and vector therapies depends heavily on their highly specific delivery to target sites in vivo. Nevertheless, in vivo tracking of therapies is difficult, and typically involves labor-intensive and time-consuming analyses that are prone to sampling error. Noninvasive imaging of therapies engineered to express or encode reporter genes holds significant promise for easy longitudinal tracking of cell and vector therapies in vivo. However, the primary reporter genes used for noninvasive imaging (optical reporters) have limited utility in the field of cell and gene therapy. Poor tissue penetration, relatively low spatial resolution, and immunogenicity, make optical reporters unsuitable for deep tissue or large animal imaging. Given these limitations, we sought to establish a versatile alternative for noninvasive tracking of cell and vector fate based on the sodium iodide symporter (NIS). NIS is a self-protein that concentrates iodide, as well as other radiotracers. The NIS gene can be engineered into cells or vectors and used to track in vivo localization via SPECT/CT or PET/CT. In order to demonstrate the advantages of using NIS for cell tracking, we first directly compared in vivo imaging of tumor cells using NIS and the optical reporter, firefly luciferase. Relative to bioluminescence imaging (BLI), NIS imaging provided more accurate localization of tumor cells in vivo. Moreover, unlike BLI, NIS imaging was readily able to distinguish and quantitate deep tissue tumors. Thus, NIS provided valuable information about cell fate, which could not be realized by BLI alone. Teratomas formed by NIS-expressing induced pluripotent stem cells were also readily distinguished in mice by SPECT imaging, further demonstrating the utility of using NIS to track target cells noninvasively in vivo. To evaluate NIS as a tracker for gene therapies, we monitored NIS expression in mice receiving NIS-encoding AAV9 vector. NIS expression was detected by PET/CT in several organs beginning on day 7 and peaking on day 14 post-administration. Correlative tests confirmed that biodistribution of NIS reporter signal corresponded closely with the sites of AAV9 transduction. Thus, NIS imaging efficiently and accurately reflected AAV9 biodistribution. Together, our data demonstrate that NIS provides a more accurate and translational alternative to BLI for noninvasive in vivo tracking of cells and vectors, and thereby has the potential to accelerate cell and gene therapy development.



265. Combination Ttherapy of OX40 Antibody and Neural Stem Cell Mediated CpG-STAT3 Antisense Oligonucleotides in Breast Cancer Animal Model

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Currently, approximately 1 in 8 women in the U.S. have a chance of developing breast cancer. Unfortunately, traditional treatments are successful only in the early stages of cancer development, in part due to signal transducer and activator of transcription 3 (STAT3)-mediated tumor immune intolerance. Treatment with CpG-STAT3 anti-sense oligonucleotides (ASOs) is a promising alternative therapy that triggers a tumor-specific immune response by inhibiting STAT3. The CpG moiety enables cellular uptake of STAT3 ASO by linking it to Toll-like receptor 9 (TLR9), an innate immune receptor commonly expressed in tumor-residing immune cells, such as macrophages, dendritic cells, and B cells. We assessed a novel approach to increase the stability and tumor-targeted delivery of CpG-STAT3 ASOs by exosome encapsulation in neural stem cells (NSCs). NSCs are tumor-tropic, making them an ideal vehicle to deliver therapeutic payloads selectively to invasive tumors. Importantly, NSCs are known to secrete large amounts of extracellular vehicles (EVs), which may increase tumor distribution of CpG-STAT3 ASOs and enhance their immunostimulatory properties. We used our clinically relevant NSC line, HB1.F3.CD21 for these experiments. *In vitro* studies demonstrated rapid uptake of CpG-STAT3 ASOs by NSCs through scavenger receptor-mediated endocytosis, which did not affect the construct or its efficacy. Following uptake, NSCs continuously released EVs containing the CpG-STAT3 ASOs (CpG-STAT3 ASO-NSCs) for 2-3 days. We hypothesized that following intratumoral administration of CpG-STAT3 ASO-NSCs in an immunocompetent breast cancer mouse model, the construct would distribute through tumor cells, attracting tumor-infiltrating lymphocytes, and generate a systemic anti-tumor effect. Moreover, because TLR9 activation induces the expression of the costimulatory receptor OX40 on T-cells, we anticipated that the addition of OX40 agonist antibodies (Abs) would lead to further anti-tumor activity in treated mice. For these *in vivo* experiments, we developed an orthotopic, immunocompetent breast cancer model by injecting female BALB/c mice with syngeneic 4T1 breast tumor cells in the left and right intramammary fat pads.

Treatment began when at least one of tumors reached 0.5 mm in longest diameter. Treatment was given only to the right side tumor, and consisted of 3 intratumoral injections of PBS, free CpG-STAT3 ASOs, or CpG-STAT3 ASO-NSCs every other day, alone or in combination with OX40 agonist Abs. Caliper measurements of both left and right tumors were recorded daily, and mice were euthanized once the largest diameter of either tumor reached 1.5 mm (in compliance with AAALAC euthanasia criteria). A subgroup of mice was euthanized 2 days after each treatment, and tumors harvested and prepared for IHC and FACS analysis. Resulting data indicated that the greatest distribution of CpG-STAT3 ASOs and increase in OX40 expression were achieved in mice that received treatment with CpG-STAT3 ASO-NSCs. CpG-STAT3 ASO-NSCs/OX40 agonist Ab combination therapy significantly suppressed tumor growth, represented by reduced volume of both the treated right side and non-treated left side tumors, and resulted in extended survival. These data support further translational development of this combined treatment approach, which has the potential to overcome immune tolerance and induce a systemic anti-tumor immune response in patients with advanced breast cancer.
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266. Mesenchymal Stem Cells as Carriers of Retroviral Replicating Vectors for Cancer Gene Therapy

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Mesenchymal stem cells (MSCs), which possess the ability to migrate to tumors and contribute to forming tumor stroma, are being investigated as a promising vehicle for delivery of anti-cancer agents, including viral vectors. In this study, we evaluated the ability of MSCs to deliver retroviral replicating vectors (RRVs), which have shown highly promising results for gene therapy of cancer in both preclinical and clinical studies. Human MSCs derived from adipose tissue (hMSC-ad), bone marrow (hMSC-bm) and umbilical cord (hMSC-uc) were all infected efficiently with RRV, but produced virus progeny less efficiently than tumor cells. We then assessed tumor cell tropism of these MSCs toward mesothelioma cells, using a Transwell plate migration assay. All of these MSC subtypes showed significant migratory activity toward the mesothelioma cell targets. Furthermore, when co-cultured, RRV were transmitted efficiently from MSCs to mesothelioma cells, thereby achieving high levels of tumor cell transduction. Additionally, treatment with 5-fluorocytosine (5-FC) prodrug after MSC-mediated delivery of RRV expressing the yeast cytosine deaminase prodrug activator gene resulted in potent cytotoxicity to malignant mesothelioma cells in vitro. Furthermore, intratumoral RRV transmission in vivo from MSCs to mesothelioma cells was confirmed by biomolecular imaging and flow cytometry. Notably, hMSC-ad and hMSC-uc showed much higher tumor transduction efficiency compared to hMSC-bm. These data indicate the potential utility of tumor-homing MSC to deliver RRV more efficiently and further enhance the efficacy of RRV-mediated gene therapy for systemic and metastatic cancers.

267. Characterization of Tisagenlecleucel, an Anti-CD 19 CAR T Cell Product Manufactured Using T Cells from Patients with DLBCL

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Tisagenlecleucel is an autologous CAR T cell product expressing a CAR that recognizes CD19, a protein found exclusively on the surface of B cells. We characterized 81 batches of tisagenlecleucel that were manufactured for the JULIET study, a phase 2 trial of tisagenlecleucel in adult patients with r/r DLBCL by immunophenotying of T cell subsets by flow cytometry. The *in vitro* functional response to CD19 expressing target cells was also evaluated by measuring cytokine release (ELISA) and cytotoxicity assay. Flow cytometry results showed that tisagenlecleucel was mainly constituted of T cells despite the very highly variable cell composition of the leukapheresis starting material. Except occasional residual NK cells, no B cells or B lymphoblasts or other cells originating from the leukapheresis starting material were detected in tisagenlecleucel. The percentage of CAR-positive cells had a wide distribution range resulting in varying mixes of non-transduced (CAR-negative) and CAR-positive CD4+ and CD8+ cells. The ratio of transduced CAR-positive CD4+ to CD8+ cells was varying and no relation to clinical outcome was observed in DLBCL. The T cells in tisagenlecleucel were predominantly less mature transduced T cells which were highly activated. Immunophenotyping showed high cellular fitness with minimal immunosenescent or exhausted phenotypes. Tisagenlecleucel demonstrated functional activity upon CD19-specific stimulation as evident by IFN-gamma release and cytotoxic capacity, however, with a wide range among different batches. The heterogeneity in regards to key product attributes such as T cell composition and functional response, did not allow for the definition of clear correlations with clinical outcome. Tisagenlecleucel resulted in DLBCL patients in 52% overall response rate; 40% of patients had complete response and 12% had partial responses.

268. Combinatorial Approach of DNA-Loaded Brain-Penetrating Nanoparticles and Tumor-Specific Promoter for Widespread yet Cancer-Selective Gene Transfer to Brain Tumors

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The potential of gene therapy as a treatment option for high-grade gliomas has grown tremendously over the past decade. However, clinical trials to date have revealed limited effectiveness, largely attributed to inability to mediate robust transgene expression throughout the highly disseminated tumor tissues within the brain.

In addition, akin to clinically-used chemotherapeutics, conventional gene therapies do not readily target cancer cells in a selective manner, thereby often accompanying off-target side effects in adjacent healthy tissues. To address these critical issues, we have schemed a two-pronged strategy, namely brain-penetrating nanoparticle and tumor-specific promoter, to achieve widespread yet cancer-selective gene therapy. We have recently developed small (sub-100 nm in diameter) DNA-loaded brain-penetrating nanoparticles (DNA-BPN) that possess unique ability to efficiently penetrate both healthy and tumor-bearing brain tissues *in vivo*. In parallel, we have screened multiple tumor-specific promoters to identify a lead candidate that drives high-level transgene expression specifically in rodent and human brain cancer cells but not in normal brain parenchymal cells *in vitro*. We then show that marriage of these two simple strategies results in widespread transgene expression exclusively in orthotopically-established brain tumor tissues, in contrast to the combination of DNA-BPN and conventional CMV promoter that lacks tumor-specificity.

269. Targeted AAVP-Based Therapy in a Mouse Model of Human Glioblastoma: A Comparison of Cytotoxic Versus Suicide Gene Delivery Strategies

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Glioblastomas are lethal intracranial tumors of the central nervous system characterized by high morbidity and mortality due to their high tumor cell proliferation rate and marked neovascularization, allowing them to infiltrate crucial structures in the brain. The delicate nature of the brain, combined with structural constraints such as the blood-brain barrier and the presence of tightly regulated, isolated physiological processes limits clinical options, particularly drug delivery, for brain tumors. Therefore, there is a grave unmet need for innovative treatment options. Given that targeted gene therapy is undergoing a renascence due to the potential efficacy of oncolytic viruses and other viral vectors in malignant gliomas and other tumor types, we hypothesized that a new class of ligand-directed gene therapy vector might be a promising strategy for overcoming the barriers to treat human glioblastoma. Over the past decade, our group has advanced a targeted hybrid vector containing cis-genomic elements from adeno-associated virus (AAV) and single-stranded M13-derived bacteriophage (termed AAVP) displaying peptide ligands to target endothelial and tumor cell surface receptors and to mediate selective internalization of ligand-directed viral particles. The broad utility of targeted AAVP constructs displaying the RGD4C motif (a double-cyclic sequence CDCRGDCFC) targeting the αv integrin subunit has previously been established with AAVP constructs containing either tumor necrosis factor (RGD4C-AAVP-TNF) or Herpes simplex virus thymidine kinase (RGD4C-AAVP-HSVtk) transgenes. However, we have yet to ascertain the relative efficacy of each transgene in the same tumor model. Since integrin subunit αv is highly expressed both in tumor cells and in angiogenic vasculature in glioblastomas, here we evaluate two parallel strategies for ligand-directed therapy with a cytotoxic agent (TNF) versus a theranostic (HSVtk) approach for imaging gene expression followed by suicide gene therapy with ganciclovir (GCV) in the same orthotopic mouse model of human glioblastoma with RGD4C-directed AAVP vectors. Our results demonstrate that RGD4C-AAVP-TNF therapy reduces tumor size in a dose-dependent manner, disrupts tumor blood vessels, and works through an apoptotic pathway. Similarly, with RGD4C-AAVP-HSVtk administration followed by GCV dosing, experimental tumors showed blood vessel damage and marked evidence of apoptosis. We conclude that the magnitude of tumor response was comparable between the two experimental cohorts evaluated. However, by administering a radiolabeled HSVtk substrate, the tumors could also be imaged during the course of the study to evaluate transgene expression over time, a transgene-specific tool potentially useful for timing GCV administration and for evaluating tumor response, a feature not currently available with the cytotoxic TNF vector.

270. Targeting Hepatic Sphingosine-1-Phosphate Transporter 2 to Control Liver Cancer Metastasis

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Liver cancer is the fourth leading cause of cancer death worldwide in 2018, with 782,000 deaths annually. Most deaths are caused by metastasis. In our previous studies, using both mouse models and clinical samples, we have established that iron-deficiency leads to liver cancer metastasis. However, the underlying mechanism and potential treatment are largely unknown. In the present studies, we observed that liver cancer metastasis was associated with elevated expression of hepatic sphingosine-1-phosphate (S1P) transporter spinster homologue 2 (Spns2). Since a large reduction in pulmonary metastasis of other cancer types in Spns2-deficient mice has been reported (*Nature*, 2017; 541(7636): 233-236), we aimed to evaluate the role of hepatic Spns2 in liver cancer metastasis. In the first set of experiments, various human and mouse hepatocellular carcinoma (HCC) cell lines were transduced with recombinant AAV vectors expressing siRNAs to knockdown Spns2. Both mRNA and protein levels of Spns2 were reduced to ~30%. It was evident that the invasion and migration ability of the HCC cell lines were significantly inhibited. In the second set of experiments, Spns2-deficient mice were generated. Mouse HCC cells were orthotopically inoculated in the mouse liver and the metastasis was then induced by iron-deficiency. Compared

to their wild-type counterparts, the Spns2 knockout mice had significantly reduced pulmonary metastasis of HCC. In the final set of experiments, we produced liver-targeting recombinant AAV vectors expressing shRNA against Spns2. Liver cancer metastasis was induced as described above. As appropriate controls, mice were either mock injected, or tail-vain injected with AAV vectors. Bio-distribution assays at 4-week post vector-administration indicated that viral genomes and transgene expression were largely limited to the liver. Western blot assays demonstrated that Spns2 expression was inhibited by ~50%. Most importantly, the pulmonary metastasis of HCC was significantly decreased, as was observed in the whole-body Spns2-knockout mice. Our results have implications in the development of new molecular targets and preventative therapies for liver cancer patients.

271. Nanoparticle-Based Delivery of CRISPR/Cas In Vivo is Able to Clear Tumours in Mouse Models

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Objectives: We have examined the use of the new gene editing technology known as CRISPR/Cas to develop a new treatment for HPV-driven cancers. CRISPR/Cas allows for the targeting and subsequent deletion or interruption of a specific gene in the host. This has resulted in a whole new field of gene editing therapies. Building on our previous RNAi work we have applied our in vivo expertise and delivery nanoparticle systems to examine the use of C/Cas in vivo both in the whole animal and vaginal tract. Methods: Using novel nanoparticles (Wu et al J Control Release, 2011. **155**(3): p. 418-26.) we introduced DNA expressing Cas and guide RNAs to target the HPV E7 oncogene. Cell viability was monitored by colony formation and viability assays. Mice were injected with tumour cells and once formed, treated with IV injections of nanoparticles containing CRISPR/Cas. Tumour formation was monitored. Results: We examined a range of guide RNAs that allowed for the cutting of the E7 gene and subsequent deletion of its function. This resulted in cervical cancer cells losing viability and to sustain long term growth by over 90%. We examined the efficiency of both gene editing using 2 different Cas family members and examined HDR verses NHEJ to understand how editing was occurring (via indel formation). Finally, pre-clinical models of cancer were used we show that CRISPR/Cas9 delivered systemically *in vivo*using PEGylated liposomes results in tumor elimination and complete survival in treated animals. Conclusion: Overall targeting of the E7 gene via CRISPR/Cas is highly effective in treating HPV-driven cancers.

272. Cargocyte™ Biofactories: A New Versatile Cell Therapy Platform for Delivery of a Wide Range of Biologics

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Cell therapies have the potential to address critical, unmet needs in the treatment of human cancer and numerous inflammatory conditions. Compared to small molecule drugs and nanoparticles, cells are highly versatile because they can respond to, interact with, and stimulate their microenvironment in unique ways. In fact, cells can also deliver an array of biologics through, cell-cell contacts, tunneling nanotubes, and the release of cytokines, growth factors, extracellular vesicles, and exosomes. Furthermore, cells detect and home to diseased tissues within the body, which is critical to their use in delivery of therapeutic agents in a precise manner with reduced off-target effects. However, cell therapies can be limited by problems such as safety, controllability, cost, consistency, and donor dependency. While cell and genetic engineering can overcome many of these issues, genetically modified cells must be rendered safe for *in vivo* administration to gain FDA approval for clinical use. This is especially true if the therapeutic cell harbors multiple genomic modifications or is a stem cell. Therefore, there is a critical need for genetically engineered cell therapies to deliver biological payloads *in vivo* in a safe, controllable, and predictable manner. To address these needs, our laboratory pioneered a new cell therapy platform technology (Cargocytes) to treat a wide range of human diseases. Cargocytes are enucleated cells genetically engineered to deliver multiple therapeutic payloads in a precise, safe, and predictable manner to damaged and diseased tissues. Cargocytes retain many desired biologic functions (endogenous and engineered), such as viability up to 72 hours, retention of cell surface markers/proteins, secretion of bioactive molecules, robust *in vitro* and *in vivo* chemotaxis, and delivery of a wide range of disease-fighting cargos. Our unique approach allows for any cell to undergo robust genomic engineering, then centrifuged at high speed through Ficoll density gradients to facilitate the rapid removal of all nuclear DNA. Following enucleation, Cargocytes can be further engineered to express exogenous mRNAs encoding a wide range of biologics (e.g. cytokines antibodies) or they can be loaded with RNA-based therapeutics (shRNA miRNA siRNA), small molecule drugs, peptides and/or gene therapy viruses. The advantages of the Cargocyte platform are an excellent safety profile, a defined lifespan (3-4 days), an optimal therapeutic window (3-4 days), and ability to be generated from multiple cell types, including endogenous or exogenously-engineered autologous or allogeneic hTERT immortalized mesenchymal stem cells (hTERT-MSCs) or cancer cells. In two independent preclinical animal models of cancer and acute-inflammation, we demonstrate the Cargocytes derived from hTERT-MSCs can be engineered with multiple chemokine and endothelial homing receptors (CXCR4, CCR2, PSGL-1) to precisely locate and travel to diseased tissues, and deliver immune modulating cytokines, and gene therapy-encoding oncolytic viruses. The cell culture and enucleation processes are readily scalable for clinical use and Cargocyte biofactories can be cryopreserved and biobanked for long-term storage and shipping worldwide. Collectively, our findings indicate that Cargocytes provide a new cell-based therapeutic platform technology to deliver a wide range of powerful biologics to diseased tissues in a safe and controllable manner.

273. Phase I-II Study Using Rexin-G, a Tumor-Targeted Retrovector Encoding a Cyclin G1 Inhibitor for Metastatic Carcinoma of Breast: A Ten-Year Follow-Up

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Background and Purpose: Rexin-G tumor-targeted retrovector encodes a cytocidal dominant negative cyclin G1 construct and has induced sustained 10-year remissions in patients with advanced chemotherapy-resistant soft tissue sarcoma, osteosarcoma and pancreatic adenocarcinoma. **Methods:** This is an open label, single arm, dose-seeking study that incorporates a modification of the standard Cohort of 3 design combined with a Phase II efficacy phase. Safety analysis used the NIH CTCAE vs 3.0 for reporting adverse events, and efficacy analysis used RECIST v1.0, International PET criteria and Choi criteria. **Results:** Twenty patients received escalating doses of Rexin-G i.v. from 8x10e11 cfu to 48x10e11 cfu/6-week cycle. Safety (n=20): Grade 1-2 treatment-related adverse events included chills (n=1), pruritus (n=2), dry skin (n=1), hot flush (n=1), dysgeusia (n=3); Grade 3 pruritic rash (n=1). No dose-limiting toxicity was observed, and no vector DNA integration, replication-competent retrovirus, nor vector-neutralizing antibodies were detected. Efficacy (n=17): By RECIST v1.0: There were 13 SD, 4PD; by PET/Choi Criteria: 3 PR, 11SD, 3PD. Combined median PFS by RECIST v1.0 was 3.0 months; combined median OS, 30 months with 1-year overall survival rate of 60%. Biopsy of residual tumor in one patient identified abundant CD35+ dendritic cells, CD8+ killer T cells, CD138+ plasma B cells, CD68+ macrophages and CD20+ B cells, suggesting a mature immune response. Two patients with pure bone metastases had >12-month PFS and OS and are the longest survivors. One patient is still alive 10 years later. **Conclusions:** (1) Rexin-G is uniquely safe, (2) exhibits antitumor activity particularly in patients with pure bone metastases, (3) PET-CT is a more sensitive indicator of early tumor responses to Rexin-G and will be used in planned Phase 2 studies, and (4) enhanced immune cell trafficking in the TME indicates that Rexin-G may be combined with other cancer therapy, including immunotherapy, and in theory, may prove to be a biochemical and/or antigen modulator.

274. Therapeutic Activity of Gene Therapy with Toca 511 & Toca FC for Esophageal Cancer

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Introduction: Despite the development in recent years of surgical technique and multidisciplinary treatment including radiation and chemotherapy, esophageal cancer (EC) remains one of the most difficult malignancies to treat. Thus, development of new therapeutics and treatment strategies are needed. Retroviral replicating vectors (RRV) are capable of highly efficient replication and transduction in cancer cells. Prodrug activator gene therapy with Toca 511 (vocimagene amiretrorepvec), an RRV encoding yeast cytosine deaminase (yCD), which converts the prodrug 5-fluorocytosine (5-FC) to the anticancer drug 5-fluorouracil (5-FU) within Toca 511-infected cells, has been shown to achieve significantly enhanced survival benefit in a variety of preclinical tumor models. In patients with recurrent high-grade glioma, Toca 511 and Toca FC (extended-release 5-FC) treatment has demonstrated promising clinical activity and an international randomized Phase 3 clinical trial is currently under evaluation. Additionally, a Phase1b clinical trial for patients with metastatic colorectal, pancreatic, breast, lung, melanoma, and renal cancers is on-going. Therefore, we evaluated the therapeutic activity of RRV-mediated prodrug activator gene therapy in human EC-derived cell lines and in a preclinical model for esophageal cancer. Materials and **Methods:** We first quantitated the replication kinetics of RRV in human esophageal squamous cell carcinoma (ESCC) cell lines (TE1, TE4, TE8 and SGF7) and adenocarcinoma (EAC) cell line (KYAE-1). ESCC and EAC cell lines were infected with RRV expressing the GFP marker gene (RRV-GFP) at a multiplicity of infection (MOI) of 0.01 (~1% initial transduction levels) and analyzed by flow cytometry. Next, we evaluated in vitro cytotoxicity by MTS assay after exposure to a series of 5-FC concentrations in these cell lines transduced with Toca 511. Finally, we evaluated in vivo therapeutic efficacy of Toca 511/5-FC prodrug activator gene therapy in TE8 subcutaneous tumor models. Cell suspension of uninfected cells (99%) mixed with Toca 511-transduced cells (1%) (5×10^6 cells/100μL total volume) was implanted in each mouse. After tumor establishment, mice were randomized to 5-FC treated group or PBS control group (n=5 per group). Tumor volumes were calculated as follows: volume = length × width^2/2. **Results:** In both ESCC and EAC lines, RRV-GFP infected at MOI of 0.01 showed rapid viral replication resulting in high levels of transduction, with the percentages of GFP-positive cells reaching nearly 90% over time. In MTS assay, viability of Toca 511-transduced cells was reduced by approximately 80% after 5 (TE8 and SGF7) or 8 (TE1, TE4 and KYAE-1) days of exposure to 0.1mM 5-FC prodrug compared to the control cells. Furthermore, in subcutaneous tumor models, tumor growth in Toca 511/5-FC treated group was significantly inhibited compared to

control group (p<0.0001) with complete tumor disappearance in the majority of mice (80% of total). **Conclusion:** These in vitro and in vivo data suggested that RRVs are highly efficient vehicles for delivering gene therapy such as yCD to esophageal cancer cells and supports evaluation of Toca 511 and Toca FC treatment as a potentially novel therapeutic for human esophageal cancer.

275. Reporting Long Term Survival Following Precision Tumor-Targeted Gene Delivery to Advanced Chemotherapy-Resistant Malignancies: An Academic Milestone

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Background and Purpose: Targeted gene delivery *in vivo* has long been considered the "Holy Grail" of genetic medicine. **Methods:** We reviewed long-term data of patients with advanced chemotherapy-resistant malignancies, previously-treated patients with two tumor-targeted retrovectors: (1) encoding cytotoxic dominant negative cyclin G1, DeltaRex-G (formerly Rexin-G), and (2) encoding cytokine GMCSF plus suicide gene HStk, DeltaVax (formerly Reximmune-C). **Results:** Ninety-nine patients received >5,000 intravenous infusions of DeltaRex-G; another 16 patients received 288 intravenous infusions of DeltaRex-G + 96 intravenous infusions of DeltaVax followed by oral valacylovir. No therapy-related bone marrow suppression, organ dysfunction or delayed treatment related adverse events were observed. Survival analysis showed **5.0%** 10-year overall survival rate for patients who received DeltaRex-G alone, and **18.8%** for DeltaRex-G + DeltaVax. **Table 1** lists the cancer type, name of targeted gene therapy/immunotherapy, and treatment outcome.

Cancer Type	Targeted Gene Therapy, Route of Delivery	Treatment Outcome
Pancreatic Adenocarcinoma, metastatic to lymph node, liver and peritoneum, resistant to gemcitabine and 5FU	DeltaRex-G, Intravenous	Alive, >10 years, no evidence of disease, no further cancer therapy
Osteosarcoma, metastatic to lung, chemotherapy-resistant	DeltaRex-G, Intravenous	Alive, >10 years, no evidence of disease, no further cancer therapy
Malignant peripheral nerve sheath tumor, metastatic to lung, chemotherapy-resistant	DeltaRex-G, Intravenous	Alive, >10 years, no evidence of active disease, no further cancer therapy
Osteosarcoma, metastatic to lung, lymph nodes, pelvic soft tissue, chemotherapy-resistant	DeltaRex-G, Intravenous	Alive, >10 years, no evidence of disease, no further cancer therapy
Intraductal carcinoma of breast, recurrent, chemotherapy-resistant	DeltaRex-G, Intravenous	Alive, >10 years, no evidence of active disease, no further cancer therapy
B-cell lymphoma, cervical lymph node, metastatic to liver and pancreas, chemotherapy-resistant	DeltaRex-G + DeltaVax, intravenous	Alive, >10 years, no evidence of active disease, no further cancer therapy
Chondroblastic osteosarcoma of maxilla, locally advanced, unresectable	DeltaRex-G + DeltaVax, intravenous	Alive, >10 years, no evidence of active disease, no further cancer therapy
Intraductal carcinoma of breast metastatic to bone, chemotherapy-resistant	DeltaRex-G + DeltaVax, intravenous	Alive, >10 years, with disease progression, on capecitabine for liver metastases/no late adverse event

Conclusion: Data analysis indicates that tumor-targeted gene delivery *in vivo*, represented by cytocidal DeltaRex-G, with or without the immuno-stimulatory DeltaVax, has induced prolonged (>10 years) sustained remissions in cancer patients presenting with advanced chemotherapy-resistant solid and hematologic malignancies—plausibly due to safety, selectivity, and immune modulation. While the curative potential of precision targeted genetic medicine necessarily remains an academic question; it is clear that these initial long-term, cancer-free (>10 year) survivors represent a major milestone in both cancer therapy and immunotherapy. Phase 2-3 clinical trials are planned for these hard-to treat malignancies.

276. A New Transgenic Rat Model & Experimental Vascular Tumors

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Background: To develop model of human cancer we have expressed the avian retroviral receptor, TVA, under a mammalian promotor in transgenic rat, thus rendering rat susceptible to infection with avian leucosis virus-derived gene vectors. TVA-based retroviral gene transfer offers advantages over current murine models of human cancer. Very little information is currently available regarding hemangioma and angiosarcoma and the experiment treatment. **Objective:** To establish novel models of **hemangioma** and **angiosarcoma** in blood vessel and several organs in TVA Tg-rats; and use these models to evaluate the efficacy of chemicals and biologicals (drugs) on associated injuries (**hemorrhages /vascular leakage / bleeding**), and survival rates in the rats. **Design/Methods:** Our strategy is based on use of the receptor for subgroup-A avian sarcoma leukosis virus(ASLVs), TVA, and allows

infection of mammalian cells by the ASLV; the tva gene was cloned from chicken and TVA transgenic rat was established by NIH. To produce higher titer recombinant virus, we have taken advantage of a Rous Sarcoma Virus (RSV)-derived replication-competent cloning vector, RCAS, and constructed RCAS-middle antigen from polyoma and an established chicken fibroblast cell line, DF-1. Newborn TVA-Tg rats were injected through the retro-orbital plexus with recombinant RCAS-middle antigen from polyoma. The drugs were admitted by oral once per day using special needle, began at 5th days after being injected the virus; water for control. RESULT: The homozygous TVA transgenic rats have been identified to cross breeding for new born rat pubs; the model of angiosarcoma (Vascular Tumors of the Head & Neck) are established in TVA rat pups by injecting RCASY-T virus through the retro-orbital plexus; It was observed the effect of the drugs improving survival proportions. **Conclusions:** Using recombinant avian retroviruses with TVA transgenic rat to generate experimental Vascular Tumors. The results suggested that the model can be used to evaluate the safety and efficacy of chemicals and biologicals (drugs) on associated injuries (**hemorrhages /vascular leakage / bleeding**) and explore for the mechanism of the tumor development.

277. Engineering and Optimization of Chimeric Notch Receptors

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Background: Chimeric Antigen Receptor (CAR) T cells have demonstrated significant promise as a cancer therapy. However, essential safety and efficacy shortcomings remain to be addressed in the field. Highly discriminatory homing instructions to tumor tissues are essential in minimizing patient toxicities. Significant challenges in meeting these specificity and potency requirements limit the therapeutic potential of adoptive T cells. synNotch is a protein technology that adds specificity and safety to engineered CAR T cells, allowing the expression of a CART to be gated by the presence of a second (priming) antigen. Originally, synNotch was composed of murine, yeast, and viral proteins; however, we are in the process of humanizing the technology for future applications. **Objective:** To present the iterative engineering and optimization of the synNotch protein from its potentially immunogenic, non-human form to a receptor composed of human proteins that may expand its therapeutic potential. **Design/Results:** We engineered synNotch and its reporter by rationally screening variant libraries in human primary T cells, and by iteratively replacing domains of the original synNotch technology with alternative components. This allows for a decrease in potential immunogenicity through humanization of the protein components while optimizing functionality through engineering of the vector and promoter components. **Conclusions:** Equipped with the capability to integrate combinatorial antigen sensing and user-defined immunomodulatory genetic outputs, the synNotch receptor is well armed to combat solid tumors and remodel tumor microenvironments. These advancements will likely allow synNotch, a much needed technology for increased control in emerging cell therapies, to further the safety and specificity of CAR-T therapy. Implementation of the synNotch technology will allow for highly tunable, persistent and selective tumor killing and will progress cell therapy's capacity to effectively treat specific cancers. Furthermore, as synNotch is a platform technology, our characterization of its functionality in various contexts will provide a toolbox for future engineers to apply to their specific needs.

278. Death Receptor-5 Targeted Delivery of Gamma-Secretase Inhibitor for Simultaneous Targeting of Cancer Cells and Cancer Stem Cells in Triple Negative Breast Cancer

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(a) Purpose of the study Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype, characterized by the higher metastasis and tumor relapse rates. Current therapies for TNBC regression target and kill differentiated breast cancer cells, which constitute the bulk of the tumor, while sparing the small population of breast cancer stem cells (BCSCs). Accumulating evidence suggest that the BCSCs, which are left behind are responsible for metastasis and tumor relapse. Recent research, therefore, focused on designing novel cancer therapeutics, which can eradicate BCSCs along with differentiated breast cancer cells (non-BCSCs). Notch signaling pathway is a regulatory pathway responsible for angiogenesis in non-BCSCS and self renewal and maintenance in BCSCs. Inhibition of gamma secretase (a regulatory component of notch pathway) is an attractive strategy to silence notch signaling. However, the clinical limitation of γ-secretase inhibitors such as N-[N-(3,5-difluorophenacetyl)-L-alanyl]-Sphenylglycine t-butyl ester (DAPT), MK-0752, BMS-708163 is their off-target effects. Death receptor-5 (DR5) has been reported to be expressed exclusively on the surface of TNBC cells (non-BCSCs and BCSCs). Current research, therefore, focused towards utilizing DR5 for site specific delivery of chemotherapeutic agent to TNBC. In the present study we propose to prepare DR5 targeted Solid lipid nanoparticles (SLNs) of γ-secretase inhibitor, DAPT and to achieve radical cure of TNBC by eradicating BCSCs and non-BCSCs. The objectives of the present study are to • Prepare and characterize solid lipid nanoparticle of γ-secretase inhibitor, DAPT (DAPT-SLNs). • Functionalize DR5 monoclonal antibody on the surface of DAPT-SLNs(DAPT-DR5-SLNs) to achieve specific targeting to TNBC cells (both BCSCs and non-BCSCs). • Study the *in vitro* and *in vivo* efficacy of developed DAPT-DR5-SLNs. **(b) Experimental procedures** Anti-DR5 antibody was conjugated on the surface of SLNs loaded with DAPT using EDC as a cross linker. The prepared DAPT-DR5-SLNs were characterized for their surface morophology, charge and size. The *in vitro* efficacy studies were performed in MDA-MB231, TNBC cell line. Cellular internalization of DAPT-DR5-SLNs was observed under confocal microscope. Cell viability was assessed using MTT assay, Annexin-V/PI staining. $CD44^{+}/CD24^{-}$ subpopulation of cells were identified by flowcytometry. Immunofluorescence analysis was performed to determine the expression of EMT markers. Cell migration was studied by scratch wound healing assay. *In vivo* tumor regression studies were performed in mice model. **(c) Results** The developed DAPT-DR5-SLNs have appreciable size of 142±1.62 nm and encapsulation efficiency of 75.60 %. DAPT-DR5-SLNs are significantly cytotoxic in MDA-MB-231 cell lines as observed by MTT assay, Annexin-V/PI assay and AO/EB staining. These nanocarriers exhibited successful internalization in

DR5 overexpressed MDA-MB-231 cell lines determined fluorescence staining method. Reduced expression of EMT markers and $CD44^+/CD24^-$ cells, implies the propensity of DAPT-DR5-SLNs to inhibit tumor relapse and metastasis. **(d) Conclusion** Metastasis and tumor relapse are the major causes of mortality in TNBC patients. Notch signaling pathway is a regulatory pathway responsible for maintainance of BCSCs, metastasis and tumor relapse. From the present study it was evident that DAPT-DR5-SLNs specifically internalized in MDA-MB231 (TNBC) cells and enhanced the therapeutic efficacy.

279. Spred2 Reduced Epithelial Mesenchymal Transition (EMT) of Colorectal Cancer Cells via Inhibiting ERK Signaling

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Colorectal cancer (CRC) is the fourth leading cause of cancer-related deaths worldwide. Although the death rate from CRC has been dropping in past decades, reduced sensitivity to conventional treatments is the major obstacle to treat advanced and metastatic CRC. Therefore, it is urgent to develop novel therapeutic strategies for CRC. Sprouty-related EVH1 domain protein 2 (Spred2) is a negative regulator of growth factor induced RAS-ERK activation. And, it has been demonstrated that reduced Spred2 expression was closely associated with high metastatic phenotypes in various tumors, such as prostate cancer and hepatocellular cancer. Here, we explored the roles of Spred2 in the development and progression of CRC. As expected, Spred2 expression was significantly down-regulated in clinic tumor tissues, compared to that in corresponding distal normal tissues. To restore intercellular Spred2 level, Ad.Spred2, an adenoviral vector expressing Spred2, was transduced into CRC cells. Ad.Spred2 transduction effectively decreased the proliferation, survival and migration of SW480 cells. Moreover, Ad.Spred2 obviously inhibited epithelial mesenchymal transition (EMT), an essential event during tumor metastasis to distant sites, such as promoting reorganization of F-actin, up-regulating E-cadherin level, reducing Vimentin protein, and so on. Importantly, extracellular signal-regulated kinase (ERK) signaling inhibition by specific inhibitor, PD98059, induced similar responses on EMT, suggesting that Ad.Spred2 could ERK-dependent regulated EMT in CRC cells. Transforming growth factor β (TGF-β), a well-known inducer of EMT, increased E-cadherin expression and decreased Vimentin expression, as well as promoted migration in CRC cells. However, both Ad.Spred2 and PD98059 had no obvious effects on the expression of SMAD2/3 and SMAD4, pivotal molecules in TGF-β/SMADs signaling pathway in SW480 cells. These results suggested that Ad.Spred2 could SMADs-independent inhibiting EMT in SW480 cells. Interestingly, Ad.Spred2 down-regulated SAMD2/3 and SMAD4 levels, while PD98059 increased SMAD4 expression in HCT116 cells, suggested that Ad.Spred2 could ERK-independent reduce SMADs signaling. We speculated that Ad.Spred2 might inhibit the EMT of HCT116 cells via both blocking ERK signaling and reducing SMADs signaling. We concluded that Spred2 could inhibit EMT of CRC cells via interfering ERK signaling, and might also through reducing SMADs signaling. Therefore, Spred2 might be a promising therapeutic target for treating CRC.

280. Targeting Small Molecule Inhibitor and microRNA for Medulloblastoma Therapy

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Medulloblastoma (MB) is the most common type of pediatric malignant brain tumor. MB is primarily classified into four subgroups: wingless (Wnt) group, sonic hedgehog (Shh) group, group 3 and group 4. Treatment of medulloblastoma (MB) is a challenge due to high heterogeneity, diverse genetic make-up, differential miRNA expression, variable prognoses, and blood brain barrier. Aberrant activation of hedgehog (Hh) pathway regulates cell growth, cancer stem cell proliferation, and tumorigenicity in 30% MB patients. Several Hh inhibitors including GDC-0449 have shown promise in cancer therapy by binding to seven-transmembrane (7-TM) domain of smoothened (Smo). However, the emergence of resistance during GDC-0449 treatment and side effects limits its utility. We synthesized GDC-0449 analog, 2-chloro-N1-[4-chloro-3-(2-pyridinyl) phenyl]-N4,N4-bis(2-pyridinylmethyl)-1,4-benzenedicarboxamide (MDB5) which had improved docking score on both wild type and mutant smoothened (Smo), confirming its tighter binding affinity. Topoisomerase I inhibitor topotecan (TPT) interrupts DNA replication inhibiting growth and proliferation. Therefore, the overall objective is to deliver MDB5 and TPT polymeric nanoparticles (NPs) for the MB treatment. Further, coating the NPs with polysorbate 80 (Tween 80) that binds apolipoprotein E (ApoE) can facilitate crossing of these NPs across the blood-brain barrier (BBB). In our preliminary study, the combination therapy suppresses colony formation, migration, invasion, and promoted apoptosis compare to monotherapy. We generated orthotopic MB tumor model by implanting DAOY cells stereotaxically in NSG mice for *in vivo* evaluation of these formulations. The electron microscopic picture of NPs coated with polysorbate 80 were well dispersed, spherical in shape and had mean particle size of less than 100 nm. Since miRNAs play an important role in the development of human cancers including MB, we carried out miRNA profiling of several MB patient tumor samples and observed only a few miRNAs (miR-217, miR-130b, miR-25) to be upregulated, but several including miR-138, miR-29b, miR-451, miR-10b and miR-129 were highly downregulated, suggesting a tumor-inhibitory function. In conclusion, application of multi-pronged approaches including small molecules, miRNA and targeted drug delivery have the potential to effectively treat MB.

281. The Composition of Dendritic Cells in Various Cancer Tissues

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Backgrounds: Dendritic cells (DCs) play a pivotal role in inducing antitumor immune responses by deliver the tumor antigens to naïve or memory T cells and secreting chemokines to recruit effector cells to tumor sites. Recently, it is reported that DCs also can enhance anti-PD-1 therapeutic effects by secreting IL-12 which licenses T cells to kill tumor cells. Although the clinical significance of DCs for anticancer immunity is well known, there is little information about composition of DCs in various cancer types. **Methods:** Single cells of breast cancer (BC, n=16), colorectal cancer (CC, n=16), and stomach cancer (SC, n=15) were obtain by dissociation of tumor tissues with collagenase/hyaluronidase. DCs and their subpopulation [conventional DCs (cDCs) and plasmacytoid DCs (pDCs)] were analyzed by flow cytometry. The proportion of DCs of different tumor types and the association of DCs with other immune cell populations and clinicopathologic factors were analyzed. **Results:** We obtained 1.5-26 x 10^5 cells/mg for CC, 1.4-9.6 x 10^5 cells/mg for SC, and 0.8-110 x 10^5 cells/mg for BC from 25-40mg of tumor tissues. SC had higher proportion of DCs than other types of tumors (SC, 12%; CC, 6.2%; BC, 3.9%). cDC was the dominant subpopulation of DCs in all types of tumor tissues, and the proportion of cDCs was not significantly different among cancer types. The proportion of pDCs was significantly higher in BC (10.6%, $p \leq 0.001$) than CC and SC (0.8% and 0.3%, respectively). The proportion of DCs in total dissociated cells was positively correlated with that of NK ($p<0.001$), NKT ($p<0.001$), T ($p=0.001$), and B ($p<0.05$) cells in all tumors. Other factors such as pathologic T and N stages, lymphovascular invasion, and the level of tumor infiltrating lymphocytes and tertiary lymphoid structures were not associated with the DCs level in tumor. **Conclusions:** The composition of DCs in CD45+ immune cells were varied from tumor types. The positive correlation of DCs with other immune subtypes supports that DC plays an important role in recruit other immune cells into tumor tissues. Thus, adoptive cell therapy utilizing DCs might be the promising therapeutic approach for the tumors with low immune cell infiltration. *This study was supported by Basic Science Research Programs through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning, Republic of Korea (NRF-2018R1D1A1B07048831).

Immunological Aspects of Gene Therapy and Vaccines

282. Induction of Regulatory T Cells and Effector T Cell Exhaustion are Essential to Establish and Maintain Liver-Mediated Immune Tolerance to Muscle-Expressed Antigens

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Recent clinical trials established the liver as an ideal target tissue for gene replacement strategies. One important advantage of the liver is its pro-tolerogenic environment that reduces potential immune responses against transgenes expressed in hepatocytes via gene therapy. This is particularly evident when compared to gene transfer in other peripheral tissues such as the skeletal muscle, where anti-transgene immune response results in partial or complete clearance of the transduced fibers by transgene-specific cytotoxic T cells. Here, we characterized liver-induced transgene tolerance after simultaneous transduction of liver and muscle with a model antigen. A clinically relevant transgene, α-sarcoglycan, a protein mutated in limb-girdle muscular dystrophy type 2D (LGMD2D), fused with the ovalbumin MHC class I epitope SIINFEKL (hSGCA-SIINFEKL), was expressed with adeno-associated virus (AAV) vectors. Intramuscular delivery of the AAV vector expressing hSGCA-SIINFEKL resulted in a strong pro-inflammatory response with expansion of antigen-specific CD8+ T cells and production of anti-transgene antibodies. Concomitant liver expression of the same antigen via AAV vectors efficiently prevented and reversed anti-transgene immune responses. We showed that synergy between expansion of CD4+CD25+FoxP3+ regulatory T cells and upregulation of checkpoint inhibitor receptors is required for the establishment and maintenance of liver-mediated peripheral tolerance. This study establishes the central role of liver-induced tolerance in the control of anti-transgene immune responses in peripheral tissues elicited in the context of gene transfer.

283. Cellular Immune Response to Subretinal AAV Gene Delivery in the Primate Retina

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Introduction: Recombinant adeno-associated virus (AAV) is a common tool for retinal gene delivery in both the pre-clinical and clinical setting. As recent reports highlighted immunogenicity towards

AAV in the retina, we evaluated aspects of adaptive immunity to AAV in the primate retina.**Purpose:** To evaluate cellular immune response in primate retina tissue following subretinal gene delivery with clinical grade AAV.PDE6A. **Approach:** Cynomolgus monkeys underwent vitrectomy and subretinal injection of clinical grade AAV8 as part of a formal toxicology study at following doses: 6 animals (3m/f) received vehicle only, 8 animals (4m/f) received 1 x 10^11vector genomes (vg, low dose) and further 8 (4m/f) received the high dose (1 x 10^12vg). Ophthalmic examinations included clinical examination of the eye, fundus photography, angiography, and optical coherence tomography (OCT). The animals were sacrificed 90 days after AAV delivery and eye sections were stained with hematoxylin and eosin. Additionally, immunostaining was performed against markers of cellular immunity. **Results:** There were no adverse changes on OCT, angiography and/or histology in the control and the low dose group. Fluorescence angiography in three out of eight animals from the high dose group revealed areas of focal leakage two weeks after surgery, that were still present at time of necropsy (d90). Histology demonstrated multiple intraretinal infiltrates, recruitment of CD20+, HLA-DR/DQ, CD38, CD3, IbaI positive cells, and focal disruption of retinal architecture in four out of eight high dose animals. The data suggest recruitment of B- and T-cells, antigen presenting cells and activation of microglia in the primate retina 90 days after AAV delivery. **Conclusions:** Collectively, these observations suggest an active and complex local immune response to clinical grade AAV8 in the primate retina with recruitment of B-/ T-cells and retinal microglia.

284. Efficient Gene Editing and Elimination of Latent Herpes Simplex Virus In Vivo by AAV-Delivered Meganucleases but Not CRISPR/Cas9

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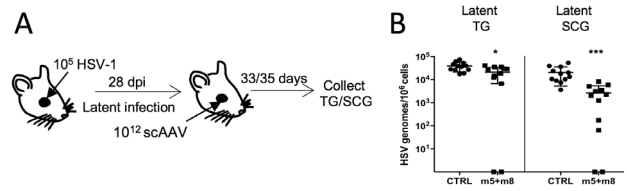
Herpes simplex virus (HSV) establishes lifelong latency in neurons of the peripheral nervous system after initial infection at mucosa. Latent HSV is the source of recurrent disease, and is not affected by current antiviral therapies, which reduce symptoms and viral shedding, but do not cure infected individuals. In contrast, gene editing using CRISPR/Cas9 or other targeted endonucleases offers the potential of lethally mutating or even eliminating latent virus. Here, we used gene editing of HSV byCRISPR/Cas9 or meganucleases (MNs), delivered via AAV vectors, to treat latent HSV in a well-established mouse model. We found that CRISPR/Cas9, despite showing highly efficient gene editing of HSV in cultured neurons, mediated only weak HSV gene editing *in vivo*. In contrast, meganucleases mediated highly efficient gene editing of HSV both *in vitro* and in latently infected mice. Treatment with a single MN resulted in up to a 65% reduction in ganglionic HSV load, with up to 30% of the residual virus showing mutation incompatible with viral replication. Using a combination of two meganucleases, the reduction in ganglionic HSV load exceeded 90%, with a similar reduction in the ability of HSV to reactivate from neurons of treated animals. Single cell RNA sequencing demonstrated that both HSV and individual AAV vector serotypes were non-randomly distributed among neuronal subsets in ganglia, implying that combinations of AAV serotype vectors will be required to reach all HSV-infected cells. These results demonstrate the power of gene editing as a therapy for chronic infectious diseases including HSV, and suggest a plausible pathway toward a cure for this widespread and important infection.



Reduction of latent HSV loads in meganuclease-treated mice. (A) Mice were infected with 10^5 PFU HSV-1 17+ in the right eye following corneal scarification, and 28 days later were administered 10^{12} genome equivalents scAAV-Rh10-CBh-MN by retro-orbital (RO) injection. Superior cervical ganglia (SCG) and ipsilateral trigeminal ganglia (TG) were collected at 33-35 days post MN exposure. (B) TG and SCG from latently infected but untreated control mice (CTRL) or mice treated with two MNs (m5+m8) were subjected to ddPCR quantification of HSV genomes by droplet digital PCR. *p>0.05; ***p>0.001.

285. Transgene Immunity Following AAV-Mediated Gene Transfer in the Macaque is Associated to Persisting Viral Genomes, Long Term Liver Inflammatory Fibrosis and T Cell Exhaustion

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The liver is a singular organ where immunity can be biased toward ineffective response and even tolerance, both in the context of allogeneic liver transplant or viral infections. When T cell immunity is unable to completely eradicate an antigen in the liver, it can result in chronic viral inflammation and even tissue injury and fibrosis that could resolve over time. Regarding liver gene transfer using recombinant Adeno-Associated Virus (rAAV), host immunity balance against the transgene product depends on multiple factors including gene product immunogenicity, vector dose and design or animal model used. Tolerance induction to different transgene products was previously reported in animal models but some other studies have shown conventional cytotoxic $CD8^+$ T cell responses with a rapid loss of transgene expression in the case of more immunogenic products such as secreted ovalbumin or GFP. We performed a long term follow up of 12 non-human primates from three different protocols in which all the individuals received a rAAV8 vector carrying the GFP transgene, at doses ranging from $2x10^{11}$ to $7x10^{12}$ vg/kg. We analyzed viral genome copies, GFP-directed immunity, GFP hepatic expression as well as liver histology. Despite the detection of an acute short-term cytotoxic

immunity with humoral and cellular anti-GFP responses leading to the loss of transgene expression in the liver, we were still able to detect persisting viral genomes until 1 year post-injection. This long term unexpected observation was associated to *in situ* liver inflammation in the majority of animals and even fibrosis in 3 macaques. Interestingly, long term inflammatory fibrogenesis was systematically correlated to T cell-associated hepatic immune regulation with the detection of PD1-positive CD8 T cells in liver infiltrates. In conclusion, our study shows for the first time that anti-transgene immunity following hepatic AAV-mediated gene transfer can lead to a non-conventional immune response in the macaque liver. Additional work is still required to better understand the mechanisms of this atypical immunity and predict its potential outcome in rAAV clinical trials.

286. AAV8-Mediated Hepatic Immune Tolerance Permits Redosing of Therapeutic Transgenes

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Recent successes with recombinant adeno-associated viral (rAAV) vectors in the treatment of certain monogenic diseases have propelled their use as the next generation of biologics. However, a critical challenge that constrains the success of rAAV-based gene therapy are the host's immune responses to both the vector capsid and transgene product. Host immunity raises concerns regarding the safety and longevity of gene expression. In addition, even though terminally differentiated tissues such as liver and muscle are preferred targets for long-term in vivo transduction of rAAV, non-replicating episomally persistent rAAV genomes may be gradually reduced over time due to the possible loss of host cells for different reasons. Thus, the development of effective therapeutic strategies for diseases may require repeated administration of vectors. One of the approaches to overcome this problem is by induction of tolerance to the transgene in the host. rAAV-mediated liver transduction can induce systemic transgene-specific immune tolerance. Considering the physiological and tolerogenic roles of the liver, it has been the favored target organ for gene therapy. In this study, we packaged into rAAVs the Ovalbumin (OVA) cDNA, a model antigen with highly immunogenic properties, and we found that generation of immune tolerance in liver is AAV serotype dependent. AAV8 blunted the CD8+ T cell response and elicited tolerance by provoking B-Reg and T-Reg cell responses and increased IL-10 production, while injections of AAV1 displayed low serum OVA levels, high anti-OVA IgG levels, and generated OVA-specific CD8+ T cells in spleen. In re-dosing experiments, where OVA was first delivered intramuscularly with AAV8 vectors followed by redosing with AAV1, the initial AAV8 injections consistently showed higher OVA levels and repressed anti-OVA IgG production followed by a boost in OVA expression upon rAAV1 redosing. This was concurrent with activation of both CD4+ and CD8+ T-Reg cells in spleen. In contrast, initial dosing with AAV1 vectors elicited a strong anti-OVA response. Re-dosing with AAV8 vectors failed to mediate tolerance. Furthermore, expression of innate and adaptive immune response genes in the liver following rAAV transduction showed that rAAV8.CB.OVA injections significantly elevated *IL1a*, *CCL5*, *Mpo*, and *CXCL1*. In addition, intramuscularly dosed AAV8 vectors that carry liver-detargeting microRNA binding sites for miR-122 reduced liver tolerance. Altogether, our results are consistent with previous studies that suggest transgene expression in the liver is an essential prerequisite for inducing immune tolerance. Moreover, we identify new immune modulatory genes in the liver whose downregulation is vital for mediating tolerance to the transgene. Our study also provides evidence that initial delivery of the transgene with AAV8 injections would ensure induction of hepatic tolerance and hence a successful second round of injections of the transgene, if required. This strategy could potentially be utilized to promote immune unresponsiveness for repeated doses of rAAV for therapeutic purposes.#Co-corresponding authors

287. Characterization of Viral Epitopes That Elicit That Elicit Response to Viral Specific T-Lymphocyte Therapy Used for Viral Disease Following Pediatric Hematopoietic Stem Cell Transplantation

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Background: Viral disease is a significant cause of morbidity and mortality during periods of profound immunocompromise following hematopoietic stem cell transplantation (HSCT). Where available, antiviral medications have suboptimal response rates while also being costly and associated with organ toxicity. An alternative approach to the treatment of viral disease has been the development of viral specific T-lymphocyte therapy (VST). At CCHMC, VST from healthy 3rd party donors are generated with activity against cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, and BK virus by pulsing peripheral blood mononuclear cells with viral peptide pools. To date, on an open phase I/II clinical trial, greater than 40 patients have received this therapy with good results and no de novo cases of graft-vs-host disease (GVHD) attributable to the infusion. Choice of VST unit is based off the degree of human leukocyte antigen (HLA)- match in conjunction with pre-clinical characterization of each cell product. However, the viral epitopes driving VST activity has been poorly understood. Objective: For CMV, EBV, and adenovirus we sought to characterize important viral epitopes presented by common HLA antigens in an attempt to better understand a bank of approximately 125 previously generated VST products. Methods: Epitope prediction was performed for three proteins from EBV (LMP2a, EBNA1,

BZLF1), two proteins from CMV (pp65, IE-1), and two proteins from adenovirus (hexon and penton) using a combination of HLA restrictor 1.2 (Technical University of Denmark) and IEDB.org in conjunction with previously published viral epitopes. 9mer to 15mer peptides were synthesized. For given VST products, predicted epitopes of interest were predicted based off of high-resolution HLA typing. VST were then pulsed with individual peptides and T-cell response was assessed by determining intracellular interferon gamma (IFNg) positivity by flow cytometry. Post-infusion analysis was performed using patient derived peripheral blood mononuclear cells (PBMC) pulsed with peptides and analyzed by IFNg ELISpot. Results: A total of 42 peptides were synthesized. EBV and CMV peptides were predicted to bind class I HLA whereas adenovirus predictions were for both class I and class II. A total of 14 VST products have been pulsed with different subsets of peptides based off of the known HLA haplotype of the donor, 9 with adenovirus peptides, 6 with CMV peptides, and 6 with EBV peptides. In 19/21 experiments, at least one peptide elicited an interferon gamma positive response by flow cytometry and 11/21 had at least two peptide based responses. In multiple cases, the response elicited by an individual peptide alone was stronger than the peptide mix utilized to generate the product which was used as a positive control. We also evaluated PBMC from 5 VST recipients by pulsing them with the peptides that elicited a response by flow cytometry in the VST product by ELISpot. Four of these showed a similar degree of IFNg positivity suggesting an *in vivo* response. Conclusion: Utilizing readily accessible epitope prediction software tools, we were able to induce antiviral T cell activity using single peptides. Our data suggests this response is polyclonal and appears similar pre-clinically and in patient samples. Further work will go towards characterizing HLA-restriction with the long term goal of creating an algorithm to adjudicate which cellular product is best for any given patient.

288. Immunogenicity of a New Flaviviral-Based DNA Launched Suicidal Replicon for Protective Vaccination Against Hepatitis C

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Chronic infections with the hepatitis C virus (HCV) can be effectively cured by antivirals. However, as cured patients can be reinfected they lack protective immune responses. Thus, to control and possibly eradicate HCV an effective prophylactic vaccine is needed. The vaccine should induce either, or both, neutralizing antibodies and protective T cell responses. We therefore developed a potent new vector system based on DNA launched flaviviral replicons that effectively prime HCV-specific T cell responses. We generated suicidal DNA-launched replicons based on Tick-borne encephalitis virus (TBEV), Kunjin virus (KUNV), and West Nile Virus (WNV) that expressed a fusion protein between the HCV NS3/4A and a stork hepatitis B virus core. In transfection studies the KUNV replicon showed superior expression levels of HCV NS3/4A. Importantly, the immunogenicity of the three suicidal flaviviral DNA launched replicons were tested in mice and were compared to a standard DNA plasmid with CMV promoter driven expression of NS3/4A. The KUNV-HCV replicon was the best replicon-based immunogen with respect to priming of HCV NS3/4A-specific T cells as determined by ELISpot, dextramer staining, and polyfunctionality. Importantly, a mutant KUNV-HCV immunogen lacking RNA replication failed to induce immune responses. Thus, the newly developed KUNV-based suicidal DNA launched repliconvaccine for HCV is a highly attractive candidate as a prophylactic vaccine against chronic hepatitis C.

289. Neutralization and Enhancement of AAV Vector Transduction by IgG and Non-IgG Factors in Plasma from Healthy Human Donors

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Adeno-associated virus (AAV) is a common nonpathogenic virus and a widely used gene therapy vector. However, humoral immunity to the viral capsid as a result of early exposure to AAV in life can negatively impact vector transduction especially following systemic administration. Anti-AAV humoral immunity is commonly measured by the presence of anti-AAV binding and or neutralizing antibodies. Of these the presence of neutralizing antibodies, as measured by the neutralizing antibody (NAb) assay, has emerged as a key exclusion criteria when recruiting patients for AAV clinical trials. The NAb assay measures reduction in AAV transduction when virus is pre-complexed with heat-inactivated serum or plasma from donors. In the present study we investigated plasma from four healthy human donors for presence of NAb to AAV6 using an *in vitro* assay. We noted that two donors had NAbs that prevented AAV6 transduction (titers >1:40). To better characterize the neutralizing activities we purified total IgGs from the donors before complexing with AAV6 in the NAb assay. As expected in two donors whose plasma prevented vector transduction, neutralization was present in the purified IgG fraction. No neutralization was observed in any of the fraction from the two donors who were NAb negative. Strikingly, among the NAb positive donors, neutralization was also noted in the IgG depleted fraction in one donor. Furthermore, in this donor vector neutralization by either fraction alone was lower than neat plasma and suggests a cooperative neutralization of vector by both IgG and non-IgG factors. Antibody dependent enhancement (ADE) of viral infection has been previously demonstrated for viral diseases including dengue, influenza, HIV, Zika and Adenovirus. ADE is mediated following increased uptake of antibody bound viral complexes by antigen presenting cells (APCs). In the present study, we observed enhanced AAV transduction when vectors were pre-complexed with plasma from the two NAb negative donors. Interestingly upon further characterization, the AAV6 enhancing activity was found mainly in the non-IgG fraction and differs from the ADE activity previously demonstrated for other viral infections. In separate preliminary studies, we also noticed robust transduction of human macrophages by AAV6 vector. It is possible that the observed ADE results in increased *in vivo* transduction of APCs within secondary lymphoid organs (SLO). Additional studies were then performed in mice passively transferred with donor plasma and administered vector to study changes in vector biodistribution

to SLO. We discuss the impact on neutralization, enhancement and biodistribution of AAV vectors in mice passively transferred with human plasma. Our data demonstrate a complex interplay of anti-AAV humoral immune responses mediated by both IgG and non-IgG plasma components that dictate neutralization or enhancement of AAV transduction. These results underscore the importance of developing novel anti-AAV NAb assays that take into consideration both IgG and non-IgG plasma components while screening patient samples to help predict the outcome of AAV clinical trials.

290. Management of Preexisting Immunity to AAV9 in Freidreich's Ataxia

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Friedreich's ataxia (FA) is the most common form of hereditary ataxias. It is caused by an inherited autosomal recessive expansion mutation of GAA repeat in the *frataxin* (*FXN*) gene. FA manifests between the ages of 8 and 16 as a multisystem disorder, primarily affecting cardiac and nervous system. There is currently no effective treatment, but AAV-based gene replacement therapy shows promise in numerous preclinical studies by our group and others. However, a crucial and unresolved challenge for the success of gene therapy is the host immune response to vector capsid proteins. Patients with prior exposure to AAV with a high titer of anti-AAV9 antibodies may have infusion reactions, raising concerns regarding safety, longevity of expression, and loss of therapeutic effect. As a result, patients with anti-AAV antibody titers above 50 U/mL, whether acquired by environmental exposure to wild-type AAV or by participating in a AAV-based clinical trial, are excluded from clinical trials. To understand the extent of preexisting immunity in the FA population, we analyzed serum of 93 patients and found that 53% had preexisting antibody titers to AAV9 that were higher than the inclusion criteria for current AAV-based clinical trials. To overcome this limitation, we evaluated immunomodulatory approaches to decrease preexisting immunity against the AAV capsid and allow for safe administration of AAV. Previous preclinical and clinical studies by our group show that dosing patients with B-cell depleting Rituximab and mTor inhibitor Sirolimus before the AAV9 infusion resulted in lower levels of circulating anti-AAV antibodies and higher transgene expression. To test this and other immunomodulatory agents (Bortezomib and Abatacept) in the case of preimmunity, we used empty AAV9 capsids to elicit defined levels of capsid specific preimmunity in mice and compared the effect of treatments on circulating anti-AAV9 antibodies, B and T cell populations, biodistribution and transgene expression. We found that all interventions we used decreased circulating anti-AAV9 by over 50% and that CD20 and Sirolimus had the largest effect on levels of B and T cells in circulation and in spleen. Ongoing studies aim to assess the effect of immunomodulatory agents on transgene expression and define the highest level of preimmunity that does not inhibit transgene expression in mice. Overall, this work aims to better understand the relationship between preimmunity to AAV9 and transgene neutralization and how to modulate it, ultimately allowing additional patients access to AAV-based gene therapies.

291. Pre-Existing Anti-Adeno-Associated Virus (AAV) Serotype 5 Neutralizing Antibodies (NABs) Titers in Minipig Serum Do Not Reflect Levels of Anti-AAV5 NABs Titers in Their Cerebrospinal Fluid (CSF)

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AAV-based therapies generated promising pre-clinical results translating into phase I clinical trials for several neurodegenerative diseases. However, it remains unclear whether naturally acquired pre-existing systemic immunity to AAV would affect the therapeutic efficacy of AAV vectors delivery to the Central Nervous System (CNS). To address this question, we investigated the levels of pre-existing anti-AAV5 NABs in serum and in CSF in a cohort of minipigs. Thirty matched serum and CSF samples from wild type minipigs were analyzed for the presence of anti-AAV5 NABs. While serum samples of minipigs had detectable levels of anti-AAV5 NABs that varied between a titer of 2 (negative) to 256 (positive), CSF samples of all the minipigs were found negative for the presence of anti-AAV5 NABs. Hence, no detectable anti-AAV5 NABs were detected in CSF of animals that had systemic pre-existing anti-AAV5 NAB titers up to 256. In order to translate our data to clinic, an anti-AAV5 NABs prevalence study was performed using serum of healthy donors (n=350). In this cohort, 88% of the subjects had anti-AAV5 NABs titers below 256. We have previously reported that serum anti-AAV5 NAB 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. Combined with the current data, we conclude that the risk for reduced therapeutic efficacy of intrathecal or intraparenchymal administration of therapeutic AAV5 vectors by pre-existing neutralizing AAV5 antibodies is very low. Further studies are needed to determine the local and systemic induction and persistence of anti-AAV5 NABs titers following intrathecal or intraparenchymal administration of AAV5 vectors to assess the possibility of re-administration of AAV5-based gene delivery.

292. Induction of Immune Tolerance Towards the Human HexM Enzyme Following Intravenous Gene Transfer in a Mouse Model of Sandhoff Disease

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Immune responses towards therapeutic proteins can diminish the effectiveness of enzyme replacement therapies and gene therapies. Here we investigated methods for induction of immune tolerance towards a foreign protein following AAV gene transfer in a mouse model of Sandhoff disease (SD). SD results from the accumulation of GM2 gangliosides in neuronal cells due to a deficiency in β-hexosaminidase A (HexA) enzyme activity. HexA is a heterodimer composed of two subunits, α and β; deficiencies of either subunit can inhibit its function. SD results from a deficiency of the β subunit. HexM is a novel, stable homodimer variant of human HexA that effectively hydrolyzes GM2 in vivo. A previous study showed that gene transfer of the HexM gene, *HEXM,* via self-complementary AAV9 resulted in an increased lifespan in SD mice, but an immune response possibly impacted the long-term efficacy of the treatment. We hypothesized that immunosuppression would allow development of tolerance towards the expressed HexM enzyme. Immunosuppressants (IS) rapamycin (R) and prednisone (P) were selected for their properties of reducing cytotoxic responses while increasing regulatory T-cell activity. Ten cohorts of SD mice were given different combinations of R and/or P, short- and/or long-term, in conjunction with the *scAAV9-HEXM* treatment. Daily IS administration via oral gavage began at 5 weeks of age, followed by an IV injection of *scAAV9-HEXM* at six weeks. Behavioural testing and blood collection was done monthly. Serum, organs and splenocytes were collected for analysis of transgene copy number, enzyme activity, histology, humoral and cellular immune response to HexM and humoral response to the AAV9 capsid. The long-term administration of both R and P showed a significant decrease in T-cell response and antibodies towards the HexM enzyme long after the end of the IS regimen. This cohort also had a significantly higher long-term liver vector distribution compared to the other cohorts. A diminished but not significant effect on the immune response to the AAV9 capsid was also observed. The *scAAV9-HEXM* treatment in combination with R and P resulted in overall reduction of GM2 ganglioside accumulation, significantly higher enzyme activity, greater long-term vector biodistribution and persistent tolerance towards HexM well beyond the 13-week regimen of IS. Survival after vector treatment was increased up to 7 weeks longer in mice administered IS compared to mice that did not receive IS. The results show the induction of tolerance towards a gene transfer expressed protein with the use of IS. The approach may be applicable to other gene therapies.

293. Merkel Cell Polyomavirus Oncoprotein-Specific T Cells Generated from Healthy Donors for Potential Adoptive Immunotherapy

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Introduction: Merkel Cell Carcinoma (MCC) is an aggressive neuroendocrine tumor driven by Merkel Cell Polyomavirus (MCPyV) in about 80% of US cases. The MCPyV oncoproteins small T antigen (ST) and truncated large T antigen (LTT) are major drivers of virus positive (VP) MCC. Sustained expression of these proteins is required for VP-MCC viability. As VP-MCC has an extraordinarily low mutation burden with few predicted neoantigens, viral oncogenes are an attractive target for cellular immunotherapy. Immune checkpoint inhibitors greatly improve patient survival, reflective of MCC being an immunogenic tumor. However, many MCC patients suffer from immune dysfunctions or iatrogenic lymphopenias that make autologous cell therapy challenging. HLA-matched healthy donors could be a potential source of cell therapy materials for such patients. Risk of graft versus host disease is low in donor-derived viral-specific T cells and this approach is currently being investigated for other viral diseases. We set out to generate MCPyV oncoprotein specific T cells from healthy donors for potential T cell adoptive immunotherapy. **Methods**: Naïve and memory T cells from healthy donors were magnetically isolated prior to co-culture. Autologous monocyte-derived dendritic cells (DCs) from healthy donors pulsed with 15-mer overlapping peptide libraries containing ST and LTT were used as stimulators. T cells were re-stimulated with peptide-pulsed irradiated, autologous PHA-blasts up to five times. Cultures were maintained in either standard growth cytokines (IL-2, IL-7 and IL-15) or a pro-inflammatory cytokine cocktail. T cell phenotype and reactivity were evaluated via flow cytometry. **Results** Standard cell culture cytokines of IL-2, IL-7, and IL-15 were unable to promote growth of significant T antigen-specific mature T cells. Altering growth conditions to include IL-1β, IL-2, IL-6, IL-7, IL-15, IL-21, IL-23, and TGFβ promoted expansion of $CD4^+$ T cells specific for MCPyV T antigen peptides producing TNFα and upregulating CD154 and CD137 upon cognate antigen exposure. Peptides from the common region of Exon 1 shared by ST and LTT proteins were recognized most frequently by reactive cells. Cells were generated from healthy donors without bias for particular HLAs. **Conclusions** We successfully generated MCC oncoprotein-specific $CD4^+$ T cells from healthy donors. These data suggest that peptide-based expansions may be a suitable platform for future adoptive T cell immunotherapies for VP-MCC patients, regardless of HLA haplotype.

294. Characterization of Pre-Existing Antibodies to Anc80 Vector in Adult and Pediatric Donors, and the Impact of ImmTOR Technology on Restoring Antibody-Compromised Transgene Activity In Vivo

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Pre-existing neutralizing antibodies (nAbs) are a major barrier for AAV-based gene therapy. Here we characterized sera from adult and pediatric donors for pre-existing antibodies that cross-reacted with Anc80, a novel in silico designed AAV vector, and evaluated the effect of ImmTOR technology (synthetic viral particles containing rapamycin or SVP-Rapamycin) on Anc80-cross-reactive nAbs in an in vivo passive serum transfer model in mice. Pre-existing IgG and IgM antibodies cross-reactive with Anc80 were screened using colorimetric ELISAs. Neutralizing antibody activity was evaluated using an Anc80-CMV-luciferase vector in an HuH-7 cell-based assay. The potent transduction activity of Anc80 in HuH-7 cells enabled the use of an MOI of 2000 to aid with assay sensitivity. A screen of healthy adult serum samples, identified 14 out of 30 (47%) samples as positive for neutralizing activity. In pediatric serum samples, the prevalence was lower with 36% (20 out of 55) of samples showing neutralizing activity. The Anc80 nAb activity correlated with the anti-Anc80 IgG levels, but not with anti-Anc80 IgM levels. This correlation was confirmed via depletion of IgG with protein A/G beads, which resulted in removal of neutralizing activity and recovery of Anc80-driven luciferase activity. Selected samples were also assessed in vivo using murine passive immunization studies. C57BL/6 mice were injected with normal human sera 24 hours prior to dosing with Anc80-CMV-secreted alkaline embryonic phosphatase (SEAP). A strong correlation of the neutralizing activity was observed between the in vitro cell based assay and the in vivo passive serum transfer studies. Notably, the addition of ImmTOR to Anc80-CMV-SEAP as an admix enhanced transgene expression, resulting in partial or complete restoration of transgene activity in mice that had received passive transfer of moderately neutralizing sera. In summary, immunogenicity assays have been developed to screen and characterize human anti-Anc80 antibodies. Anti-Anc80 IgG antibodies drive neutralization of the vector. Using a murine passive transfer model, ImmTOR technology has been observed to recover transgene activity even in the presence of moderately neutralizing antibodies.

295. Study of the Neutralizing Antibody after rAAV.TL65 Transduction in Ferret Airway

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Recombinant adeno-associated viral (rAAV) vector-based gene therapy has the potential to cure cystic fibrosis (CF) lung disease regardless of various CFTR genotype. Recently, we developed a novel AAV capsid (AAV.TL65) variant highly tropic to the apical membrane of human airway epithelial cells. Additionally, the rAAV vector AV.TL65-SP183-fCFTRΔR with this capsid is capable of transducing ferret airway to express a R-domain-partially-deleted ferret CFTR mini gene. Thus, this vector can be used to evaluate the efficacy of CFTR gene transfer on lung function in CF ferrets. Although rAAV has demonstrated long-term genome persistence and stable transgene expression in well-differentiated airway epithelium, repeat dosing of the gene transfer agent may be needed in vivo, but little is known about rAAV-mediated immune responses in the ferret lung. In this study, we investigated the neutralizing antibodies (NAbs) elicited by AAV.TL65 capsid after single or repeated dosing, and compared the transgene expression between those two different dosing regimens. In the repeat-dose group, ferrets were intratracheally injectedwith the AV.TL65-SP183-fCFTRΔR (ferret CFTRΔR) at age one week and intratracheally sprayed with the AV.TL65-SP183-gLuc reporter vector at 4 weeks of age. Both doses contained rAAV of $1x10^{13}$DRP/kg. In the single dose group only the reporter vector was intratracheally sprayed in 4 weeks old animals. Age-matched untreated ferrets were included in the experiment to serve as a background control. Blood samples were collected at age 7, 28, 33, 38 and 42 daysto measure plasma NAbs, as determined by rAAV infection of A549 cells and indexed by the half maximal inhibitory concentration (IC50). Bronchoalveolar lavage fluid (BALF) was also collected when the animals were sacrificed at 14 days post-dosing of 4-week-old animals. Blood samples collected at 33, 38, and 42 days of age and BALF were used to assess the secreted Gaussia Luciferase (gLuc) transgene expression. ELISA-based procedures were established to determine the titer of total IgG and IgM binding to AAV. TL65 capsid in plasma and also the IgA of BALF. The levels of total anti-capsid IgG and IgM in plasma increased after the repeat dose, but the total anti-capsid IgA levels in BALF decreased after the repeat dose. At 14-days post repeat-dosing, there was a 13-15-fold increase in the IC50 of plasma NAbs, which was 3-6-fold higher than that of the single dose group. Furthermore, NAbs IC50 of the BALF from the repeat-dose group was 2-3-fold higher than that from the single dose group. However, transgene expression in the lung revealed only 2-3-fold reduction in plasma and in BALF gLuc expression in single vs repeated administration groups at 6-weeks of age. These results suggest that the capsid of AV.TL65-SP183-fCFTRΔR after the first dosing does elicit a neutralizing response, but to a lesser extent than in the periphery.

296. Developing Strategies to Inhibit Host T Cell Response During Viral Vector Mediated Gene Therapy

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Viral vector mediated gene-transfer based therapies have a promising future to treat many human diseases; however, host immune responses to viral vector and its components can present a safety risk, limit vector and transgene persistence and reduce efficacy of these therapies. Thus, novel strategies to design viral vectors to evade host immune responses are critical for advancing these therapies. T cell activation (TCA) is

required for generation of T cell response against viral vector during gene therapy. We hypothesized that incorporation of factor(s) that inhibit TCA in the design of 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 short 20 amino acid (aa) immunomodulatory peptide from the hepatitis C virus (HCV) NS5A protein that has been previously shown to inhibit Lck, a key enzyme required for TCA. GFP expression and inhibition of T cell function were compared between LVs either expressing NS5A peptide with GFP (LV-NS5A) or GFP alone (LV). 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. LV-NS5A did not induce impairment of global TCR signaling as TCR-independent TCA was unaffected in LV-NS5A transduced T cells. Current studies are underway to assess the ability of LV-NS5A to modulate T cell response *in vivo* using murine models. If we find that NS5A peptide modulates T cell response against LV *in vivo*, its ability to inhibit vector-specific T cell response will be also tested in context of other viral vectors such as AAV. Since TCA is also critical for development of humoral response, inhibition of TCA by gene vectors may also reduce vector-specific humoral immunity. Acknowledgements: This work was supported by the Intramural Research Program of the Center for Biologics Evaluation and Research, U.S. FDA. 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 US Department of Energy and US FDA.

297. Insulin Blunts Interferon and Pro-Inflammatory Cytokine Responses in an In-Vitro, Co-Culture Model Developed to Assess Immunity Against rAAV

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30 years of viral vector research to treat genetic disorders has yielded FDA approved therapies with rAAVs. These advances overcame significant hurdles, however there are still challenges, particularly in regards to cytotoxic immune responses which make corrected cells vulnerable to destruction by host immunity to viruses. In this post-FDA era of AAV gene therapy, developing an in-vitro model that measures both interferons and pro-inflammatory cytokines in the corrected cells (i.e cultured hepatocytes) as well as cultured macrophages will be beneficial in screening pharmaceutical approaches for limiting immune responses to AAV vectors. In this research, Hep3B liver cells and differentiated U937 monocytes (assessed by levels of cd11b and TLR-9) were co-cultured in trans-wells and stimulated with self-complementary AAV vectors. Trans-wells allowed us to measure gene expression of IL-12, TNF-a, IL1-b IL-6, and IFNa / IFNb separately in the two cell lines following co-culture for 2 hrs. Our previously studies showed that stimulating Hep3B cells alone had no response, while stimulation of differentiated U937 cells resulted in a modest increase in gene expression of pro-inflammatory cytokines (3 - 5 fold) but not interferons. For this study we initially co-administrated TLR-9 agonists with sc-AAV vector with the goal of generating a more substantial pro-inflammatory cytokines and interferon response in differentiated U937 cells and/or Hep3B cells without co-culturing. A data tread showed adding the TLR-9 agonist boosted the pro-inflammatory cytokine levels in differentiated U937 cells (5-8 fold compared to 3-5 fold with sc-AAV alone), however addingTLR-9 agonist showed no interferon response in U937 cells and Hep3B cells were still unresponsive. Therefore, we tested co-culturing. Two co-culture models were used. First, differentiated U937 cells were stimulated with sc-AAV vector for 2 hrs, the stimulation media was removed and then the U937 cells were added to unstimulated Hep3B cells using trans-wells. This resulted in a robust increase in both cytokines and interferons in the Hep3B liver cells (6 to 11 fold). We were also able to measure a 3 to 5 fold increase in interferon gene expression in the U937 cells (which was not observed when these cells were not co-cultured). Additionally, the co-culturing enhanced the pro-inflammatory cytokine signal in U937 cells compared to no co-culturing. Interestingly, we also saw a modest increase (2.5 to 3.5 fold) in cytokines and interferons in Hep3B cells when they were co-cultured with unstimulated U937 cells. In the second co-culture model the two cells were first co-cultured together in trans-wells and then sc-AAV was added for 2 hours. The results were similar to the first co-culture model where U937 cells were stimulated prior to co-culture. We had previously shown that insulin improves AAV transduction of liver and skeletal muscle in vitro and in vivo but the improvement was better in the animal models which suggested that insulin may impact more than transduction. We therefore co-administered insulin with AAV vector which prevented the modest increase in cytokine gene expression in stimulated U937 cells without co-culture. Additionally our data showed that insulin could blunt the increase in cytokines and interferons in our more robust co-culture model. This study clearly demonstrates that stimulating and co-culturing differentiated U937 monocytes with Hep3B cells can generate a vigorous cytokines and interferon response to sc-AAV vectors and that this model can be used to assess pharmaceutical approaches to limit or prevent these responses.

298. Anti-Idiotypic Monoclonal Antibodies Targeting the Germline Variant of a Broadly Neutralizing HIV-Specific Antibody VRC01

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To be effective, HIV vaccine must elicit maturation of rare antibody lineages towards breadth and potency. However, ENV-based vaccines have so far been unable to stimulate such responses. Major efforts aimed at structure-guided reverse engineering ENV molecules to bind the germline and intermediate maturation forms of broadly neutralizing antibodies (bnAbs) have produced highly promising candidates now entering clinical trials. It remains unclear whether such monomorphic HIV-based vaccines will be capable of producing durable and potent bnAb responses without unwanted non-neutralizing antibodies.We report on the production of two monoclonal anti-idiotypic antibodies (mAIAs) raised against the predicted germline variant of a CD4bs-

targeting bnAb VRC01 (gl-VRC01). The antibodies recognize the cognate antigen with picomolar affinity (SPR and BLI measurements). mAIAs do not react with the mature form of VRC01 (m-VRC01), pooled human IgGs or chimeric mAbs with swapped heavy/light chains. Furthermore, the mAIAs were observed to specifically and robustly activate sensor B cells expressing gl-VRC01, but not m-VRC01 in the form of functional B cell receptors.Thus, we expect that our mAIAs may serve as a priming agent of the HIV vaccine to selectively expand the rare population of naïve B cells that can ultimately mature into bnAb-producing cells by HIV-based boosting molecules. This approach may help guide the maturation along the bnAb pathway without running the risk of antibody repertoire freeze.The work was supported by the grant 18-29-08051 from the Russian Fund for Basic Research. AVT was supported by the Basic Scientific Research Program project 0310-2019-0012.

299. Exploring the Messenger RNA Capping Code: CleanCap Co-Transcriptional Capping Allows Synthesis of Cap 0, Cap 1, Cap 2 and $^{m6}A_m$ Capped RNAs

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Messenger RNA (mRNA) therapy is an increasingly popular platform technology for expressing proteins in cells or *in vivo*, since there is minimal risk of insertional mutagenesis. mRNA transfection is being utilized to express proteins for genome editing (Cas9, ZFNs and TALENs), protein replacement, vaccines, and antibody expression. Exogenous mRNAs can be recognized as foreign by the innate immune system and one approach to avoiding innate immune responses is to mimic the structure of endogenous mRNAs. One important feature of mRNAs is the 5' cap structure. During RNA capping, Cap0 (^{m7}GpppN) is formed as an intermediate. Methylation of the 2' position of the first cap proximal nucleotide forms Cap1 (^{m7}GpppN$_m$N$\cdots$) which is found in all eukaryotic transcripts. In ~50% of transcripts, the 2' position of the second cap proximal nucleotide is also methylated to form Cap2 (^{m7}GpppN$_m$N$_m\cdots$). Another frequently found cap modification found in conjunction with Cap1 (and potentially Cap2) is N6-methylation of adenosine at the first cap proximal nucleotide (^{m6}A$_m$N$\cdots$). ^{m6}A$_m$ is the second most frequently found modification in mRNA. The role of mRNA cap structures remains to be fully elucidated. RNA viruses frequently encode methyltransferases that convert their Cap0 structures to Cap1. Deletion of methyltransferase activity frequently leads to attenuation of these viruses. It is thought that IFIT proteins, and potentially other pattern recognition receptors, recognize Cap0 structures as foreign, activating an antiviral state that represses translation. Thus, Cap1, and potentially Cap2 structures license endogenous mRNAs as "self" RNAs. The role of Cap2 and ^{m6}A$_m$ is poorly understood since it has not been possible to produce such mRNAs synthetically at scale. A recent study suggests that ^{m6}A$_m$ caps may increase stability and translation while decreasing de-capping of mRNAs (Mauer et al., Nature 2016). Traditional co-transcriptional capping methods utilize ARCA (anti-reverse cap analog) and yield Cap0 structures which are immunogenic. ARCA capping results in low yields and poorly capped material (~70% capped). Post-transcriptional capping by Vaccinia virus capping enzymes can yield Cap0 or Cap1 structures, but is expensive and capping can be incomplete due to inaccessibility of structured 5' ends. Enzymatic capping also requires a purification step between transcription and capping. Previously, methods for producing Cap2 RNAs have not been commercially available. We recently developed a novel co-transcriptional capping method called CleanCap® which can yield Cap 0, Cap1, Cap2, ^{m6}A^m, or un-natural caps. Capping is robustly reproducible and is highly efficient (90-99% capping), is less expensive than enzymatic capping, and is carried out in "one pot" reactions without additional purification. mRNAs with different cap forms were formulated with Lunar® lipid nanoparticles and injected by tail vein into mice. Expression *in vivo* was influenced by the mRNA cap form. *In vitro* de-capping assays showed ^{m6}A$_m$ may de-capped more slowly, shedding light on the pattern of *in vivo* activity. In addition, the identity of the first two 5' nucleotides altered the rate of de-capping.

300. Gene-Bbased Prime-Boost Immunotherapy for BockingViral Entry to Hepatocytesin Chronic Hepatitis B and D Infections

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Chronic hepatitis B and D virus (HBV/HDV) infections cause cancer and are treated either life-long with reversed transcriptase inhibitors (HBV), or with a two-year interferon therapy curing 25% of HDV infections. A sustained therapy response most likely involves an activation of the host immune response. We here developed a unique immunotherapy targeting viral entry to complement existing antivirals that inhibit viral maturation. The strategy consists of a DNA-prime and protein boost immunotherapy that induce receptor blocking antibodies to the HBV PreS1 domain and HBV- and HDV-specific T cells. We found that the prime-boost strategy was superior in inducing both antibodies and T cells, as compared to either DNA or protein alone. Importantly, the prime-boost strategy induced superior levels of neutralizing antibodies to HBV. This is a highly promising prime-boost strategy as an immunotherapy for use in combination therapies for treating chronic HBV to obtain a functional cure.

Cell Therapies

301. Measles Vectors as a Tool to Study the Role of OCT4 in Somatic Cell Reprogramming to Generation of Induced Pluripotent Stem Cells (iPSC)

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Gene delivery/expression vectors are fundamental technologies in gene and cell therapy. These vectors have also been used in regenerative medicine as tools to reprogram somatic cell to a pluripotent state. Rapid progress in regenerative medicine has facilitated the development of more sophisticated gene delivery/expression technologies. Our lab has previously developed Measles Virus (MeV) as recombinant viral vector to generate induced pluripotent stem cells from somatic cell. This safe and efficient system is based on the vaccine strain of MeV, a non-integrating RNA virus that has with a long standing long-standing safety record in humans. Moreover, the reprogramming factors (RFs- OCT4, SOX2, KLF4 and cMYC) are delivered via a single "one-cycle" MeV vector (MV4F), decreasing the need for multiple vectors at the time of transduction. Here, we introduce a microRNA (miR) controlled MeV vector, optimized for studying the role of OCT4 during reprogramming. We engineered two sets of MeV vectors, one set expressing OCT4; the other with four RFs. These vectors were further designed to have three target sequences of miR-375 in the 3' untranslated region of the P gene. After verification of OCT4, SOX2, KLF4 and cMYC expression, and that the presence of the miR-375 target sequences did not affect vectors propagation or phosphoprotein cofactor activity, we assessed the ability of the vectors to reprogram human fibroblasts into iPSCs. We first compared the reprogramming of MV(OCT4) with MV(OCT4)^{PmiR375} in combination with three lentiviral vectors encoding SOX2, KLF4 and cMYC. Reprogramming using MV(OCT4)^{PmiR375} occurs faster and more efficiently than with MV(OCT4), indicating that the reduction/elimination of expression of OCT4 after initial induction could enhance reprogramming efficiency. We next compared the reprogramming efficiency of MV4F with MV4F^{PmiR375} vectors. While MV4FP efficiently reprogrammed human fibroblasts to fully mature iPSCs, MV4F^{PmiR375} couldn't, indicating that the elimination of all four RFs is detrimental. Supplementation of MV4F^{PmiR375} with three lentiviral vectors (LV), each expressing one of the three factors - SOX2, KLF4, cMYC (MV4FPmiR375 +3LV) reprogrammed as efficiently as MV4FP, but combination of MV4F^{Pmir375} with 2LV or 1LV only led to intermediate or inefficient reprogramming. The results of our study indicate that a continuous expression of SOX2, KLF4 and cMYC is essential for "stabilization" of the iPSC clone after initial reprogramming, while exogenous OCT4 is required in the initiation and maturation stages of iPSC reprogramming. Our results not only demonstrate the use of our MeV vector as a tool to study reprogramming process but also offer a safe reprogramming platform for genomic modification-free iPSCs amenable for immediate clinical translation.

302. Amelioration of Acetaminophen-Induced Liver Inflammation via Delta-Opioid Receptor Activated Human Mesenchymal Stem Cells-An In Vivo Approach

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Background: Liver injury (inflammation) poses to be a prevalent and persistent problem at a global scale and liver transplantation using human mesenchymal stem cells (hMSCs) being the frequent respite to overcome it. Although there has been a plethora of advancements in liver transplantation studies, failures of a successful transplant remain difficult to evade, majorly due to lack of hMSCs survivability at the injured site. Hence, in this study the effect of delta opioid receptor (DOR) activated umbilical cord blood borne hMSCs, reported to have shown a pronounced increase in hMSCs survivability *in vitro* under different stress conditions, have been elucidated on an acute liver injury model of mice. **Methods:** Acetaminophen, a commonly used paracetamol for induction of liver inflammation was administered intraperitoneally at a dosage of 500mg/kg to the treatment groups of mice. The control groups included without treatment phosphate buffer saline (PBS) injected. For the transplantation of hMSCs tail vein injection route of administration was followed at a dose of $5X10^5$ cells/animal. After 48h, the liver tissues and blood samples were collected for determination of the ALT-AST activity and the alterations in the levels of inflammatory cytokines. Alongside, liver tissues were fixed using 10% formalin and observed for portal and lobular inflammation. **Result:** The transplantation of hMSCs significantly prevented increase in the levels of serum ALT (1900 U/L vs 26.5 U/L) and AST (2101 U/L vs 33.5 U/L), respectively on comparison with the acetaminophen-treated groups. There was an additional repression observed in their levels upon transplantation with DOR-activated hMSCs (ALT: 330 U/L vs.742.6 U/L, AST: 196 U/L vs 516 U/L). Analysis of the inflammatory cytokines post-induction and transplantation of hMSCs and DOR-activated hMSCs revealed a prominent mitigation of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α by over ~2 folds and a significant upregulation of anti-inflammatory cytokine IL-10 by about ~4 folds when compared to the acetaminophen-treated. Histological evidences of the liver tissue samples also followed a similar trend wherein maximum necrotic tissues were observed in the groups treated with acetaminophen. The groups transplanted with hMSCs showed potential recuperation from inflammation and subsequent hepatic necrosis, which were further curbed down in animals transplanted with DOR-activated hMSCs. **Conclusion:** This study corroborates the potential benefits of transplantation of DOR-activated hMSCs in a liver-injury mice model and implies that the recuperation of the groups with DOR-administered hMSCs was majorly due to the amelioration in the inflammatory cytokines, along with subdued levels of ALT-AST enzymes. Therefore, DOR-activation on hMSCs could prove to be a successful prospective therapeutic of numerous such liver-failure models.

303. GMP Compliant Manufacturing of Induced Pluripotent Stem Cells (iPSC) for a Phase I Clinical Trial for "Dry" Age Related Macular Degeneration

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Induced pluripotent stem cells (iPSC) are adult cells that have been generated by forced expression of factors important for maintaining pluripotency. These cells have the ability to propagate indefinitely and differentiate into cells representative of all three germ layers, thus, they have the potential to revolutionize the field of regenerative medicine. The National Eye Institute aims at using iPSC derived retinal pigment epithelial cells (RPE) to treat "dry" AMD in a phase I clinical trial. To accomplish this, we have developed a GMP compliant process to generate iPSCs using patient whole blood from which PBMCs are separated by manual density gradient and cryopreserved at 40e6 cells per vial. 1-2 vials of PBMC are thawed to yield approximately 0.5e5 to 5e5 CD34+ cells. After expanding CD34 cells for 4 days, 12 transfections are performed using 1e4 cells per transfection to generate iPSC. Based on their morphology, iPSC colonies are selected manually and cultured to passage 10 in feeder free conditions. These cells are then tested for purity, plasmid loss, karyotyping, STR and absence of pathogenic variants. iPSCs that meet release criteria are differentiated into RPE using a defined cocktail of cytokines and reagents. This clinical trial will be open and enrolling patients in first quarter of 2019.

304. Freshly Thawed Mesenchymal Stem Cell Product Shows Comparable Immunomodulatory Potency to Freshly Cultured Cells In Vitro and In Vivo

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Introduction: Mesenchymal stem cells (MSCs) have been shown to exert important immunomodulatory effects in both acute and chronic diseases. However, in acute inflammatory conditions, like septic shock, immunomodulatory cell therapy must be administered within hours of diagnosis. While "off-the-shelf" cryopreserved allogeneic cell products are best suited for sepsis therapy, the immunomodulatory potency of freshly thawed cells injected immediately has not been well documented. In this study, we compared the abilities of "freshly cultured" versus "freshly thawed" MSCs in modulating immune responses.

Methods & Results: MSCs from bone marrow donors were either: 1) thawed and cultured for 2 days before being re-suspended as the "freshly cultured" (FC) cell product; or 2) thawed directly as the "freshly thawed" (FT) cell product. FC and FT donor-matched MSCs exhibited similar surface marker expression profiles by flow cytometry; both were positive for CD73, CD90, CD105, and negative for CD14, CD19, CD34, CD45, and HLA-DR. MSCs suspended in the final container showed similarly high levels of cell viability as evidenced by live cell counts both by Trypan blue assay (0hr: 92% ± 2% vs. 93% ± 2%=) and by Annexin V/Propidium iodide analysis (0hr: 95% ± 0.6% vs. 92% ± 2%). MSC *in vitro* potency was assessed by their ability to suppress proliferation of activated inflammatory cells, enhance phagocytotic capacity of peripheral blood mononuclear cells (PBMCs) and reduce endothelial cell (EC) permeability using *in vitro* potency assays. Similarly, both FC and FT MSCs inhibited T cell proliferation (30% ± 3% and 27% ± 3%, respectively), enhanced PBMCs' phagocytic ability (38% ± 8% and 37% ± 10%, respectively), and restored endothelial permeability after injury (31% ± 10% and 35% ± 13%, respectively) . The *in vivo* potency of the MSCs was assessed by their ability to improve phagocytic activity of peritoneal lavage cells in an animal model of sepsis. Administration of either FC or FT MSCs significantly improved the phagocytic ability of the peritoneal lavage cells in a comparable level (with 2-fold increase in phagocyting cells).

Conclusions: Our results show that a freshly thawed MSC product exhibits similar surface marker phenotype, viability, *in vitro* and *in vivo* immunomodulatory efficacy compared to freshly cultured cells.

305. Synergistic Effect of Small Molecules: A Potential Alternate to Bmp-2 for Enhanced Osteogenesis of Human Mesenchymal Stem Cells- An In Vitro Study

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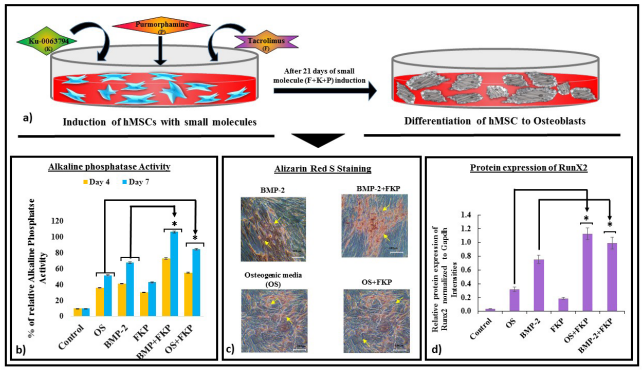
The use of bone morphogenic protein-2 (BMP-2) to accelerate the healing of bone defects has resulted in side effects such as ectopic bone formation, soft tissue swelling and cyst like bone void formation. An equipotential and a cost effective replacement for BMP-2 is therefore required. In this study different combinations of small molecules like FK506 (F) - an immunouppressent, purmorphamine (P)- hedgehog agonist and Ku-0063794(K) an mTOR inhibitor were evaluated on human mesenchymal stem cells (hMSCs) to assess the osteoinduction behavior. Optimum concentration of individual small molecule (F -5nM, K-10nM and P-2μM) was determined based on the MTT and ALP activity. Small molecules (F+K+P) with osteogenic media (OS) showed 1.5 fold higher ALP activity with respect to OS and a comparable expression of Runx2/osteopontin/collagen genes as BMP-2. Thus, small molecules may have the potential to substitute or enable use of reduced dose of BMP-2 for enhanced osteogenesis of progenitor cells. **Fig. 1** Osteogenic potential of small molecules. (a) A diagrammatic representation of differentiation of hMSCs to Osteoblasts after 21 days of incubation on treatment with small molecules such as Ku-0063794(k)- an mTOR inhibitor, Purmorphamine (P) - a Hedgehog signaling agonist and Tacrolimus a.k.a FK506 (F)- an immunosuppressant. (b) Increase in Alkaline phosphatase activity with addition of all three small molecules FKP (F+K+P). (c) Alizarin Red S staining of hMSCs after 3 weeks of culturing with BMP-2/ OS and with & without small molecules. On addition of small molecules

visibly increase red color of calcium ion deposits can be observed. (d) Protein expression of RunX2 shows a significant increase in the RunX2 expression after addtition of small molecules with BMP2/OS.



306. Bringing Autologous Cellular Therapy from the Academic Lab to a Multicenter Trial. Overcoming Logistical Complexities of Transporting Cells

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The technology to safely modify ex-vivo human cells for therapeutic intervention has become considerably more obtainable over the past decade. Manufacturing costs are becoming commercially viable as the industry has seen continued growth. Regulatory uncertainties have been reduced with first marketing authorization of modified T-Cell therapies by the FDA. More than 200 autologous gene therapy trials have entered the clinic, yet few of those have progressed to multicenter trials. Multicenter trials demonstrate the reproducibility of therapeutic benefit across clinical sites in an environment more typical of future commercial use; however, they introduce complexities that can present challenges unique to this technology. Here we attempt to elucidate the logistical complexities of cellular therapy trials and provide a process to ensure traceability compliance of modified cellular therapies. Autologous cellular therapy trials necessitate specific requirements around the chain of custody and traceability process. We have developed a procedure to ensure the documentation and collaborative management of cells through the implementation of standardized methods that guide the collection process, shipment, cell-manufacturing, return shipment and site preparations. This process has been used to successfully infuse dozens of patients with autologous genetically modified cells across multiple sites. Multicenter programs impose detailed oversight requirements and documentation to support the collective tracking and control of a subject's cells from procurement, manufacturing, and infusion. In our programs we have implemented a series of procedures to provide sites with designated forms to ensure tractability and accountability for each party that touches the cells. The subject's cells are collected via leukapheresis and packaged in a temperature-controlled cryoshipper with a GPS tracking device and shipped to a specialized manufacturing facility. We dictate that constant communication between the manufacturing facility, study site, Contract Research Organization and any vendor who is involved in the shipping logistics, is specified in advance and monitored to ensure the cells arrive at each facility on time and without temperature excursions. Included in the shipment are COC forms which provide the subject's information, number of cells collected and any other relevant information required for the manufacturing and manipulation of the product. Throughout the manufacturing to infusion process, we have introduced a number of standardized forms to document quality testing, chain of custody, product receipt confirmation, and viability of the product prior to infusion in the patient. We believe we have developed a process that standardizes a complex process and ensures the safety of the product for the patient.

307. Comparative Analysis of Umbilical Cord Tissue Mesenchymal Stromal Cells Obtained from Digestion and Explant Outgrowth

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Background: Umbilical cord tissue is rich in mesenchymal stromal cells (MSCs) and other stem and progenitor cell populations with potential therapeutic and research value. The umbilical cord tissue, like umbilical cord blood, can be collected in a non-invasive manner following delivery. Given the potential utilization of MSCs, from a variety of perinatal and adult tissues, in regenerative medicine applications, a growing portion of newborn stem cell banks now offer cryopreservation of umbilical cord tissue, alongside cord blood, for future cell-based applications. With the rapid development of cord tissue cryopreservation as a storage offering, it is not surprising that there exists a considerable breadth of approaches for preparation and cryopreservation from institution to institution. Approaches for isolating MSCs from cord tissue include manual and mechanically assisted methods and can be either enzymatic or non-enzymatic in nature. Here we describe a series of studies to characterize MSCs isolated after thawing cord tissue cryopreserved as composite cord tissue (c-CT MSCs) and MSCs isolated from previously-cryopreserved, heterogeneous, single-cell suspensions of enzymatically-digested cord tissue (d-CT MSCs). **Methods:** Ten research donated umbilical cords were collected from consenting mothers. Each cord was divided into two segments to allow for comparison of c-CT MSCs and d-CT MSCs from biologically identical material. From one segment, composite material was prepared for cryopreservation and later isolation of MSCs by explant outgrowth as previously described (Skiles et al, 2018). Concurrently, tissue from the second segment was digested using a cGMP-grade collagenase and hyaluronidase cocktail following a previously-validated protocol. After performing a series of filtration steps, the resulting heterogeneous singe cell suspension was cryopreserved. All material was stored in the vapor phase of liquid nitrogen for at least one week prior to thawing. **Results:** c-CT MSCs were isolated from thawed tissue by explant outgrowth over a two week period (Skiles et al, 2018). Heterogeneous cell suspensions were thawed and plated in MSC selective media to allow for establishment of MSC cultures during the same time period. The end of passage 0 was defined as day 14 for c-CT and for d-CT when cultures reached confluence. Purity of c-CT and d-CT cell populations from the same donor was evaluated by the percentage of cells expressing MSC markers

CD73, CD90, CD105 and hematopoietic markers CD34 and CD45. Additional release criteria of the post-thawed product, including sterility and viability, were also compared. **Conclusion:** This study presents different strategies employed by newborn stem cell banks for cryopreservation of umbilical cord tissue when the intended application of MSCs is unknown at the time of storage. We highlight logical points for standardization that can be implemented after thawing of a cell suspension or, in the case of whole tissue cryopreservation, recovery of cells by explant outgrowth from thawed material. As an industry, newborn stem cell banks have the opportunity to consider a panel of agreed upon assays to confirm that an umbilical cord tissue-derived MSC product meets specified acceptance criteria, regardless of the cryopreservation approach, before moving to ex vivo expansion required to prepare clinically relevant cell doses.

308. Neuropotency and Neurotherapeutic Potential of Human Umbilical Cord Stem Cell's Secretome

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Background: Regenerative medicine has gained momentum in the past decades due to the scientific advancements of cell therapy strategies. Recent studies have revealed that the therapeutic effect of MSCs is due to their secretome. Secretome is considered to be advantageous over cells, because of less chances of teratoma formation and no cell genome variability of donor and recipient. Among MSCs, human umbilical cord stem cells are neonatal discarding tissue origin with less ethical issues. The current study aimed to screen the secretome of cord lining (CL) and Wharton jelly (WJ) of human umbilical cord stem cells and their functionality. **Methods:** Human umbilical cord tissue was obtained from donors with their informed consent. Tissues were washed with PBS and blood vessels were removed. CL and WJ were separated and cultured in MEM-alpha with 10% FBS. Cultured cells were characterized for classical MSC markers by flow cytometry and for the expression of pluripotency as well as for the germ layer expression. These stem cells were screened for the expression of trophic factors (BDNF, GDNF, CNTF, NT3, NT4, NGF, FGF1, FGF2, VEGF, HGF, IGF, EGF, PlGF), and cytokines such as IFN gamma, TNF α, IL-1b, IL-4, IL-10, IFN α1, TGF-β, IL-8, IL-6, IL-3, IL-7. Cells were cultured without FBS media for 48 hours and the conditioned media (CM) were collected. CL and WJ cells were studied for neurosphere assay and neuronal induction. Functional effect of CM was analysed on SHSY5Y cell line for proliferation, differentiation, and anti-inflammatory response. In addition, effect of CM on endothelial cells was also carried out. **Results:** CL and WJ cells were positive for MSC markers (CD29, CD90, CD105, and CD73) and negative for haematopoietic lineage. These cells demonstrate mild expression of Sox2, Oct4, Nanog, Klf4, c-Myc, all of which are responsible for pluripotency and also showed ectoderm and endoderm germ layer expression. Both stems cells exhibited highly proliferative phenotype with normal karyotype even after several passages. Gene expression studies showed the expression of all growth factors such as BDNF, GDNF, NT3, NT4, NGF, FGF1, FGF2, VEGF, HGF, IGF, EGF, PlGF with varying levels, except CNTF. In addition, the cells illustrated the expression of TNF α, IL-1b and IL-6 as pro-inflammatory cytokines and anti-inflammatory cytokines such as IL-4, IFN α1, TGF-β except IL-10. Sox1, Sox2, NCAM, Nestin and Notch were highly expressed in respective neurospheres when compared to WJ and CL cells alone. However, Hes1, Hes2, Mash1 were predominantly expressed only in neurospheres generated from CL and WJ stem cells. This shows that the cells are primed to neural stem cell like phenotype. Upon neuronal induction, these cells also expressed mature neuronal markers like GFAP, Olig and NF. CM from WJ and CL could enhance SHSY5Y (neuroblastoma cells) proliferation and differentiation. Moreover, anti-inflammatory effect was also elicited by the CM on SHSY5Y cell line, by increased expression of IL-4, IL-10, TGF-beta, IFN-alpha when compared to LPS treated cells alone. Anti-apoptotic Bcl2 gene expression was also enhanced in CM treated cells. Moreover, CM from both WJ and CL were able to induce invitro tube formation in endothelial cells *in vitro*. Thus, CM from MSCs isolated from both CL and WJ has potential angiogenic, anti-inflammatory, proliferative and neuro-differentiation effects. **Conclusion:** Easily accessible human umbilical cord stem cells have neuropotency and their secretome can have therapeutic potential in neurodegenerative diseases.

309. PureQuant™ Real-Time PCR-Based Assay for Quantitative Determination of Immune Cell Identity and Purity

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Significant progress has been made in harnessing the power of immune system, in particular, T cells, to treat certain kinds of cancers. One of the key challenges in developing immune cells as therapeutic agents is the accurate estimation of their identity and purity. Current methods used for the characterization of T cell types and other immune cell products rely on flow cytometry. Flow cytometry can accurately estimate CD8+ T lymphocytes and other surface markers, but the method is challenging to implement in a GMP manufacturing environment posing logistical challenges such as requirement for live cells, variability leading to difficult in standardizing and high throughput. In addition, cytometric methods are not accurate for specific intracellular targets that positively identify Regulatory T (Treg) cells and T Helper 17 (Th17) cells. Therefore, there is an emerging need for alternative assay methods that address these challenges. Methylation state is known to be unique for specific cell types and can thus be used as an identifier in heterogeneous population of cells. Capitalizing on these differences in methylation patterns, we developed assay kits that quantify the percentage of Treg and Th17 by detecting methylation status of FoxP3 and IL17A via qPCR of bisulfite converted genomic DNA. In contrast to flow analysis, sample requirement is minimal and the assay works with fresh/frozen cells or genomic DNA. The combination of accuracy, low sample requirement and flexibility provides an ideal measurement system for confirmation of identify and purity of T cell types critical for therapeutic applications.

310. Vesicular Stomatitis Virus Engineered to Express Human CXCL10 Does Not Increase Intratumoral Infiltration of Activated Human T Cells in the FaDu-Luc Squamous Cell Carcinoma Xenograft Model

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Oncolytic virotherapy is an emerging treatment modality using replication competent viruses to specifically kill cancer cells. Vesicular stomatitis virus (VSV) is a potent oncolytic virus due to its rapid replication and lysis, tumor specificity, and absence of neutralizing antibodies in the general population. After initial replication, the innate immune system is a barrier to VSV therapy, clearing the virus before it kills the entire tumor. We aim to increase T cell infiltration into the tumor by encoding human T cell chemokine, CXCL10 (hCXCL10), into VSV. *Chemo*tactic cyto*kines*, or chemokines, orchestrate immune cell movement throughout the body. Of approximately 50 chemokines, CXCL10 was chosen due to its chemotactic effects on CXCR3+ CD8+ T cells, Th1 cells, and NK cells, angiostatic properties, and conversed function between humans and mice. Furthermore, hCXCL10 is not induced at high concentrations after VSV-M51R control virus infection of FaDu-Luc squamous cell carcinoma cells *in vitro* or *in vivo*. We engineered hCXCL10 into VSV-M51R, an attenuated VSV which is suitable for immunocompromised mouse tumor models such as FaDu-Luc xenografts. *In vitro* hCXCL10 was specifically secreted from VSV-M51R-hCXCL10 infected FaDu-Luc cells by ELISA. In *in vivo* FaDu-Luc xenograft studies, hCXCL10 was specifically increased and retained in the tumor through 96 hours after VSV-M51R-hCXCL10 infection. At 24 hours post virus infection, activated human CXCR3+untransduced T cells (UTDs) were administered intravenously and tumors were harvested 3 days later to quantify immune cell infiltration via flow cytometry. There was no specific increase in hCD3+ T cells in the tumors of infected mice, suggesting the chemokine concentration was not large enough to by physiologically relevant or the T cells were not retained in the tumor because they were not antigen specific. Ongoing studies in immunocompetent mouse models are exploring whether VSV-mCXCL10 can increase the trafficking of T cells in the B16-OVA tumor model with adoptively transferred, activated OT-1 T cells. In this model the timing of chemokine concentration and receptor expression are controlled, in addition to the T cells being antigen specific.

311. Considerations for Qualifying VCN in Cell-Based Therapies

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Recombinant lentiviral vectors (RLVs) are engineered replication-deficient viruses that have been used to deliver transgenic material for gene and cell-based therapies. Vector integration into the host cell genome is necessary for RLV-delivered transgene expression, but it can possibly contribute to cellular transformation. Presently, regulatory guidance documents state that vector integration and the risk of cellular transformation is poorly understood. Because of the lack of understanding, reducing target cell vector copy number (VCN) has been proposed as the only way to reduce the probability of cellular transformation. The US Food and Drug Administration (US FDA) has proposed that the VCN should not exceed five. This assessment reviews the publicly available regulatory guidance and safety data on RLV VCN with the objective of identifying: (1) the basis of the US FDA limit, and (2) a potential alternative VCN limit. Based on the available regulatory guidance and clinical, *in vivo*, and *in vitro* experimental data, we found limited support for the US FDA value or any generic limit. Instead, the data indicate that a target VCN should be experimentally and empirically qualified for any permutation in the vector design, target cell, and dosing regimen, as these may individually and collectively impact the probability of transformation at the clonal and/or population level. Further, the data suggest that simply reducing the VCN could alter or reduce treatment efficacy and potentially place replicative stress on a subset of the RLV-modified cells.

312. Tools and Strategies for Improving Confidence in Image-Based Cell Counting Measurements

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Cell count and viability measurements are critical for the characterization of cells and cell-based therapeutic products. Robust, precise, and accurate cell count and viability measurements are needed at all levels of cell therapy product (CTP) development, from research to manufacturing control and product release. Automated image-based cell viability analyzers, utilizing dye exclusion principles, are often selected as a rapid, simple, and cost-effective method for cell counting. These counting methods can reduce operator variability while increasing statistical power of the analysis by increasing the number of images and cells analyzed. Due to the diversity of cell samples that are counted across the industry (e.g. different cell types, different suspension media and formulation), users must often define thresholds or parameter settings which allow the instrument's software to detect, identify and enumerate live and dead cells based on trypan blue membrane permeability or other nuclear vital stains. Optimization of settings used for image analysis in automated cell viability analyzers is necessary and critical to obtaining high quality measurements. In one example, we demonstrate that identical data sets analyzed using slightly different image analysis parameter settings yield viability results that differ by 50%. Here, we use a design of experiments (DOE) approach to develop a parameter setting protocol for an automated, trypan blue image-based cell viability analyzer. The image-based cell viability analyzer uses 8 user-adjusted parameters to identify live and dead cells. Our approach was to vary these 8 parameter settings as part of an orthogonal fractional factorial experimental design, where the eight input factors were tested at two levels (low/high) to study the sensitivity of cell viability and cell concentration measurements to each factor and to assess interactions between different factors. 65 sets of parameter settings (protocols) were tested across 8 different NIH3T3 cell conditions, including healthy and compromised cell samples. Additionally, spike-in conditions using dead cells or beads specifically spiked into a healthy cell mixture were tested. Five response variables were measured: % viability, total cell count, viable

cell count, % error in total cell count, and % error in % viability. A manual counting analysis was conducted by four expert users using ImageJ in order to establish expert % viability and cell count for a subset of images in each sample group. Percent error in concentration and viability measurements for each protocol were evaluated across all cell samples. For some compromised cell conditions, we observed a range in reported % viability of more than 50% across the 65 protocols, while for healthy cell conditions, the range in % viability measured based on protocol number was less than 10%. Using this DOE approach, we identified the parameter settings that significantly affected overall viability measurement output and we observed two-way interactions between several parameters. Through this approach, we ranked the 65 protocols according to mean % error in viability for all eight conditions, and were able to identify several parameter setting protocols which would minimize the amount of error observed in the % viability measurement across all conditions. Our goal was to establish a framework for establishing robust and sensitive image analysis parameter settings for trypan-blue based cell viability measurements. DOE type approaches will be a valuable tool in the development of analytical methods with complex sources of variability and large sets of influencing factors. This study provides a use-case for the application of DOE strategies in method optimization for critical cell viability and cell count measurements. Additional work in the area of automated image-based cell count and viability includes identifying measurement assurance strategies for determining quality of image acquisition, and exploring strategies to verify quality of images acquired prior to post-processing image analysis.

313. Cell Therapy on Sciatic Nerve Injury in Mice: Insights on Simplification

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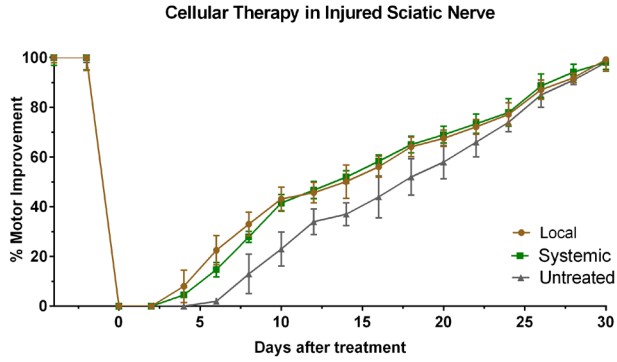
Traumatic injuries of the peripheral nerves, common at all ages, have been the subject of numerous studies towards understanding the process of repair; these injuries have also been a challenging problem worldwide. Despite advances in surgical techniques, functional results have been quite disappointing, as they depend on the time interval between injury and treatment. For rapid and efficient treatment of nerve injuries, cell therapy has improved nerve regeneration, thereby increasing the success of nerve repair. The Cell Therapy is the use of cells to replace or repair tissue injury, for tissue regeneration or even cure of diseases. In this scenario, stem cells are the protagonist ones, because their great capacity of proliferation, self-renew and differentiation potential that can be widely applied to the regenerative medicine. Hematopoietic stem cells (HSC) are the most famous cells used a long time to improve disorders, especially by bone marrow transplantation. Besides HSC, mesenchymal stem cells (MSC) is another group extensively explored on the regenerative medical research. Both are much emphasized mainly because of their capacity of differentiation on different tissues and by their capacity to produce and manage anti-inflammatory responses. Here, we let their importance aside and explored the potential of normally unwanted cells for therapy. We used short-term hematopoietic cells, characterized by flow cytometry as most lineage positive cells, derived from bone marrow to evaluate the treatment of peripheral nerve injury. We explored an alternative protocol for derivation of cells from bone marrow by differential centrifugation and we showed an improvement in the functional response of an injured sciatic nerve after treatment with short-term cells administrated locally or systemically (Figure). We treated mice with surgically injured sciatic nerve with GFP+ cells and analyzed the sciatic nerve recover by Sciatic Functional Index (SFI). Moreover, we confirmed the regeneration level of the nerve by measuring the presence of neutrophin receptor p75(NTR) and neurophilament, and also by nerve structure. We had success with a fast, effective, and simple protocol based on cellular therapy, using cells normally unwanted for therapy. This work brings us insights to the potential of treatment of peripheral nerve using cells from bone marrow in the treatment of sciatic nerve lesions 'roughly' purified as a simple and effective type of therapy. It also might raise questions whether we are losing any information regarding cell therapy excluding some cells and favoring others.



314. Direct Differentiation of Tonsillar Biopsy-Derived Stem Cells to the Neural Linage

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Human multipotent stromal cells (MSC) reside on blood vessels, and therefore can be found in any tissue throughout the body. In normal conditions, adult MSCs are quiescent and play a role in the proper function of the blood vessel. However, in response to tissue injury, hypoxia or inflammation, they replicate and have the potential to integrate within their tissue of origin and repair bone, cartilage, fat, muscle and promote tissue regeneration. Several studies reported the capacity of bone marrow MSCs to differentiate to immature neurons. However, unlike embryonic stem cells it is not clear whether MSCs can develop into mature neurons. We recently reported that tonsillar biopsies, which can be attained by a minimaly invasive procedure, as a rich source of MSCs, which are equivalent in markers and multipotency to bone marrow derived MSCs. We have generated 14 human tonsil-derived MSCs (T-MSCs) lines and fully assessed their growth, markers and potency. Here we describe for the first time the differentiation of

tonsillar biopsy derived MSCs (T-MSCs) into post-mitotic neuron-like cells by defined xeno-free conditions and growth factors. We show our novel procedure leads to a rapid neural lineage commitment and loss of stemness potential within as little as four days. Our data is the first to show that tonsil derived stem cells can efficiently differentiate into post-mitotic neural-like cells without genetic manipulation. Given that immature neural cells have shown a superior therapeutic potential than undifferentiated MSCs, our study may have the potential to improve the use of adult stem cells for the treatment of neuropathologies.

315. Acoustic Concentrate and Wash with the FloDesign Sonics Ekko™Platform

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Acoustic Cell Processing is a unique acousto fluidics platform technology for shear-free 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 and 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, FloDesign Sonics (FD Sonics) has been developing a key applications for cell and gene therapy manufacturing, an Acoustic ConcentrateWash, ACW, system for closed and shear free Cell and Gene Therapy manufacturing, namely CAR T immunocellular therapies. The ACW technology has been applied to primary T Cell cultures of 1 Liter with two ranges of cell concentrations, a low cell concentration of between 1 to 2 million cells per milliliter (ml), and a second range with cell concentrations ranging from 30 to 40 million cells per ml. Thus, the number of total viable CAR T cells processed ranges from 1 to 40 billion. The process flow rate varies from 2-5 L per hour with average cell recoveries of more than 80% in less than 1 hour. At low cell concentrations, a 150 fold volume reduction has been achieved with cell concentrations in excess of 250 million cells per ml. At high cell concentrations, a 20 fold volume reduction and more than 500 million cells per ml were measured. The efficiency of the buffer exchange process is higher than 90%. ACW is a powerful acoustic-based cell processing technologies that lower cost and risk while enabling a modular, automation-friendly manufacturing process for cell and gene therapy manufacturing.

316. A Novel, Single-Use Bioreactor System for Expansion of Human Mesenchymal Stem/Stromal Cells

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There is a significant need for efficient systems that can be used to generate primary cells and stem cells which can be readily implemented in research labs and expedite process development studies, pre-clinical testing, and large-scale expansion. Although various platforms for expansion of sufficient cell numbers exist, these systems are rarely comprehensive enough to allow for rapid implementation by researchers. These systems are also not easy to use nor are they robust enough to reproducibly supply high quality cells. A complete system for the generation of sufficient cell numbers that utilizes a bioreactor platform which is robust and easy-to-use is therefore highly desirable. We have previously demonstrated efficient expansion of human mesenchymal stem/stromal (hMSC) in the PadReactor® single-use bioreactor system to the 40 L scale. This system utilized commercially-available, well-characterized cells, specially-formulated growth media and supplements, SoloHill microcarriers, and a single-use bioreactor platform that could be used to generate tens of billions of cells within 7 days of culture. Here we extend these finding by employing these components in a novel bioreactor type which contains a bottom-mounted impellor. The Allegro™ STR bioreactor is a new single-use stirred tank bioreactor platform which is scalable, compact, ergonomic, and designed to maximize usability and process assurance. hMSC and medium were cultured at 30 L scale in microcarrier-based, fed-batch cultures in the Allegro STR bioreactor for 5 days. Cells harvested from microcarriers retained critical quality attributes when examined in standard cell characterization assays. The results obtained in this study lay the groundwork for a complete system for efficient generation of high quality cells for process development studies and future large scale cell expansion. Together, the Allegro STR bioreactor and the SoloHill microcarriers provide a practical manufacturing solution for large-scale expansion of adherent cells.

317. Development of a Bioprocess Xeno-Free Cell Culture Medium for hMSCs

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R&D, RoosterBio, Inc., Frederick, MD

Biomanufacturing media design is changing in response to growing numbers of clinical trials and an increasing need for high-quality animal origin-free materials compatible with limited downstream processing. A variety of media-development strategies allow process-development optimization; in particular, eliminating the need for substrate coating and media replacement is highly desirable. We successfully developed a batch culture bioprocess xeno-free media that requires no coating step and supports high rates of hMSC proliferation. Using high-throughput formulation DOE and multiple and single factorial analysis, we were able to identify critical components in the media formulation. We analyzed multiple media and cell types (xeno-free, serum-cells, adipose-derived, umbilical-derived, and

bioreactor cultures), using a panel of characterization assays to evaluate cell health and functionality. The panel included expansion kinetics (cell density), cell surface marker expression (flow cytometry analysis for CD14, CD34, CD45, CD73, CD90, CD105, and CD166), cytokine secretion (FGF, HGF, IL-8, TIMP-1, TIMP-2, and VEGF), trilineage differentiation potential (osteogenesis, adipogenesis and chondrogenesis) and immunomodulatory function (response to IFNγ stimulation). Our results demonstrate that the new RoosterBio bioprocess xeno-free cell culture medium supports high rates of proliferation across multiple hMSC product types while maintaining cell robustness and functionality.

318. StemPro™ HSC Expansion Medium (Prototype) Enables Superior CD34⁺ Cell Expansion, CRISPR-Cas9 Gene Editing and iPS Reprogramming for Use in Gene and Cell Therapy

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The use of CD34⁺ hematopoietic stem cells (HSCs) for clinical transplantation and gene therapies has been limited due to the low CD34⁺ cell numbers in tissue sources such as peripheral blood and cord blood. A major limitation of current culture systems used for the expansion of CD34⁺ cells is that *ex vivo* culture leads to differentiation of HSCs, at the expense of the most primitive pluripotent long-term repopulating HSC. Development of a culture media system that expands, both short term progenitor cells and long-term repopulating HSC would enable immune protection during the early phase of recovery, and provide a suitable solution for transfusion-independent hematopoiesis. Moreover, the utilization of this media system and expanded CD34⁺ cells for gene therapy and inducible pluripotent stem cells (iPSC) reprogramming would facilitate the generation of cellular diseases models for applications in cell and gene-editing research. Indeed, we have developed a xeno-free, serum-free medium - StemPro™ HSC Expansion Medium (Prototype) that when supplemented with FLT3L, SCF, TPO, IL-3, and IL-6 (FST36) expanded CD34⁺ cells from mobilized peripheral blood, cord blood, and bone marrow. For example, culture of primary human CD34⁺ cells from mobilized peripheral blood for 7 days in FST36-containing StemPro™ HSC Expansion Medium (Prototype) 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. Expanded CD34⁺ cells expressed high levels of aldehyde dehydrogenase and formed erythroid and non-erythroid cell colonies *in vitro*. By utilization of Neon electroporation to deliver Cas9 RNPs and donor (500 ng 1.4 kb GFP construct), we observed indel rates of up to 60% and a knock-in rate of 10% in CD34⁺ cells. CD34⁺ cells demonstrated a ~0.3% reprogramming efficiency with the CTS™CytoTune™-iPS 2.1 Sendai Reprogramming Kit. We are enthusiastic to introduce StemPro™ HSC Expansion Medium (Prototype), which enables a complete xeno-free and serum-free work flow for utilization of CD34⁺ cells in gene and cell therapies.

319. Characterization and Quality Assesment of Neural Stem Cell Generated by Direct Reprogramming

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Direct reprogramming(direct conversion) is the use of differentiated cells to differentiate into other specific cells bypassing unstable intermediate pluripotent state. Research is actively underway to develop cell therapeutics using direct reprogramming technologies because it reduces the risk of tumorigenecity. In this study, a direct reprogramming technology was introduced to induce neural stem cells using human fibroblast. Oct4, Sox2, Klf4, c-Myc was introduced using sendai viral vectors and induced neural stem cells by adjusting environmental factors such as media and growth factors. To identify the characteristics of induced neural stem cells, we analyzed cell morphology, gene expression analysis through RT-PCR, and related marker expression through immunocytochemistry. And we confirmed genetic stability through karyotyping analysis. We will analyze differentiation ability of neural stem cell to neuron, astrocyte, oligodendrocyte. Based on this study, we will provide basic data and considerations on the quality assessment of cell therapy products based on the direct reprogramming technology.

Pharmacology/Toxicology Studies or Assay Development

320. Challenges and Solutions for AAV2 Cell-Based Neutralizing Antibody Assay Development and Validation

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Gene therapy is an emerging area of drug development that brings the promise of effective, sustained treatment for a variety of genetic diseases. A common transgene delivery method is adenovirus or adeno-associated virus (AAV), a non-pathogenic virus that can infect humans and preclinical species. Pre-existing immunity to AAV or dose related immune response can limit effective gene transfer, and neutralizing antibodies (NAb) produced against the virus or expressed transgene pose a safety and efficacy liability. Therefore, an effective and robust strategy to measure pre-existing and/or generated NAbs are an important component of preclinical and clinical AAV-based gene therapy programs. We developed a GLP compliant cell-based NAb assay against AAV2

to support preclinical cynomolgus NHP studies. The NAb method was designed entirely with commercial reagents to measure the luminescence signal emitted from HEK-293 cells transduced with an AAV2-Luciferase control virus. The presence of AAV2 NAbs would result in decreased luciferase signal. The assay was developed to characterize screening and confirmatory cut points using a 5% and 1% false positive rate, respectively, along with accuracy and precision, sensitivity, stability, specificity and selectivity of the NAb response utilizing a commercial positive control anti-AAV2 NAb. To ensure the assay was developed and validated with true naïve matrix for regulatory compliance, we pre-screened individual serum lots during early development. The results showed 60% seropositivity across 110 serum lots tested, and negative serum lots were combined to create a naïve study pool. The method was validated with naïve pooled serum and cut point statistics resulted in a Normalization Factor of 6093 Relative Light Units and assay sensitivity of 714 ng/mL. A second production lot of AAV2-Luc was bridged into the assay. AAV2-Luc Lot2 demonstrated more robust cellular transduction and higher binding affinity of the positive control antibody such that endogenous pre-exposure was identified in the study serum which was not previously detected. Therefore, new individual serum lots were screened to re-establish naïve serum for the validation pool. We observed higher rates of serum anti-AAV2 NAb pre-exposure, likely due to the increased transduction efficiency of the new viral production lot. The assay was re-developed utilizing the new study serum pool, and was partially validated to re-establish Cut Points, Sensitivity and Accuracy and Precision. The subsequent partial validation resulted in a more sensitive assay (234 ng/mL) with a Normalization Factor of 34010 Relative Light Units. We successfully validated an AAV2 cell-based Nab assay using commercial reagents and assessed assay robustness to critical reagent production lot changes. We found that production lot variation in the AAV control virus had a significant effect on transduction efficiency, thus lot changes should be closely monitored throughout the assay lifetime. Furthermore, our results demonstrate a high level of pre-existing AAV2 seropositivity in a sample NHP population. This highlights the importance of NAb prescreening prior to study placement. Finally, these data suggest that NAb bioanalysis is an essential component of AAV-based gene therapy programs and should be implemented early to help ensure safety and efficacy of the asset.

321. Single Dose, AAV2-aBLyS Parotid Gland Administration in Cynomolgus Macaques Results in Dose Dependent Transduction, Accompanied by a Lack of Serum Transgene Product and Anti-Transgene Antibody Responses

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Primary Sjogren's Syndrome (pSS) is characterized by dry mouth and eyes (due to lacrimal and salivary duct damage), pain, and fatigue (1). Appearing at frequency of 1 in 1000 people, and at a female:male incidence ratio of 9:1, pSS is a relatively common systemic autoimmune disease . Dry mouth and eyes are thought to be the result of auto-antibody mediated exocrine glandular destruction, and recent findings of plasma cells in the salivary glands and elevated levels of BLyS (B-lymphocyte stimulator) support this understanding (2). A gene therapy strategy using an Adeno-associated virus (AAV) vector expressing a monoclonal antibody (mAb) targeting BLyS for the treatment of pSS was devised. An AAV2 vector expressing an αBLyS mAb, was administered to cynomolgus macaque parotid gland in a three cohort (1 control, 2 dosed), unilateral, single administration pilot toxicology study. Specifically, cynomolgus monkeys (1 animal/sex/group) were given either 0 (vehicle control), 1E+11 genome copies, or 1E+12 genome copies of AAV2-αBLyS by temporary cannulation of one parotid gland. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Q-PCR analysis revealed even vector distribution across the administered parotid gland in a dose-dependent fashion. Contralateral, non-infused parotid glands showed signal below the lower limit of quantitation (LLOQ), as did non-target tissues including brain, heart, kidney, liver, lung, and mandibular lymph node. In situ hybridization studies showed dose dependent vector transduction of a proportion of parotid gland ductal cells, and overall serum free BLyS levels were initially reduced in dosed animals, fluctuating thereafter. However, transgene expression was not quantifiable in the saliva, serum, or salivary gland homogenate at all timepoints, save for 2 high dose animals at week 4 that showed saliva concentrations near the LLOQ (LLOQ 10ng/mL, animal concentrations were 12.2 and 13.2 ng/mL). In addition, vector related mRNA was not demonstrable by in situ hybridization. As expected, neutralizing and total anti-AAV2 capsid antibodies were generated in all dosed animals post vector administration. Somewhat surprisingly, all animals that were observed over a 13 week duration screened positive for ADA against the αBLyS mAb transgene product, beginning at week 4. These ADA were subsequently confirmed to be specific for the αBLyS mAb transgene product. In addition, dosed animal glands had a significant increase in mononuclear cell infiltrate at weeks 4 and 13. Overall, single administration of AAV2-αBLyS

by temporary cannulation of cynomolgus parotid gland resulted in safe, non-toxic vector transduction and DNA persistence, yet did not produce detectable transgene expression levels in the face of robust anti-transgene antibodies. 1. Mariette X, Criswell LA. Primary Sjogren's Syndrome. N Engl J Med. 2018;378(10):931-9. 2. Quartuccio L, Salvin S, Fabris M, Maset M, Pontarini E, Isola M, et al. BLyS upregulation in Sjogren's syndrome associated with lymphoproliferative disorders, higher ESSDAI score and B-cell clonal expansion in the salivary glands. Rheumatology (Oxford). 2013;52(2):276-81.

322. Fast-Seq, a Universal Method for Rapid and Inexpensive Genomic Validation of DNA Virus Gene Therapies in Preclinical Settings

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Adenoviral and adeno-associated viral (AAV) vectors have shown great clinical promise in gene therapy as evidenced by recent FDA approvals. However, despite considerable efforts to optimize vector manufacturing for GMP productions, few preclinical labs have the resources to rigorously assess many aspects of vector lot composition. One critical component of vector quality is the fidelity of packaged vector genomes and relatedly, the level of spurious DNA contamination. Errors in viral genome replication and packaging can result in the incorporation of faulty genomes and contaminating DNA, such as those from production plasmids or packaging cells. Genomes with mutations, truncations or rearrangements, as well as DNA contaminants, could compromise the safety and potency of gene therapy vector lots. Thus, sequence validation of vector lot DNA composition is an important quality control, even in preclinical settings where resources are often low. We developed an end-to-end method for extraction, purification, sequencing and analysis of packaged viral DNA genomes intended for non-GMP environments that is inexpensive, simple, fast and reliable, known as Fast-Seq. We validated our method on the two most popular DNA viruses for gene therapy, AAV and adenovirus, with genomes spanning 5-38kb. When compared to several other recently published NGS methods aimed more toward GMP settings, Fast-Seq has several unique advantages: Tn5 transposase-based fragmentation rather than sonication (no expensive sonicator required), up to 125x less input DNA, simpler adapter ligation, compatibility with more commonly available sequencing instruments like MiSeq and iSeq, fewer magnetic bead purification steps enabling greater sample retention, and free open-source code for analysis. Fast-Seq can be completed in 1-2 days, is more cost-effective than other NGS methods geared towards GMP productions, and is both cheaper and more accurate than traditional Sanger sequencing which generally only provides 2x sequencing depth. Fast-Seq is a rapid, simple, and inexpensive methodology to validate gene therapy vector DNA composition in academic and preclinical settings.

323. Integration Site Analysis in a Pre-Clinical Trial of Lentivector-Modified hPLC Gene Therapy for Hemophilia A Shows Polyclonality and No Signs of Genotoxicity

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Prenatal transplantation (PNTx) has vast potential for dramatically improving the standard of care for many patients with genetic disorders, including hemophilia A (HA). HA is an ideal disease to treat by PNTx, since 75% of HA patients have a family history of HA, prenatal diagnosis is feasible and available, and successful PNTx could assure FVIII tolerance and long-term therapeutic levels. We previously demonstrated that human placental cells (hPLCs) constitutively produce low levels of FVIII and can be modified by lentiviral vector (LV) transduction to produce and secrete high levels of functional FVIII. Moreover, hPLCs endogenously express vWF, which greatly enhances the stability/half-life of FVIII, and inhibits its uptake by antigen-presenting cells. As such, hPLCs are an ideal cellular platform for delivering a FVIII transgene to treat HA. In recent studies, we demonstrated that PNTx with hPLCs transduced with a SIN LV encoding a myeloid codon-optimized ET3 (mcoET3) FVIII, (mcoET3-PLC), resulted in curative plasma levels of FVIII for 22 months post-PNTx in a large preclinical animal model (sheep). Because product safety is paramount for the translation of these studies to the clinic, here we assessed the relative safety of hPLCs as gene delivery vehicles, by performing integration site analysis on culture-expanded mcoET3-PLC used in PNTx studies, and by performing long-term observation and histological analysis on recipients of PNTx with mcoET3-PLC. Following transduction (MOI=7.5), hPLCs were passaged a minimum of 4 times in culture, and vector copy number (VCN) was determined to be stable at 0.35/per diploid genome equivalent. hPLCs maintained a normal morphology and normal phenotype during expansion and did not upregulate expression of stress-induced ligands such as MICA, MICB, or ULBP1-6.Integration site analysis of mcoET3-PLC was performed using Lenti-X Integration Site Analysis. The resultant PCR products were TOPO-cloned and the insert present in 270 individual bacterial colonies was sequenced commercially. All sequences were analyzed for the presence of the correct cloning "landmarks" to confirm they were legitimate insertion sites, and were then mapped to the human genome using BLAT. Redundant sequences were excluded from further analysis. We identified 22 unique integrations on chromosomes 1, 2, 3, 5, 7, 9, 10, 11, 13, 14, 16, 17, and 19. Of these 22 integrations, 16 were located within the body of protein-coding genes, and 6 fell in intergenic space, demonstrating maintenance of a high degree of polyclonality. To address the safety of mcoET3-PNTx, 2 sheep were euthanized at 4 months post-PNTx, and the levels of liver engraftment quantitated by mcoET3 qRT-PCR. Quantitation of cell engraftment was extrapolated from a standard curve constructed with known percentages of mcoET3-PLCs mixed with control sheep

cells. mcoET3-PLC comprised approximately 9% of the liver of each recipient, which corresponds to an *in vivo* expansion of the transplanted cells of at least 7 population doublings considering the total cellularity of the liver at this stage ($20x10^9$cells). Integration site analysis in liver tissue is underway. Histopathological evaluation of all organs examined revealed no tissue alterations, confirming that no overt genotoxicity occurred in the hPLCs as a result of transduction, and attesting to the safety of this therapy.

324. An Investigation of an Ultra-Sensitive Immunoassay Platform for Detection of BMN270 in Plasma

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"Shedding assays" for monitoring the presence of vector in biological matrices are commonly used to assess the risks of post-dose horizontal transmission in studies of Adeno-associated virus (AAV) based gene therapies. Current PCR methods to detect vector DNA can inform its presence in plasma, but positive PCR results may not indicate the DNA is biologically active or encapsidated. We investigated two ultra-sensitive immunoassay platforms, the Meso-Scale Discovery (MSD) QuickPlex and the MilliporeSigma Erenna® Immunoassay System, to evaluate their ability to detect the presence of intact AAV5 capsids in plasma over time, using antibodies from a commercial ELISA kit. The initial ELISA kit sensitivity was $1x10^{10}$ molecule/mL of plasma which was improved in a custom-developed MSD assay to a lower limit of quantitation of $1x10^9$ molecules/mL in plasma. The Erenna® assay quantified capsid particles as low as $1x10^8$ molecules/mL pooled human plasma, increasing sensitivity up to 100-fold. Despite a substantial improvement in sensitivity, low AAV5-antibody tolerance and variable selectivity made the current assay formats unsuitable for their intended purpose. This work demonstrates that further improvements to current capsid detection assays are needed before they can be used to characterize low amounts of capsid particles corresponding to the level of residual vector DNA in blood, and in the presence of anti-capsid antibodies.

325. Functional Vector Safety Studies for Immune Gene- and Cell-Therapies

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As part of the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) CRACK-IT Challenge, our InMutaGene consortium set up the basis for a standardized human induced pluripotent stem cells (iPSc) based assay for Individualized Genotoxicity Testing (InGeTox) of vectors used in gene therapy. We determine the risk of potential genotoxicity by performing rigorous molecular 'omics-' analyses in vector transduced hepatoblasts and hepatocytes differentiated from iPSc. Integration site analyses, transcriptome and non-coding gene studies as well as methylome analyses link vector integration profile to functional consequences of biological (and potentially clinical) relevance. While the InGeTox assay can rank the potential genotoxicity risks of each individual gene therapy vector, further development of this assay will allow the assessment of combined preclinical safety (genotox and immunotox) and efficacy of immune gene therapy approaches, such as chimeric antigen receptor (CAR) T cell therapy. In this combined approach, we will dissect factors that may influence the efficacy and safety of engineered T cells such as gene delivery approaches, vector type and in vitro culture conditions. The rationale of our approach will be to undertake comprehensive molecular omics analyses to directly link viral vector integration events with the function of T cell bulk populations, T cell sub-fractions (memory, regulatory, effector etc.) and individual T cells. Such tracking of engineered T cells linked to immunological repertoire composition and dynamics may substantially improve our understanding and the performance of successful CAR T cell gene transfer studies. Here, we will outline the concept of our new InGeTox assay dedicated to immune gene therapy approaches.

326. Quantitative Trilineage Differentiation QC Assays for cGMP Manufacturing of Human MSCs

Joseph Takacs, Katrina Adlerz, Bill Hooker, Jon Rowley, Taby Ahsan

RoosterBio Inc, Frederick, MD

Human MSCs are an attractive reagent for cell-based therapies and tissue engineering due to their self-renewal, low immunogenicity, and multipotent differentiation capabilities. There are currently more than 800 ongoing clinical trials associated with hMSCs registered at clinicaltrials.gov. As these trials progress to Phase 2 and beyond, the requirements related to cGMP biomanufacturing of hMSCs become more stringent. In particular, there is an unmet need for standardized assays of cell products to ensure consistency, safety and potency of clinical products (the National Cell Manufacturing Consortium Roadmap). The potential to differentiate into osteogenic, adipogenic, and chondrogenic cells is a critical quality attribute of hMSCs, and the current standard of assessment is histological staining of adherent cell layers (Alizarin Red stains calcium deposits in osteogenic cells, Oil Red O stains lipid vacuoles in adipogenic cells, and Alcian blue stains glycosaminoglycans in sections of chondrogenic cell pellets). A subjective assessment of positive staining is then taken as an indication that the hMSCs have the potential to differentiate down that lineage. These subjective assays can serve as for-information only (FIO) characterization, but quantitative assays are required to provide a product release specification for cGMP testing of hMSCs. Of the quantitative methods currently available, PCR offers multiple advantages over other technologies (Table 1). Here we present quantitative RT-PCR assays for the expression of genes diagnostic of

differentiation along osteo-, adipo-, and chondrogenic lineages. We have identified multiple markers for each lineage and will validate the use of these markers in ΔΔCT and standard-curve based assays, using characteristics defined by FDA and ICH guidance documents (precision, specificity, limit of quantitation/detection, linearity, range and robustness). In addition, this work will be proposed as a standard, allowing wide dissemination of the differentiation protocols to address a pre-competitive technical challenge in the adult stem cell industry.

Table 1. Comparison of Differentiation Assays

	Colorimetric Staining	Flow Cytometry	qRT-PCR
Reproducible	++	++	++
Quantitative	-	++	++
Sensitive	-	-	++
High Throughput	-	-	+++
GMP Transfer Capability	not favorable: subjective assay	not favorable: scarcity of GMP MSC antibodies	favorable: already used for virus testing

Staining is currently used as a FIO assessment of hMSC differentiation. Other assays like image analysis and flow cytometry offer improvements in various areas, qRT-PCR offers multiple improvements over staining in characteristics important for tech transfer.

327. Development and Validation of Quantitative Real-Time PCR for Detection of Oncolytic Herpes Simplex Virus from Shedding and Germline-Transmission Samples

Songhee Han, Soyeon Lee, Sang-Jin Park, Ji-Young Kim, Saegyeol Na, Geon Yoo, A-Ram Lee, Min Heui Yoo, Dong Oh Kim, Kyoung-Sik Moon

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Oncolytic virus is able to replicate selectively in cancer cells and lysis the tumor without harming normal cells. Since the talimogene laherparepvec (Imlygic®, T-VEC) first approved by the FDA for treating melanoma, a number of oncolytic viruses are being evaluated as potential treatments for cancer. Considering biological properties of the viral vectors especially oncolytic viruses, potential risk associated with spreading or vertical transmission to untreated subjects or animals should be carefully assessed. As a part of preclinical safety evaluation, it is necessary to investigate the persistence of oncolytic virus vector DNA in shedding and germline transmission samples. In general, quantitative polymerase chain reaction (qPCR) method is highly suggested to evaluate biodistribution of viral vector. Therefore, in this study, we set out qPCR validation for detecting oncolytic herpes simplex virus (oHSV) from shedding and germline transmission samples. Genomic DNA (gDNA) extraction protocols were optimized in urine, feces, semen, mouse fetus, mouse fetal blood and liver from mouse and rabbit animals for further qPCR analysis. We also optimized oHSV vector-specific primers to get the lowest limit of detection (LOD). The qPCR validation was conducted to evaluate for specificity, linearity, LOD, accuracy, and precision criteria. There were no interfering reacts with the targeted sequence between true positive and negative (no template) control samples. The correlation coefficient (R^2) was greater than 0.996 in all samples. The LOD was attained below 100 copies per a range of 50 to 500 gDNA in each sample. The accuracy and precision were verified by intra- and inter-assay analysis. In summary, the analytical qPCR method was successfully performed in validation parameters. Further investigations into shedding and germline transmission studies in mouse and rabbit animals are ongoing in our group.

328. Evolution of Nonclinical Dose Extrapolation to Support Clinical Dose Selection for Advanced Therapy Medicinal Products

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Clinical dose extrapolation based on safe and effective nonclinical doses is an integral part of the benefit:risk analysis regarding initiation of a first-in-human clinical study for any new medicinal product. Information on the pharmacologically active dose levels, toxicological dose response, and pharmacokinetics (PK) is critical for determining the maximal recommended starting dose (MRSD). However, because of inherent differences in the composition and putative mechanism of action, nonclinical data packages used to support advanced therapy (ie, cellular, gene, and tissue construct products) development are often limited compared to those supporting traditional small molecules or therapeutic proteins. Moreover, currently there is no uniform guidance regarding dose extrapolation of advanced therapies. Historically, dose extrapolation has been driven by identification of the no observed adverse effect level (NOAEL) in an appropriate animal species and conversion to the human equivalent dose using scaling factors. However, it can often be challenging to determine the NOAEL in nonclinical studies for advanced therapies because these products may not be biologically active in nonhuman species, necessitating further reliance on in vitro studies and the use of analogous or surrogate products. Furthermore, information on PK is often limited to distribution. Nevertheless, scientific justifications for the dose selection and projected multiples of human exposure (safety factor) are needed, and these can vary with each class of biotechnology-derived medicinal product. For example, MRSD selection in gene therapies depends on the rate of transduction of target cells, transgene expression, and the expected duration of activity (durability), while cell therapies are impacted by the maximum feasible dose that can be administered in the nonclinical animal model. MRSD selection for bioengineered tissue constructs should consider cell number, cell distribution within the graft, and the overall size of the graft compared to the target organ/tissue. Clinical dose extrapolation and benefit:risk assessments for advanced therapies are further complicated by other factors including patient population (which could include individual variability, limited numbers, and pediatric patients), clinical indication, and product source (eg, autologous, allogeneic, and/or xenogeneic cells). An estimation of pharmacologically active dose and the minimum anticipated biological effect dose levels can inform prospect of direct benefit to support inclusion of pediatric patients. These strategies are particularly important in rare disease indications and gene therapies involving viral vectors, where a patient may only have a single opportunity for treatment. A review of FDA Guidance for Industry, Summary Basis of Approval, Advisory Committee Meeting materials, and approved product labeling was performed. This review

revealed an evolution of nonclinical dose extrapolation approaches for advanced therapies. Several emerging trends were identified that should be considered in product development strategy for advanced medicinal therapies.

329. ATMPs - Science Led Development and Analytical Challenges

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The analytical tools required to support the development of cell and gene therapy molecules cover a range of development phases and disciplines, each with different development, validation and regulatory requirements. From the initial proof of concept pharmacology, toxicology and biodistribution studies, the CMC assays required to support the IND/IMPD submissions, clinical trial support and commercialization - the analytical tools range from simple pharmacopoeia qualifications to the complex and time consuming cell based potency methods and critical titre assays. The analytical approaches used are be based on the understanding of the product and the phase of development, its critical quality attributes, safety profile and the proposed mechanism(s) of action that relate to clinical effect. The poster/oral presentation will focus on the in life nonclinical work and analytical assays used to bring these therapies to the market with a focus on the nonclinical and CMC analytical activities. This will include the development and clinical phase-appropriate validations that are required - detailing the current understanding of assay validation requirements to support the different phase of clinical trials up to and including the commercial release of cell and gene therapy products. This will include design and validation consideration for a number of assays - e.g. QPCR/ddPCR, ELISA and cell based Potency assays, and a break down of the analytical expectations when performing transfer, development, qualification, and limited and full GMP validations. In addition general considerations for performing the inlife Biodistribution studies will be presented, and the many precautions and procedures that are put in place to prevent the risk of contamination, including inlife and when tissues are being processed for analysis in these critical studies.

330. Determinants of Patient Response to Clinical Trial Recruitment Advertisements in Gene Therapy: A Pilot Ethnographic Study and Trend-over-Time Simulation Model

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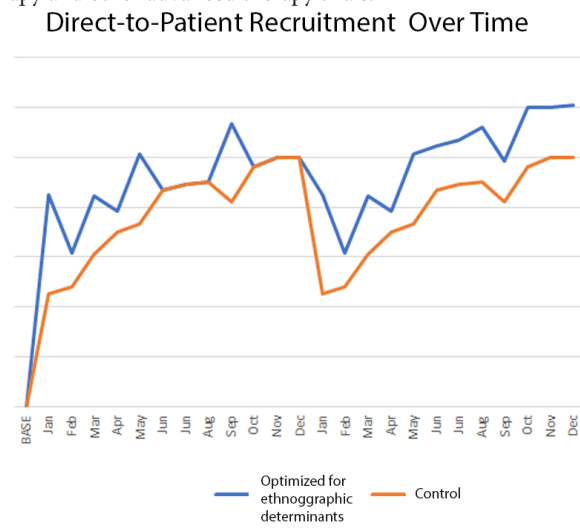
Background: While the number of gene and advanced therapy trials is rapidly increasing, few empirical studies have examined the determinants that affect recruitment in advanced therapy clinical trials, particularly in the early translational setting.

Objective: This prospective pilot study employs prospective ethnographic observation of a representative lay population to direct-to-patient gene therapy clinical trial recruitment materials to empirically document positive and negative determinants specific to these trials. **Methods:** Representative lay population ($N = 97$) positive and negative responses to gene therapy trial direst-to-patient recruitment flyers were observed in a field ethnographic setting. A 2-year first-order autoregressive trend-over-time clinical simulation model was generated using known determinants previously reported for interventional multicenter trials (starting, seasonal, and launching effects on recruitment) and observed positive and negative (+/-) determinants. **Results:** 32 positive and 24 negative determinants in response to direct-to-patient recruitment materials were coded heuristically, with the most frequent positive determinants being self-perceived non-availability of alternative treatments (96%), presence of immediate caregiver support (80), urban research site (48%), non-academic medical center, (32%), opinion of non-study clinician (32%), and perceived safety (36%); notably, perceived knowledge of genetic or technical considerations was a positive determinant in 24% and a negative determinant in 24%. Perception of possible germline effects was a negative determinant (no study impacted germline) in 32%. Simulation modeling demonstrated that when negative determinants were minimized, trial enrollment could be significantly improved by 21% ($P < 0.05$). **Conclusions:** This pilot study provides initial empirical support for patient response to direct-to-patient recruitment materials in advanced therapy trials, with possible implications for selection bias in these trials and practical application for optimization of recruitment numbers.

Figure 1. Two-year simulation model of observed determinants on direct-to-patient clinical trial advertisement effectiveness showing improvements possible when positive determinants are maximized alongside seasonal peaks, notably improv recruitment at launch and higher enrolling summer seasons. These findings could be utilized to inform site selection and optimization of recruitment materials in gene therapy and other advanced therapy trials.



331. Optimizing Residual DNA Detection for Gene Therapy Programs

Rebecca Cooper
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The detection and quantitation of residual host cell DNA has been established as an important aspect of impurity characterization for biologics. As the field of gene therapy expands, specific concerns for accurately measuring host cell DNA levels for viral vector-based programs have arisen. Assay specificity, sensitivity, and identification of

encapsidated host cell DNA are of utmost importance when designing residual host cell DNA assays for gene therapy programs. Here, we explore DNA standard preparations, measurement technologies, and sample treatments to overcome detection challenges faced while designing residual DNA assays for viral production cell lines.

332. US Pharmacopeia Standards for Cell and Gene Therapy

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The United States Pharmacopeia is actively engaged in standard setting activities relevant to the growing fields of cell and gene therapies. In this presentation, we will cover the existing USP general chapters and physical standards that are relevant for developers. These include Chapters: ⟨1027⟩ Flow Cytometry, ⟨1043⟩ Ancillary Materials for Cell, Gene, and Tissue-Engineered Products, ⟨1044⟩ Cryopreservation of Cells, ⟨1046⟩ Cellular and Tissue-Based Products, and ⟨1047⟩ Gene Therapy Products. We will also discuss USP efforts to develop additional documentary and physical reference standards to assist developers of cell and gene therapy products, such as the USP CD34+ Cell Enumeration System Suitability RS.

Vector and Cell Engineering, Production or Manufacturing

333. Exploiting Adenovirus Mechanisms for the Enhanced Production of rAAV Vectors

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Adeno-associated virus (AAV) has shown great promise as a vector for gene therapy. However, current manufacturing strategies are unable to scale to support the considerable demands for rAAV vectors for clinical and pre-clinical applications. Here we describe a new strategy for the manufacture of AAV using Adenoviral vectors based on the modulation of the major late promoter (MLP). We show that by strategic insertion of tetracycline repressor binding sites into specific loci of the MLP and encoding the tetracycline repressor under transcriptional control of the virus MLP, we enabled doxycycline-dependent controlled expression of Adenoviral structural proteins, with enhanced adenoviral DNA amplification in cells and reduction of adenovirus contamination by up to ~2.5 million-fold. Using this negative feedback self-repression system, we demonstrated delivery of adenoviral 'helper' functions and AAV DNA to yield >4-fold increases in AAV production without any adenovirus or small drug contamination. Moreover, by infecting AAV packaging cells this novel approach allows for a scalable, single agent, rAAV manufacture system that is free of plasmid DNA and contaminants, with considerable advantages over standard production approaches.

334. Improved Two-Plasmid Packaging System for Manufacturing of AAV Vectors with High Quality and Consistency

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The rational design of recombinant AAV (rAAV) plasmid packaging systems is an indispensable factor contributing to rAAV production processes with improved vector quality and high vector yields. The complexity of the rAAV formation process mediated by the required precise interplay of the AAV rep and cap genes, the complementation with helper virus functions and the efficient replication and encapsidation of the vector genome adds to the necessity of an efficient plasmid packaging system. We developed a two-plasmid packaging system for manufacturing of rAAV vectors in mammalian cells containing the adenoviral E1A and E1B genes, wherein the helper virus gene functions are combined with AAV rep on one plasmid, and AAV cap is combined with a pair of ITR sequences flanking a heterologous nucleic acid of interest on a second plasmid. The advantage of the physical separation of rep and cap on two different plasmids is that the likelihood of replication-competent AAV generation upon non-homologous recombination of the plasmids is minimized as repeated sequential recombination steps are required to generate pseudo wildtype AAV consisting of AAV ITRs flanking rep and cap. Furthermore, we observe that the two-plasmid packaging system enables adjustment of the ratio of vector-genome containing particles to total particles through a wide range. This is of high relevance for clinical application of rAAV vectors as the payload of excess empty particles can be either advantageous or detrimental for clinical efficacy and safety depending on the route of application and target of gene transfer. This optimization, driving increased vector genome yields, leads to a decrease in the payload of process-derived impurities. The improved two-plasmid packaging system displays high flexibility, allowing efficient packaging of vector genomes containing different nucleic acids of interest. Flexibility is also reflected by the fact that high virus yields can be obtained with this system using different natural AAV cap serotypes and synthetic cap variants. Applicability of the system was demonstrated from small-scale dish format up to bioreactor format (see abstract "A robust commercial rAAV-vector platform process using the iCELLis® 500 fixed-bed bioreactor" for details). In general, multi-product manufacture is streamlined as each product switch only requires modification of the plasmid containing the AAV cap gene and the vector cassette. The second plasmid serves as a platform plasmid which can be employed regardless of which AAV vector product is manufactured. In summary, we demonstrate substantial improvements in the rAAV plasmid packaging system leading to production processes with increased vector quality and vector yields.

335. Generation of an Inducible Producer Cell Line for Adeno-Associated Virus Vectors

Bingnan Gu, Caitlin M. Guenther, Anandita Seth
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Adeno-associated virus (AAV) has emerged as the vector of choice for gene therapy worldwide with more than 160 ongoing clinical trials for various indications. Given the fast-growing demand of recombinant AAV with the potential to cure indications that require high quantities of the vector, there is an urgent need for highly efficient, scalable and robust manufacturing platforms. To address this, we previously established a fast and scalable manufacturing process using plasmid-based triple transfection system in suspension HEK293 cells. For this platform, we isolated a highly transfectable suspension HEK293 clonal cell as "super-clone" 5B8 that supported high titer AAV production. This process was scaled up to 50L scale stirred tank bioreactors in our development lab for different serotypes. The platform was further optimized for processes including both upstream production and downstream purification for a novel synthetic ancestral AAV vector (Anc80). The clonal susHEK293 cell 5B8 will be available as GMP grade Master Cell Bank for future use. In our attempts to further develop a next-generation AAV production platform, we describe here a new plasmid/helper virus-free AAV producer cell line that would support efficient AAV manufacturing in long-term at significantly reduced labor and cost[1]. This cell line was created in our clonal suspension HEK293 cell 5B8 by stable integration of all essential Helper and Rep-Cap genes that were under the tight control by inducible promoters. In addition, in another version, the AAV transgene was also stably integrated in these cells. In proof-of-concept experiments, we validated the controlled induction of Rep proteins and AAV production at the titer that was comparable with triple transfection method. Also, the performance of rAAV produced from these cells shows similar characteristics to rAAV generated using standard transient transfection process, as tested by different analytical methods. These cells will be further used to develop a manufacturing process that is high producing, scalable and GMP compliant. This AAV producer cell line will provide a cutting-edge platform for both clinical and commercial AAV manufacturing that can support the demand and impact patient lives significantly.

[1] Patent pending.

336. Scalable rHSV Platform Development for AAV Gene Therapy Vectors

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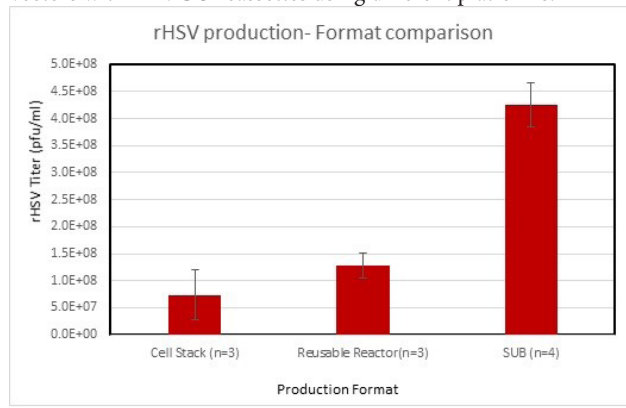
Gene therapy holds promise for treating a wide range of diseases, from cancer and diabetes to rare genetic disorders, potentially resulting in curative treatments in monogenic disorders. Scalable, highly productive manufacturing systems hold the key to bringing AAV-based gene therapeutics within reach for large population, monogenic diseases at commercial scale. Transfection-based AAV production methods will likely be challenged to keep up with the predicted commercial demand for systematic diseases. Recombinant herpes simplex virus type 1 (rHSV)-assisted recombinant adeno-associated virus (rAAV) vector production provides a highly efficient and scalable method for manufacture of clinical grade rAAV vectors. For Cell stack process, on an average 3-4-fold volumetric expansion is obtained from infection of rHSV to produce rAAV vector but with microcarrier process 14-15 fold volumetric expansion is observed. Large quantities of infectious HSV stocks are requisite for these therapeutic applications, requiring a scalable vector manufacturing and processing platform comprised of unit operations which can accommodate the fragility of HSV. Here, we present scale up efforts of a replication deficient rHSV-1 vector bearing the rep and cap genes or ITR-gene of interest (GOI) cassette of AAV-2. Adaptation of rHSV production from CS10 vessels to a microcarrier bioreactor permitted a 3-fold increase in cell density and a proportional increase in infectious vector concentration (pfu/mL). The fed-batch microcarrier bioreactor system at 3 L scale afforded an upstream rHSV vector production of nearly 4.5×10^8 pfu/mL in single-use bioreactors (SUBs), which is amenable to scale-up. Figure 1. Production of rHSV vectors with ITR-GOI cassettes using different platforms.



337. Rational Design and Characterization of a Human Serum Albumin-Binding AAV9 Vector

Quan Jin, Chunping Qiao, Jianbin Li, Juan Li, Xiao Xiao
Division of Pharmacoengineering and Molecular Pharmaceutics, Eshelman School of Pharmacy, The University of North Carolina at Chapel Hill, Chapel Hill, NC

Owing to an exceptional safety and efficacy profile, adeno-associated viral (AAV) vectors are frequently used for liver-directed gene delivery in preclinical and clinical studies. However, a high AAV titer is still required for effective liver transduction, especially in larger mammals. Therefore, improvement of AAV transduction efficiency is an important step towards improving the clinical utility of AAV vectors. It was recently reported that an interaction between human serum albumin (HSA) and AAV9 led to improved liver transduction *in vivo* (Wang et al., Gene Ther, 2017). However, we were not able to observe AAV9 binding to HSA by an enzyme-linked immunosorbent assay (ELISA), which suggests that the interaction between AAV9 and HSA is weak or transient. In order to determine the impact of HSA binding on vector transduction, we modified the AAV9 capsid to bind HSA with high affinity. To achieve this, a non-natural albumin binding domain with high affinity for HSA (ABDcon; Jacobs et al., PEDS, 2015) was fused to the N-terminus of the AAV9 VP2 to generate the AAV9-ABDCon vector. AAV9-ABDCon was capable of packaging a 4.6 kb transgene

cassette containing a β-galactosidase (LacZ) reporter gene. Vector yield of AAV9-ABDCon-LacZ after cesium chloride density gradient ultracentrifugation and dialysis was approximately 70-80% of the yield of unmodified AAV9-LacZ, as determined by DNA dot blot. Western blot analysis of AAV9-ABDCon-LacZ capsid proteins confirmed incorporation of the modified VP2 with the ABDCon insertion. AAV9-ABDCon-LacZ binding to HSA was verified by an ELISA using ADK9 (anti-AAV9 intact particles; Tseng, et al., J Virol Methods, 2016) detection antibody. In the same assay, unmodified AAV9-LacZ and free VP2-ABDCon protein did not elicit a signal. To assess the impact of ABDCon insertion on AAV9 tropism, male and female 6-week old C57BL/6J were injected with AAV9-LacZ or AAV9-ABDCon-LacZ at a dose of 2E+11 vg/mouse, and tissue LacZ levels were analyzed 4 weeks later. Liver LacZ levels were 2.57-fold higher in mice injected with AAV9-ABDCon-LacZnls versus AAV9-LacZ-treated controls as determined by a quantitative LacZ detection assay kit. However, LacZ levels were 1.43-fold lower in heart tissue from the AAV9-ABDCon-LacZ group. These results were corroborated by LacZ staining in liver and heart tissue sections. Interestingly, we also observed a higher amount of LacZ staining in splenic tissue sections of mice treated with AAV9-ABDCon-LacZ. No significant difference was seen in skeletal muscle LacZ levels, and LacZ was barely detectable in the brain and spinal cord across all the groups at the vector dose tested. From these results, we determine that insertion of the ABDCon peptide at the N-terminus of the AAV9 VP2 is tolerated and permits the formation of genome-containing vector particles. This AAV9-ABDCon vector appears to have improved liver transduction over AAV9, although the mechanism by which this occurs is not yet understood. Currently, we are investigating whether AAV9-ABDCon vector pre-incubation in HSA could further improve liver transduction, and animal studies are ongoing. Future directions include evaluating modified AAV9-ABDCon capsids with reduced binding affinities for HSA, as it may be more effective to have the vector less tightly bound to HSA. The ABDCon peptide can be easily adjusted with specific single point mutations to fine-tune its binding affinity to HSA over a range from 75 pM to 860 nM (Jacobs et. al., PEDS, 2015). We may also assess the feasibility of ABDCon peptide insertion on other AAV serotypes.

338. Development of a Scalable Platform for GMP Production of High Quality, Novel Clade F rAAV Vectors Following Comparison of HEK293 Mammalian and the Sf9-Baculovirus Systems

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Recombinant adeno-associated virus (rAAV) is one of the leading viral vectors in the gene therapy field, attributed to its low immunogenicity and therapeutic persistence. We have identified 15 novel Clade F rAAVs derived from human hematopoietic stem cells (AAVHSCs). Multiple methods have been developed to produce rAAVs based on introduction of packaging genes by helper viruses, stable cell lines, or plasmid transfection. To determine the optimal method for manufacturing our AAVHSC vectors, the production of an AAVHSC15 encoding a human phenylalanine hydroxylase (*PAH*) gene was evaluated in Sf9-baculovirus and HEK293 triple transfection systems. Both methods showed similar virus productivity as measured by vector genome titer and percentage of full capsids; however, increased *in vitro* infectivity and *in vivo* efficacy were observed with purified triple transfection produced vectors in the Pah^{enu2} murine model of phenylketonuria (PKU). Linear scalability of our transfection-based platform was demonstrated up to 400L in a serum-free, suspension cell line system. This triple transfection platform was utilized to produce other AAVHSC capsid serotypes and package multiple different therapeutic constructs all demonstrating similar virus productivity and quality.

339. Novel Antibiotic-free Nanoplasmid™ Vectors for Gene Therapy and Viral Vector Production

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Typical plasmids used in gene therapy and immunotherapy contain 1.5 to 3.5 kilobases of bacterial sequences in a bacterial backbone spacer region flanking the transgene expression cassette. If these prokaryotic sequences are more than about 1 kb, they can trigger transgene silencing in target cells and tissues, and often disrupt regulation of large numbers of cellular genes. We report development of the high manufacturing yield Nanoplasmid™ backbone, the smallest plasmid bacterial replication and selection backbone (<500 bp) comprising a R6K mini-replication origin and an antibiotic free RNA selection marker, RNA-OUT. Nanoplasmid™ vectors are conditionally replicative due to the requirement for a proprietary strain of *E. coli* K12 that expresses the R6K replication protein necessary for Nanoplasmid™ propagation. Nanoplasmid™ vectors are unable to replicate outside of this strain, enhancing safety. Both the R6K origin of replication and the RNA-OUT marker have been tested in multiple clinical trials with no serious adverse events reported. The RNA-OUT (antisense RNA) selectable marker substitutes sucrose tolerance for antibiotic resistance. Additionally, RNA-OUT appears to provide an enhancer effect, boosting gene expression significantly over plasmids with antibiotic-resistance markers. Easily fermentable to gram/liter yields in *E. coli*, there are no downstream modifications or antibiotics required during Nanoplasmid™ manufacture. Nanoplasmid™ vectors, in addition to the expected resistance to transgene silencing, also reduce transfection associated toxicity and have much higher expression levels compared to canonical plasmid vectors. These attributes confer Nanoplasmid™ vectors with additional utility beyond improving performance of non-viral expression vectors, such as:(**i**) improving transposon vector activity for engineering CAR T-cells; (**ii**) increasing Lentiviral vector production titers; (**iii**) eliminating antibiotic marker contamination of ***Adeno-associated virus*** (***AAV***) particles during AAV production. The Nanoplasmid™ is a dramatically improved vector backbone (<500 bp) that is designed to replace previous generation bacterial

backbones. It has application for gene and cell therapeutics, DNA vaccines, immunotherapeutics, and for improving the performance and/or titers of existing viral and transposon vectors.

340. Activation of the CMV Promoter: Considerations for Viral Vector-Mediated Transgene Expression

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The transcriptional promoter of the cytomegalovirus (CMV) immediate early genes has been extensively exploited in mammalian vectors used for *in vitro* and *in vivo* transgene delivery. Transcriptional activation by the CMV promoter is dependent on the presence of certain cellular transcription factors, several of which are known to be altered in response to cellular stimuli. However, the stability of transgene expression in model systems that depend on coincident stimuli has not previously been addressed. Here we monitored transgene expression from two different CMV-based promoters in AAV vectors targeting primary cortical neurons *in vitro* and rodent brain *in vivo* after they were treated with various stimulatory compounds or the activation of G-protein coupled receptors. Our results indicate that a general increase in neuronal activity, whether through excess of excitatory amino acids, activation of G protein-coupled signaling, or pharmacological stimulation of dopaminergic signaling can have a strong activating effects on the CMV promoter. These results have important implications for studies where stable constitutive expression of a transgene from the CMV promoter is assumed. Given these findings, caution should be applied when using the CMV promoter to drive expression of effectors of neuronal activation.

341. Generation of cGMP-Compliant Stable Packaging and Producer Cell Lines for Inducible Lentiviral Vector Production

Qian Liu
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Lentiviral-mediated gene transfer remains the method of choice for stable, long-term genetic manipulation of a range of celltypes, as exemplified by ex-vivo modification of T-cells for CAR-T therapy. Despite recent positive clinical data in this field,vector manufacturing technologies are largely focused on fully transient, adherent processes, bringing associated issues ofprocess robustness and scalability, as well as high cost-of-goods. Here, Oxford Genetics has generated a cGMP-compliantsuspension-based Lentiviral packaging cell line using a proprietary Tet inducible system. High-titre Lentiviral production hasbeen achieved with single plasmid transfection in both shake flask and bioreactor. Lentiviral producer cell lines have also beengenerated based on the packaging cell line, and enable high-cell density processes and alternative manufacturing modes.Further upstream and downstream process development, and associated analytics are currently in progress.

342. Stability of rAAV Vectors: Response to Various Biochemical and Biophysical Stresses

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As the field of gene therapy matures, greater consideration is being shown to drug product aspects of vector development such as formulation and in depth understanding of product stability. The use of "off the shelf" formulations, such as PBS and biological fluid mimics, can lead to manufacturing and stability concerns, and fails to realize the true potential of a vector as an optimized drug product. Careful attention to drug product development can offer many benefits. Highly stable formulations can enable high concentration vector solutions, opening up administration areas once limited by low administration volumes. Identification of solution properties amenable to vector stability can support the development of improved purification techniques. Further, vector formulations stable in the liquid state for extended time periods enable effective manufacturing, where stability at room temperature and robustness to physical stresses are key. And most tantalizing of all: formulation development may ultimately enable viral vector drug products that do not require freezing for long term storage. Such a prospect would ease demands on clinical and commercial logistics, avoid the need for careful planning around freeze-thaw cycles, and would ease clinical administration procedures - all while curtailing overall costs from generation through administration. Once a stable formulation has been identified, as with any biopharmaceutical product, rAAV drug products require rigorous stability data to support a defined expiry. Such data sets can be especially challenging to generate, in part due to limited material production, but also to the distinctive physicochemical features of rAAV vectors. Analytics can also present a challenge for both formulation development and long term stability assessment. Assays, often limited in number, may have high variability, be non-quantitative, and labor intensive. Furthermore, as frozen storage is intended, the representativeness of liquid state studies may be questioned, but frozen samples would require unacceptably long time points. Using current tools and testing limitations, we have generated a stable, high-concentration rAAV drug product formulation for clinical/commercial use. Here we present data on rAAV vector stability under a variety of conditions, including intended and accelerated scenarios, and under those mimicking worst-case storage and handling situations. This work demonstrates the importance of investing time and effort early in drug product development including stability planning to optimize experimental use of materials and number of studies to be run.

343. Improving AAV Quality Attributes and Process Robustness Through Molecular Redesign

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The manufacturing of recombinant AAV relies significantly on the underlying production technologies to support the robustness and scalability of clinical grade AAV material. To date two main production platforms are utilized to generate clinical grade rAAV material. One is the chemical transient transfection of HEK293 cells and the other is utilizing the baculovirus expression vectors to deliver genetic material to insect cells. The baculovirus production system has been shown to be capable of large scale rAAV manufacturing (500L) in stirred tank reactors while maintaining the high quality of the rAAV attributes. That said, large scale processes require a significant improvement in process robustness. Process robustness is dependent on the timing, quantity and quality of the precursor substrates where scaling of the processes can lead to difficulty in controlling these key parameters. Here we will highlight the results from molecular biology approaches in attaining higher process stability. Key improvements were obtained for the % full capsids (minimally 70% full, up from 30%), crude lysed bulk titers (2-fold improvement) and a process that is significantly less sensitive to the starting and raw materials used for production. In conclusion, molecular engineering of the BEVS constructs facilitated significant process-related improvements and robustness.

344. Replication Competent Retrovirus Testing for Gene Therapy Vectors: Current Methodology and Regulatory Expectations

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In recent years there has been a dramatic increase in the use of virus vectors to produce ground breaking gene-based therapies. Ensuring the biosafety and quality of vectors used in gene therapy is achieved through a multi-tiered approach that examines several factors to establish product safety and manufacturing consistency.

For retrovirus-based therapies, the generation of replication competent particles through recombination at various stages during the production process and subsequent treatment of target cells is a potential risk. Although improvements in vector design and manufacturing methods have decreased the probability of generating replication competent viruses, regulatory agencies have provided guidelines to test for the presence of these viruses at multiple stages of the production process. This presentation reviews the strategies for monitoring replication competent retroviruses. We describe current methods with a discussion on sample types, testing volumes and expected results based on regulatory guidelines.

345. Abstract Withdrawn

346. The Growing Gene and Cell Therapy (GGACT) Cooperative

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The Growing Gene and Cell Therapy (GGACT) cooperative was established in 2016 with funding from NIH NCATS. The founding collaborative centers have a history of working together to implement pioneering gene and cell therapy pediatric trials, and their experiences have required the development of both expertise and significant infrastructure at each institution that focuses on preclinical models of disease and testing, process development, GMP vector production and cellular manufacturing, protocol development and implementation, trial and data management and regulatory expertise that is specifically focused on early phase biologics in a manner suitable for transferring to industry for later phase studies. Its aim is to support academic investigators to rapidly translate complex gene and cell therapies to early phase, investigator-initiated clinical trials. In our collaborative cross-CTSA structure the GGACT will meet the recent IOM recommendation that the CTSAs "enhance the transit of therapeutics…along the developmental pipeline." A long-term goal will be to increase participation to even larger numbers of institutions, such that these innovative therapies become available, across a wide spectrum of pediatric institutions via funded CTSAs. The GGACT provides support services in 7 specific areas:
1. IND-enabling pre-GMP process development guidance.

2. Pre-GMP vector production and transduction manufacturing process and product development.

3. Trial-supporting assay development/implementation.

4. Regulatory pathway planning and assistance.

5. Development of reliance agreements to allow rapid implementation of new protocols via a simplified IRB review process.

6. Assistance in clinical trial protocol development, implementation and data management including packaging of data for potential licensing opportunities.

7. Assistance in clinical trials compliance, monitoring and reporting. Since inception, 6 rounds of applications have led to receipt of 12 letters of intent and 8 full applications. The GGACT is currently supporting 6 projects across 6 institutions that are distributed along the translational spectrum from completing proof-of-principle studies to IND submission. These are focused on treatments for: Hemophilia A, Fanconi Anemia, Glioblastoma, Pulmonary Alveolar Proteinosis, and two on Sickle Cell Disease. Five are using lentiviral vectors, four of these to CD34+ cells and one for CAR T cells, and one trial will use CRISPR/Cas9 for *BCL11a* gene disruption. GGACT maintains a web-

site (http://www.ggact.org) describing the organization and leadership of GGACT, the available core services, the supported projects and the application procedures, and also has useful links to relevant regulatory and educational sites. Letters of intent to submit applications for potential GGACT support for new projects are solicited quarterly and 2-3 new projects can be taken on per year, depending on the services requested. The GGACT may provide a valuable resource to assist with the advancement of gene therapy applications to human diseases. GGACT is supported by NCATS U01TR001814 ("Disseminating Curative Biological Therapies for Rare Pediatric Diseases").

347. Increasing Lentiviral Transduction Efficiency: Towards Cost-Effective T Cell Therapy Manufacturing

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Introduction: Chimerical Antigen Receptor (CAR) T cells are a promising new class of advanced therapies, although high-cost manufacturing processes render these treatments prohibitively expensive. Due to the cost of virus, transduction is a major cost driver in CAR T cell manufacturing. Nevertheless, limited research has been performed on identifying and optimising transduction process parameters. Therefore, the aim of this study was to build a small-scale test-bed to investigate sensitivities of various bioprocess parameters upon T cell transduction. **Methods:** A cryopreserved $CD3^+$ T cell bank was generated from fresh apheresis. Over a period of four days, transduction of cells was achieved in 36 parallel culture conditions using GFP lentiviral vectors (MOI=1). Investigated process parameters included (1) working volume (1-3mL); (2) agitation frequency (0-8 cycles/day); (3) cell seeding concentration (0.5-1.0 viable cells/mL), and (4) activation agent (0.5-1.5 U/mL). After cell counting and Fluorescence-Activated Cell Sorting (FACS) analysis, time course profiles and multivariate screening models were generated. **Results:** Transduction efficiency, total transduced cells, and cost per transduced cell were significantly sensitive ($p<0.05$) to variations in agitation, culture volume, and seeding density. Agitation was found to have a negative effect on both transduction efficiency ($p=0.0014$) and the total number of transduced cells ($p=0.0012$). A 2.6-fold higher transduction efficiency was observed for static cultures (73.1%) after 4 days in culture, compared to frequent agitation (27.8%). In addition, Figure 1 shows a 6.9-fold difference of *total* transduced cells between static ($1.17x10^6$ cells) and frequently agitated cultures ($0.17x10^6$ cells).

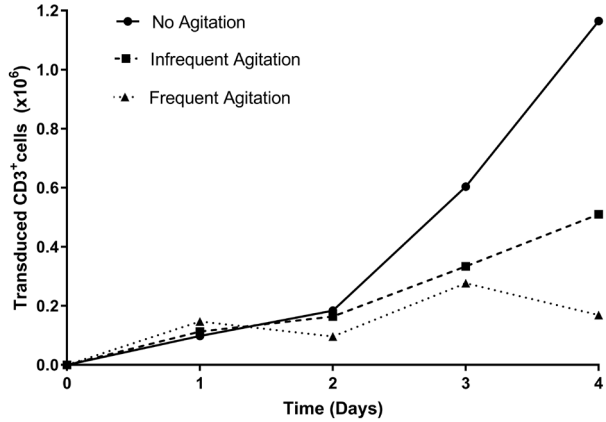

Figure 1: Transient, total number of transduced cells through: no agitation; infrequent agitation (4 cycles/day); and frequent agitation (8 cycles/day).

Volume was found to have a negative effect on transduction efficiency ($p>0.001$). Time course profiles showed 2.0-fold higher levels of transduction efficiency between low (59.8%) and high (30.1%) volume cultures. Within the investigated design space, the lowest virus cost per transduced cell was achieved at (1) no agitation, (2) high initial cell concentration, and (3) high culture volume (Figure 2). This corresponded to a 4.6-fold cost-reduction relative to the highest observed virus cost per transduced cell (3.0 vs. 13.8 viruses per transduced cell).

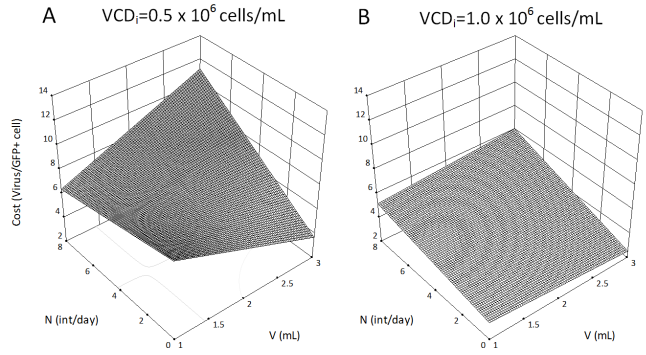

Figure 2: Fitted response surface model for virus cost per transduced cell due to variation of agitation cycles (N) and working volume (V) at (A) low seeding density (VCD_i) and (B) high seeding density

Discussion: This work shows that high cell seeding densities and lower working volumes, with little to no agitation allows maximum transduction of T cells, perhaps due to congregation of cells and virus. Furthermore, virus cost per transduced cell can be dramatically lowered through bioprocess optimization.

348. Optimization of PiggyBac Transposon-Based Gene Delivery by the MaxCyte Scalable Transfection System for Manufacturing Chimeric Antigen Receptor-T Cells

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Background: Chimeric antigen receptor (CAR)-T cell therapy has become a breakthrough therapy for patients who are refractory to standard therapy. Currently two CAR-T cell products have been marketed for the treatment of refractory hematologic malignancies, and several CAR-T pipelines are being tested for approval. Gamma retroviral gene transfer is widely accepted as the commercial-scale manufacturing method of CAR-T cells However, the high cost of the engineering process and the complicated regulatory restrictions associated with the use of viral vectors make the product notoriously expensive. CAR-T cells engineered by non-viral gene transfer, including *piggyBac* transposon (PB)-based gene transfer by electroporation, can be produced at an extremely low cost as compared to virally engineered CAR-T cells. In this study, we developed PB-based CAR-T cells by the MaxCyte (MC) Scalable Transfection System and evaluated the phenotype, anti-tumor efficacy, and exhaustion of CAR-T cells. **Material and Methods:** To optimize the electroporation protocol, we transduced plasmids containing the GFP gene into unstimulated peripheral blood mononuclear cells (PBMCs) by MC using 9 different electroporation protocols. The viability and the number of GFP positive cells were checked 48 hours after electroporation. Next, using EPHB4 receptor-specific CAR-T cells as a model (Yagyu et al. ASGCT2018), we transduced the PB-based EPHB4-CAR transgene together with the PB transposase gene into unstimulated PBMCs by MC, and cultured them for 14 days with defined T cell media, supplemented with IL-7 and IL-15. The phenotypes and the expression of exhaustion markers on CAR-T cells were assessed using flow cytometry. To evaluate the cytotoxic activity, the CAR-T cells were co-cultured with tumor cells at different effector:target ratios, and the number of live tumor cells were analyzed by real time cell analyzer or flow cytometry. **Result:** By using the optimized electroporation protocol, the GFP gene could be transduced and expressed in unstimulated T cells, with 32.9% of GFP positive cells and >90% cell viability being observed 48 hours after electroporation. We successfully generated EPHB4-CAR-T cells by MC with 48±7% of CAR positivity. These EPHB4-CAR-T cells predominantly showed the naïve/central memory phenotype and demonstrated sustained killing activity against EPHB4 positive tumor cells, even in multiple tumor re-challenges. **Conclusion:** PB-CAR-T cells engineered by MC showed acceptable CAR positivity, stable CAR expression, favorable phenotype, and strong antitumor efficacy. Since MC is designed for clinical use and has high scalability, production of PB-CAR-T cells by MC can be easily translated into clinical practice at an affordable cost.

349. Enabling Stem Cell Based Therapies: End-To-End Solution for Human Pluripotent Stem Cells Manufacturing

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Enabling stem cell-base therapies requires innovative solutions to close the gaps existing between research and commercialization. Allogeneic cell therapy indications that target large patient populations will necessitate the use of flexible cell production platforms to meet required cell quantities. Here we will show how moving away from conventional 2D culture platforms and developing a truly scalable, controlled bioreactor platforms for cell expansion enables meeting cell quantity demand for clinical applications while allowing comparability between the various scales. Likewise, it enhances process automation and allows integration of online monitoring systems. These bioreactor platforms are flexible cell production platforms, applicable to various cell types. Utilizing many common components, such as bioreactor controllers and centralized up-stream and down-stream hardware, while being able to quickly and easily change components such as vessels, media and microcarriers. The capability of effectively culturing adherent stem cells, namely pluripotent stem cells, will be presented. Human pluripotent stem cells (hPSCs) have unique characteristics of self-renewal and pluripotency that make them a powerful, though intermediate, cell therapy product. Conventional large-scale manufacturing platforms of hPSCs utilize 2D cell culture methods, which are open, uncontrolled, labor-intensive and involved large footprint. Here we present an efficient and scalable platform for large-scale expansion of hPSCs. The platform take advantage of 3D, stirred tank bioreactors, expanding hPSCs in suspension. Our data shows that hPSCs expand rapidly and efficiently in a 3L bioreactor, while retaining normal karyotyping and hPSC-associated characteristics such as marker expression and differentiation potential. hPSCs are fed with an animal-free hPSC media, perfused into a closed, monitored and controlled, bioreactor. 80-120 fold expansion (cell line dependent) can be reached in 10-16 days of continuance culture, with no need for cell passaging. Our platform supports expansion of hPSCs seeded in a bioreactor either when harvested from 2D cell culture or when thawed directly into the suspension vessel. Thawing cryopreserved cells directly into a suspension vessel eliminates the need for a 2D seed train and, therefore, greatly reduces labor, time and contributes to process aseptic, monitoring and automation. Furthermore, we show that 3D seed train from one suspension vessel to another is a feasible and viable solution for large-scale expansion of hPSCs. We show that hPSCs expanded in a bioreactor could be harvested and cryopreserved. Likewise, hPSCs expanded in a bioreactor are shown to have the capability to be directly differentiated in suspension. This expansion platform and associated attributes provides end-to-end, cryo-to-cryo, solution for hPSC manufacturing. Expanding hPSCs in suspension, in a controlled bioreactor, results in high fold expansion without compromising cell quality, and the capacity of the cells to be further differentiated. This platform avoids 2D cell culture steps, reduces footprint, labor and cost, while enhancing process control and cell product quality.

Cancer Targeted Gene Cell Therapy

350. Toward a Hematopoietic Stem Cell Gene Therapy Approach for Cancer Prevention

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Our ultimate goal is to develop a long-lasting, cost-efficient, and technically simple approach that allows for the immuno-prophylaxis of cancer in patients with high-risk for tumor recurrence and, ultimately, in carriers of cancer-predisposing inherited mutations. Our approach is based on *in vivo* genetic modification of hematopoietic stem cells (HSCs) that give rise to all blood cell lineages, including tumor infiltrating leukocytes that support tumor growth. Our *in vivo* HSC transduction approach 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. In preliminary studies we used an HDAd5/35++ vector expressing GFP and $mgmt^{P140K}$. Short-term exposure of *in vivo* transduced mice to low O^6BG/BCNU doses resulted in stable GFP expression in 80% of peripheral blood cells and, in mice with implanted syngeneic tumors, in 80% of tumor infiltrating leukocytes. The predominant GFP-positive cell type in two syngeneic tumor models was tumor-associated neutrophils (TANs) (Ly6G+) and macrophages (TAMs) (F4/80+/MHCII+). GFP+ TANs and TAMs were detectable at very early stage of tumor development. To avoid adverse reactions with cancer prophylaxis protocol, the expression of therapeutic transgenes has to *i)* be localized to the tumor, *ii)* be automatically activated only when the tumor begins to develop, and *iii)* cease when the tumor disappears. To develop such an expression system, we determined (by miRNA-Seq and miRNA-array) the micro-RNA profile in GFP^+ cells isolated from HSCs, spleen, PBMCs, and tumors of *in vivo* transduced mice. We found four miRNAs that were expressed at high levels in HSCs, splenocytes and PBMCs, but were absent in tumor-associated leukocytes. By inserting four copies of the corresponding target sites into the 3'UTR of the transgene, the corresponding mRNA is degraded in all cells except tumor-associated leukocytes allowing for tumor-restricted transgene expression. We performed a pilot therapy study with an HDAd5/35++ vectors expressing a mouse PDL1-targeted immune checkpoint inhibitor (αPDL1-γ1) under miRNA control in mice with syngeneic tumors, including tumors derived from mouse mammary carcinoma (MMC) cells. MMC cells were isolated from a spontaneous tumor in neu-transgenic mice. In *in vivo* HSC transduced mice with implanted MMC tumors, after initial tumor growth, 6 out of 7 tumors regressed and did not recur until the end of the observation period (100 days). Treated mice rejected another challenge of MMC cells given 8 weeks after the first injection. We also found about 20-fold higher amounts of αPDL1-γ1 in the tumor than in PBMCs, bone marrow, and spleen. This suggested that miRNA-regulation of αPDL1-γ1 expression attenuated its expression in hematopoietic cells other than tumor infiltrating myeloid and lymphoid cells. Serum αPDL1-γ1 became detectable after MMC cell injection and declined once tumors had disappeared, indicating a functional autoregulation of αPDL1-γ1 expression. Starting from week 2 after MMC cell injection, we observed auto-immune reactions reflected by inflammatory infiltrates in the kidneys and other tissues as well as fur-discoloration. Autoimmune reactions subsided once the tumors had disappeared and αPDL1-γ1 was no longer expressed. The efficacy of the *in vivo* HSC αPDL1γ1 gene therapy approach is remarkable considering that in the neu-tg/MMC model, other immunotherapy approaches including anti-PDL1 antibodies, vaccines, or T-cell therapies resulted in only transient tumor control. We are currently testing whether our approach can prevent the growth of spontaneous tumors in mouse models.

351. Tumoricidal Stem Cell Therapy Enables Killing in Novel Hybrid Models of Heterogeneous Glioblastoma

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Background: Tumor-homing tumoricidal neural stem cell (tNSC) therapy is a promising new strategy that recently entered human patient testing for glioblastoma (GBM). Developing strategies for tNSC therapy to overcome the intratumoral heterogeneity, variable cancer cell invasiveness, and differential drug response of GBM is essential for efficacious treatment response in the clinical setting. The aim of this study was to investigate the impact of GBM heterogeneity on tNSC therapies using novel hybrid tumor models. **Methods:** We used organotypic brain slice explants and distinct human GBM cell types to generate heterogeneous models *ex vivo* and *in vivo*. Tumor morphology and growth/invasion kinetics were measured using live animal optical imaging and *post-mortem* fluorescence imaging, and were compared to human patient MRI. We then tested the efficacy of mono- and combination therapy with primary NSCs and fibroblast-derived human iNSCs engineered with TRAIL or enzyme-prodrug therapy. **Results:** Optical imaging, molecular assays, and immunohistochemistry revealed the hybrid models recapitulated key aspects of patient GBM, including heterogeneity in TRAIL sensitivity, proliferation, migration patterns, hypoxia, and blood vessel structure. To explore the impact of heterogeneity on tNSC therapy, testing in multiple *in vivo* models showed tNSC-TRAIL therapy potently inhibited tumor growth and significantly increased survival across all paradigms. Patterns of tumor recurrence varied with tNSC-TRAIL dose and route of administration, with tNSC-TK outperforming tNSC-TRAIL in optimized treatment models. **Conclusions:** These studies demonstrate the ability of tNSC mono- and combination therapy to overcome important aspects of GBM heterogeneity in newly designed hybrid GBM models. Our results have begun to serve as a guide for optimizing and selecting the most efficacious tNSC therapy for human patient testing, with these new models continuing to be integral in testing multiple types of targeted GBM treatments.

352. Gene Therapy for Chronic Eosinophilic Leukemia

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Eosinophils, highly specialized bone marrow-derived granulocytic cells that carry a variety of cytotoxic mediators in cytoplasmic granules, normally play a role in combating parasites and other pathogens. In healthy individuals, eosinophils represent <5% of peripheral leukocytes (300-500/μl), persist in the circulation 8 to 12 hr and survive in tissues 8 to 12 days. However, if blood eosinophil levels are high, eosinophils release cytotoxic mediators that cause significant organ damage and dysfunction. Chronic eosinophilic leukemia-not otherwise specified (CEL-NOS), a subtype adult chronic eosinophilic leukemia characterized by persistent elevation of blood eosinophils (>$1.5x10^3$/μl) of unknown cause, is characterized by dysfunction of organs infiltrated with eosinophils and is unresponsive to any therapy. The disease is characterized clinically by weight loss, cough, weakness, diarrhea, splenomegaly, hepatomegaly and cardiac and lung dysfunction and survival of approximately 2 yr. Since the pathogenesis of this disease is unknown, the most direct therapy is to suppress the number of eosinophils in blood, thus suppressing eosinophil tissue invasion and organ dysfunction. Based on this concept, we hypothesized that a one-time gene therapy for CEL-NOS using an adeno-associated virus (AAV) vector coding for an anti-eosinophil monoclonal antibody (AAVrh.10anti-Eos) will provide sustained expression of the transgene, resulting in lower blood and tissue eosinophil levels *in vivo*. AAVrh.10anti-Eos codes for an anti-eosinophil receptor [Siglec-F (mouse)] monoclonal antibody that induces eosinophil apoptosis and reduces numbers of blood, bone marrow, and tissue eosinophils in mouse models of hypereosinophilia. To evaluate the effectiveness of AAVrh.10anti-Eos, a CEL-NOS mouse model using NOD-*scid IL2rγ*^{null} (NSG) mice was created using a vector expressing the cytokine IL-5 (AAVrh.10mIL-5) which induces bone marrow differentiation of eosinophils. AAVrh.10mIL-5 administered intravenously to NSG mice (5×10^{10} genome copies) stimulated the bone marrow to persistently generate high blood levels of eosinophils (>10^4 eosinophils/μl), with tissue eosinophil infiltration in heart, lung and liver and eventually death. AAVrh.10anti-Eos (10^{11} genome copies) administered 4 wk after establishment of the CEL-NOS murine model expressed persistent blood levels of the anti-eosinophil antibody for at least 24 wk and induced apoptosis of circulating eosinophils *in vivo*. Strikingly, there was concomitant markedly lower blood eosinophil levels in the CEL-NOS mouse model ($p<0.05$, both female and male mice) and reduced mortality 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 CEL-NOS, a fatal malignant disorder. It could also be considered for therapy of other chronic hypereosinophilic disorders that currently have unmet treatment needs.

353. In Vivo Activity of CD33/CD3-Directed Bispecific Antibody and Protection of Normal Hematopoiesis by CRISPR/Cas9-Mediated Gene Editing

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Background: The myeloid differentiation antigen CD33 has long been pursued as immunotherapeutic target in acute myeloid leukemia (AML) as it is displayed on a subset of the AML blasts and possibly leukemia stem cells. Improved survival in some AML patients with the CD33 antibody-drug conjugate gemtuzumab ozogamicin (GO) has validated CD33 as immunotherapeutic target and sparked interest in developing new, highly potent CD33-directed therapeutics. A promising new approach is built on small fragment bispecific antibodies such as Bispecific T-cell Engagers (BiTEs), which kill target cells by tethering with effector T-cells. An important limitation of these drugs is the display of CD33 on normal myeloid cells, which causes significant on-target/off-leukemia effects that manifest as prolonged cytopenias. In this study, we are evaluating the *in vivo* activity of the CD33/CD3 BiTE AMG 330 against normal hematopoietic cells in a humanized mouse model and, specifically, test whether CRISPR/Cas9-CD33 gene editing can protect these cells from AMG 330-induced cytotoxicity. **Methods**: NSG neonate mice were infused with $5.0x10^5$mock-treated (Cas9 only) or CRISPR/Cas9-treated human CD34+ cells. CRISPR/Cas9-editing was conducted using two gRNAs directed at intronic sequences for precise excision of exon 2, which encodes the V-set domain of CD33 that is recognized by AMG 330. At 23 weeks post transplantation, AMG 330 was administered intravenously daily for 7 days at a dose of 0.2mg/kg. **Results**: Consistent with our previous findings, CD33-edited CD34+ hematopoietic stem and progenitor cells (HSPCs) showed comparable multilineage engraftment relative to mock-treated HSPCs, with substantially reduced CD33 surface expression in peripheral blood monocytes. Intravenous AMG 330 administration was well tolerated and substantially reduced the number of circulating CD14+ monocytes only in the mock-treated cohort (**Fig.1**). In comparison, CD14+ cells (which were largely CD33-) were not affected by AMG 330 in the cohort that received CD33-edited HSPCs, (**Fig.1**), suggesting that these cells were protected from AMG 330-mediated cytotoxicity. Specificity of AMG 330 was demonstrated by the decrease exclusively in the number of CD14+CD33+ cells without affecting CD14+CD33- number present in these animals. The AMG 330 effect was transient, and CD14+ cell numbers were partially restored at 2 weeks post-treatment. **Conclusions**: We demonstrated activity of the CD33/CD3 BiTE AMG 330 in the humanized mouse model, which specifically targeted CD33-expressing hematopoietic cells. These results confirm normal function of *in vivo* differentiated CD3+ T-cells in this model, which will allow for the testing of novel BiTE molecules and chimeric antigen receptors. We also show complete protection of CD33-edited hematopoiesis from AMG 330, thus corroborating our previous data that used GO treatment. This study should facilitate the clinical translation of CD33/CD3-directed bispecific antibody therapy and help improve safety of this type of CD33-directed immunotherapy.

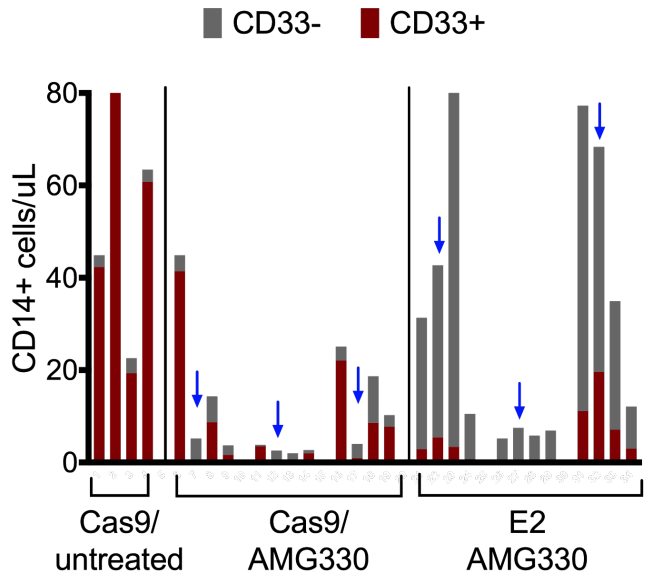

Figure 1:Quantification of CD14+/CD33+ and CD14+/CD33− cells from peripheral blood in Cas9 only (n=3) and CRISPR/Cas9-treated mice (n = 3) before (left bar), 24h post- (middle bar) and 14 days post-AMG330 administration

354. Neural Stem Cells Secreting Bispecific T Cell Engagers Improve Survival in a Preclinical Model of Glioblastoma

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Background. Glioblastoma (GBM) is the most aggressive primary adult brain tumor. Despite surgical resection, radiation, chemotherapy, and electric field therapy, GBM remains incurable. The invasive nature of glioblastoma preventing a complete resection, immunosuppressive microenvironment, and limited ability of therapeutics to penetrate the blood-brain barrier (BBB), collectively are responsible for poor treatment outcomes. Mobilization of an immune response within the tumor is highly desired. Bispecific T cell engagers (BiTEs) are molecules comprised of two single chain variable regions (scFvs) against CD3 and tumor-associated antigen (TAA) connected via a flexible linker. Upon engagement with BiTEs, T cells bridged with cancer cells undergo activation and mobilization of cytotoxic potential against cancer cells. However, BITE proteins have a short half-life in circulation and only a small fraction of systemically delivered BITEs reach the tumor in pre-clinical GBM models. Cellular carriers like neural stem cells (NSCs), which possess an inherent ability to pass the BBB and migrate to the tumor, may overcome this obstacles and serve as a platform for continuous release of therapeutic proteins within the tumor bed. We **hypothesized** that NSCs secreting BiTE proteins specifically targeting TAA interleukin 13 receptor alpha 2 (IL13Rα2) can activate a local immune response within the tumor and improve the survival of glioma-bearing mice. **Methods.** Two BiTE constructs containing short (SL) or long (LL) flexible linkers connecting scFv against CD3ε and scFv47 against IL13Rα2 were generated. A BiTE molecule with modified light chain in scFv47 was developed in order to abrogate an interaction with IL13Rα2 and served as a negative control. NSCs were engineered to secrete BiTE ($NSCs^{BiTE}$) molecules by lentiviral transduction. The ability of IL13Rα2-specific BiTE proteins to activate T cells was assessed in a Cr^{51} cytotoxicity assay as well as flow cytometric analysis for T cell activation markers and the production of cytokines, IFNγ and TNFα. The potential of $NSCs^{BiTE}$ to migrate to glioma cells was assessed *in vitro* using invasion chambers and *in vivo* by magnetic resonance imaging (MRI) of iron nanoparticle-labeled $NSCs^{BiTE}$ injected into the glioma-bearing mice. Therapeutic potential was assessed in the NSG mice bearing GBM12 patient-derived xenograft tumors treated locally with $NSCs^{BiTE}$ and healthy donor $CD3^+$ T cells. **Results**. The analysis of BiTE interaction with IL13Rα2 protein in ELISA revealed that LL BiTE is superior to SL BiTE. Also, LL BiTE was more potent in lysing IL13Rα2-expressing GBM cells and it was chosen for all subsequent experiments. We determined that $NSCs^{BiTE}$ secreted BiTE proteins at a level sufficient for activation of T cells *in vitro*. $NSCs^{BiTE}$ robustly stimulated T cells to proliferate and produce pro-inflammatory cytokines, INFγ and TNFα. The activation, proliferation, and cytokine production by T cells was observed only in co-culture with IL13Rα2-expressing GBM cells. Engineered $NSCs^{BiTE}$ cells retain their ability to migrate to GBM6, GBM12 and U251 glioma cells *in vitro*. T2* brain maps generated from longitudinal MRI studies demonstrated that iron-labeled $NSCs^{BiTE}$ injected into the contralateral hemisphere migrated robustly to the glioma xenograft over the course of 2-3 days. Finally, mice bearing GBM12 xenografts had a significantly improved survival after a local intra-tumoral injection of $NSCs^{BiTE}$ with $CD3^+$ T cells in comparison to negative control groups. **Conclusions.** We demonstrated that NSCs secreting BiTE proteins can locally engage T cells to respond against glioma cells, which warrants further investigation of the $NSCs^{BiTE}$ in various therapeutic settings.

355. Feasibility of Gene Replacement Therapy in Neurofibromatosis Type 1 (NF-1)-Related Tumors

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Background: Neurofibromatosis Type 1 (NF-1) is a RASopathy that represents a major risk for the development of cutaneous and plexiform neurofibromas and the malignant peripheral nerve sheath tumor (MPNST), in which biallelic-inactivating *neurofibromin 1* (*NF1)* mutations in Schwann cells result in tumor development due to Ras hyperactivation. MPNSTs are very difficult to treat and current therapies have shown little long-term benefit. In in the treatment of NF-1-related MPNSTs, gene therapy remains a largely unexplored area despite the known uniform monogenic alterationas underlying cause of tumor formation and the fact that clinical gene therapy has been increasingly successful owing to improved gene delivery technologies. Among these, adeno-associated viruses (AAVs)-based delivery vectors have emerged as safe and effective. However, AAV's limited packaging capability prohibits the use of full length NF1 molecule owing to its very large size. Hereby, we initiated the development of an AAV-based treatment using the NF1-GAP-related domain (NF1-GRD) to deactivate

Ras activity in MPNSTs and in pre-cancerous cells in NF-1 patients. **Methods**: NF1-GRD domain was cloned in viral expression vectors and tested for inhibition of Ras activity and growth in MPNST cells. Thirteen AAV serotypes expressing EGFP were produced and tested for transduction in MPNST and human Schwann cells. NF1-GRD was further optimized for anti-Ras activity by fusing with a membrane-attaching motif. **Results**: We first demonstrated the ability of NF1-GRD in suppressing the Ras activity and cell growth in MPNST cells using a lentivirus vector. Following, we systematically assessed 13 AAV subtypes for their capacity of transducing three human NF-1-derived MPNST cell lines and human Schwann cells. Among 13 different strains tested, 5 AAV serotypes appeared particularly promising, with very efficient transduction rates in MPNST and Schwann cells. Finally, to further optimize the therapeutic efficacy of NF1-GRD, we created a novel NF1-GRD construct with cell membrane-targeting resulting in superior and specific inhibition of MPNST cells. **Conclusions**: This approach has the potential to add a new dimension to the treatment of NF-1-related MPNSTs and neurofibromas and sets the foundation of engineering a novel AAV vector with higher transduction efficiency in MPNST and Schwann cells. The scope of such a therapy could ultimately be extended to a preventative application for *NF1* haploid individuals who are at high risk for a higher tumor burden/cancer or NF-1 patients in general.

356. Novel Strategy to Enhance In Vivo Expression of Adeno-Associated Virus Vectors in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide. Current therapeutic options for HCC are very poor as only early HCC stages are suitable to receive curative treatments, but the majority of patients are diagnosed at advanced stages (>80 %). Therefore, HCC has emerged as a target for novel therapeutic strategies such as AAV vector-based cancer gene therapy. Specifically, AAV vectors may be used to deliver suicide genes, tumor-suppressor genes or immunotherapeutic transgenes specifically and efficiently to the tumor site. However, current AAV vectors show poor transduction efficiency of HCC *in vivo*. Here, we investigated whether co-injection of receptor-binding peptides improve AAV transduction efficiency *in vivo* in a transgenic HCC mouse model to test the hypothesis that we can use receptor-binding peptides to change AAV's interaction with the tumor microenvironment. Indeed, we show that application of a receptor-binding peptide led to significantly improved transduction of tumor nodules as compared to controls. Interestingly, despite enhanced transduction, vector genome content was similar between cohorts. However, we observed a trend for enhanced transduction in the inner tumor layers in the peptide-co-injected cohort, which argued for deeper tumor tissue penetration. In summary, our study describes a simple strategy that may significantly improve current AAV vector-based cancer gene therapies and possesses a high potential for transfer into clinical application.

357. Therapeutic Effect of Extracellular Matrix Degradation Enzyme Delivered by Combination Engineered Bacteria with Anticancer Drugs

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To overcome limitations associated with bacteria-mediated cancer therapy, the engineered bacteria that encoded therapeutic drug have been reported. Several attenuated bacteria such as *Salmonella typhimurium* could target tumor tissue and regress the tumor mass. However, engineered bacteria have some hurdle to apply the clinical applications. We designed the combination therapy with bacteria and anti-cancer drugs. Extracellular matrix (ECM) of tumor tissue is one of targets for tumor therapy since the growth of tumor cells and tissues depends on its formation. Hyaluronic acid (HA) is major polysaccharide component of ECM and increases the interstitial fluid pressure (IFP) within the tumor tissue by absorbing significant amounts of water, resulting in an inhibition of anti-cancer drugs diffusion to tumor tissue. In this study, we assessed a possibility that ECM breakdown would increase the activity of tumor therapeutic drugs. We constructed plasmid encoded highly expressing hyaluronidase (HAase) under the controlled constitutive promoter originated from *Staphylococcus aureus*. The transformed attenuated *S. typhimurium* ΔppGpp (SL-HAase) exhibited the enzyme activity that degraded HA in agar plates. The bacterial lysates showed enhanced enzyme activity over 16-fold compared to control bacteria. In order to measure in vivo degradation of HA in tumor tissue, we injected SL-HAase into 4T1 and PC3 xenograft models intraveneously resulting in observation of significant decrease of ECM in both 4T1 and PC3 tumor tissue after 5 days compare to control group. To assess the combination therapy of an engineered bacteria with a chemotherapeutic drug, we injected ECM degrading bacteria and then doxorubicin every 2 days (a half of dose, 5mg/Kg) in 4T1 and PC3 xenograft models, resulted in inhibition of tumor growth compared to control group. The ability of ECM degrading bacteria to enhance chemotherapy efficacy is likely due to increased drug perfusion by reduced IFP.

CAR T Cell Therapy

358. Long-Term Remission of CLL Sustained by Oligoclonal CD19-Specific Chimeric Antigen Receptor T Cell Clones

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We recently demonstrated that sustained remission in (chronic lymphocytic leukemia) CLL patients treated with the CD19-specific, 4-1BB/CD3zeta-signaling chimeric antigen receptor T-cells (CTL019) correlated strongly with the expansion and persistence of the engineered T cells (Fraietta et al., 2018, Nat Med 24:563). We here report two advanced, chemotherapy-resistant CLL patients with the longest (8+ years) follow-up on any trial of CART19 cells. Both patients had received five therapies before being treated at the University of Pennsylvania with autologous CART19 cells (tisagenlecleucel) cells in 2010. Both patients have persistence of CAR-engineered T cells and both patients are still in remission as determined by flow cytometry and deep sequencing of IgH rearrangements for over eight years.To understand the fate of the infused CAR-T cells we determined the phenotype, function, and clonal nature of the persisting CTL019 cells. Flow and mass cytometric CART19 cell analyses demonstrated that early during the anti-leukemia response, activated, HLA-DR-expressing CD8+ CAR-T cells rapidly expanded, followed by similarly activated CD4+ CAR-T cells. With tumor clearance the CAR-T cell population contracted and maintained at low levels. An activated CD4+ CAR-T cell population was dominated the CAR-T cell repertoire at later time points and was still detectable at the last follow-up of 8+ years. The CD8+ CAR-T cell pool remained present at low frequencies. Deep immunophenotyping of CAR-T cells by mass cytometry (cyTOF) demonstrated that the long-term persisting CAR-T cells actively cycles and maintained high levels of activation markers in addition to PD1 and TIGIT. These data suggest that the initial tumor clearance was mediated by CD8+ CAR-T cells, but sustained by a CD4+ CAR-T cell population that still actively engages with target cells. TCR-seq analysis of early post-infusion time points demonstrated a) that the circulating CAR-T cell populations consisted of hundreds to thousands of distinct clones which in patient 1 and 2 displayed clonal focusing by 21 and 1 month post-infusion, respectively, with some clones making up as much as 12% (patient 1) and 48% (patient 2) of the CAR-T cell repertoire and b) that the CAR T cell repertoire stabilized early (1 month; patient 2) and late (21 months; patient 1) after infusion. Lastly, clonal analysis of the CAR T cell repertoire in the infusion product up to the latest time point for patient 1 (8 years) and 2 (7 years) via vector integration site sequencing of the infusion products identified a very diverse, non-clonal make-up, containing over 8,000 and 3,700 integration sites in patients 1 and 2, respectively. The higher degree of clonality in patient 2 but not 1 CAR-T cells as seen by TCR-seq was confirmed by integration site analysis, as was the sharing of CAR-T cell clones over time. Importantly, whereas the CAR integration site repertoire in patient 1 was diverse in the first two years, it stabilized and trended towards oligoclonality 21 months after infusion. Lastly, CAR integration site analysis revealed a high degree of clonal persistence, suggesting that tumor control and B cell aplasia were maintained by few, highly functional CD4+ CAR-T cell clones. In summary, we demonstrate that in both patients with the longest persistence of CAR-T cells reported thus far, early and late phases of the anti-CLL response are dominated by highly activated CD8+ and CD4+ CAR-T cells, respectively, largely comprised of a small number of persisting CD4+ CAR-T cell clones.

359. Genomic and Epigenetic Analysis of Patient-Derived Pediatric B Cell Acute Lymphoblastic Leukemia (B-ALL) to Define New Mechanisms of Resistance to CD19 CAR-T Cell Therapy

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Our recent clinical results have demonstrated that durable responses to CD19-directed CAR-T therapy in pediatric pre-B-ALL (acute lymphoblastic leukemia) are associated with the time of persistence of CAR-T cells in the periphery and the percentage of CD19-positive cells in marrow biopsies prior to CD19-CAR T infusion. However, in a small number of cases where these criteria were met, patients were resistant to CD19-CAR-T therapy. Little is known about the molecular factors that predict responsiveness to CAR-T therapy. These are important to define now that the response rate at one year is 50% or less. Our hypothesis is that patients that evidence CAR-T resistance in the short-term, despite the presence of positive prognostic indicators, have a distinct disease subtype that can be identified in pre-treatment marrow biopsy specimens. We used advanced exomic, and single-cell genomic and epigenomic analysis techniques to define signatures present in CD19-CAR-T resistant disease, in comparison to CD19-CAR-T responsive disease, using material from patients enrolled in a phase I clinical trial at Seattle Children's Hospital (PLAT-02, NCT02028455). Bone marrow aspirates from patients with leukemias resistant to therapy (4 pre-treatment with 2 paired post-treatment) were analyzed and compared to patients with therapy sensitive leukemias (5 pre-treatment). We performed bulk whole-exome sequencing and RNAseq, single cell (sc) RNAseq, sc B-cell receptor (BCR)-seq, methylation array, H3K27ac ChIPseq, and ATACseq. Initial genomic analysis revealed a total of 5 hotspot mutations in *ABL1*, *IKZF1*, *EP300,* and 2 in *KRAS*. RNAseq

analyses identified actionable fusions in 2 *ABL1*, 2 *ETV6*, 2 *ETV5*, and 1 *KMT2A*. Interestingly, a therapy-sensitive leukemia harbored a *KMT2A-AFF1* fusion that was previously shown to predispose patients treated to leukemic plasticity and lineage switching when treated with blinatumomab. Additionally, we identified *CREBBP*-fusions in all leukemias that failed to achieve CD19-CAR-T cell induced B cell aplasia. *CREBBP* perturbations have previously been associated with relapsed and refractory ALL. We are continuing to analyze patient biopsy material in order to define gene expression and epigenetic pathway differences. Single cell RNAseq and scBCRseq data are being analyzed for the existence of mixed lineage and gene expression-based heterogeneity that may predict clonal selection under CAR-T pressure. Similarly, ATACseq and methylation data is being analyzed for lineage specification in CAR-T resistant samples. Functional Epigenetic Module analysis (Jiao and Teschendorff, 2018) has identified distinct pathways present in resistant disease prior to therapy, that may lead to new approaches to potentiate CD19-CAR-T-based therapy. This study establishes one of the most comprehensive approaches to genomic profiling for leukemia patient samples. Although our analysis is preliminary and sample number is small, we believe these in-depth analyses will highlight crucial differences in leukemia that predict responsiveness to CAR T therapy.

360. Rational Combinatorial CAR Designs for Effective Immunotherapy

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Chimeric antigen receptors (CARs) are synthetic receptors for antigen that reprogram T cell specificity, function and persistence. Patient-derived CAR T cells have demonstrated remarkable efficacy against a range of B cell malignancies, and early trial results suggest activity in multiple myeloma (MM). Despite high complete response rates, relapses occur in a large fraction of patients, some of which are antigen-negative and others antigen-low. Whereas mechanisms resulting in complete and permanent antigen loss have been identified, those leading to antigen-low tumor escape remain obscure. We have found in a murine model that a decrease in CAR target expression may precede relapse and therefore begun to explore dual-targeting strategies as one approach to prevent antigen-low tumor escape. Extending the CAR "stress test" we previously described, we generated variants of the NALM6 acute lymphoblastic cell line that constitutively express wild-type or decreasing levels of CD19, which we termed CD19 high, med, and low. All of these express the same intermediate level of CD22 as well as CD38 (a known MM target). These tumors were treated in the in vivo CAR stress test using different CAR combinations schemas. In a xenograft NSG model, treating $CD19^{high}$ with 2e5 19-BBζ or 19-28ζ CAR T cells afforded significantly superior survival benefit compared to mice bearing $CD19^{med}$ or $CD19^{low}$ tumors. In the latter instances, 19-28ζ CAR T cells consistently induced longer survival than the 19-BBζ CAR T cells, confirming the former's greater sensitivity to low antigen density. Targeting CD22 antigen with 2e5 22-BBζ or 22-28ζ CAR T cells did not afford long-term tumor control, similar to the outcome observed with $CD19^{med}$ or $CD19^{low}$ tumors. However, targeting CD22 proved to be effective in preventing relapse of $CD19^{high}$ after infusion of 2e5 19-BBζ CAR T cells when 5e5 22-28ζ or 22-BBζ CAR T cells were administered 10 days later. Re-infusion of a higher CAR T cell dose afforded a better survival benefit, more significantly with 22-28ζ. When T cells were engineered to express two CARs, co-targeting CD22 in association with the 19-BBζ CAR afforded the best survival benefit if CD22 was targeted with a 28ζ CAR rather than a BBζ CAR. Under lower antigen density conditions ($CD19^{med}$ or $CD19^{low}$), the 19-28ζ/22-BBζ combination afforded the most durable responses. In contrast, the targeting of $CD19^{med/low}$ by the BBζCAR could not be rescued, regardless of which CD22 CAR was used. These results indicate that combinatorial targeting in conjunction with thoughtful pairing of CAR signaling features is critical to mitigate escape of tumors with low baseline target expression. The above data showed that targeting two antigens expressed at low density poses a greater challenge (eg. failure of 19-BBζ/22-BBζ co-targeting against $CD19^{low}$). As dual-targeting with two 28ζ CARs may promote tonic signaling or shortened T cell lifespan, we made use of an attenuated 28ζ module (28ζ1XX), which we previously found to reduce exhaustion by balancing effector and memory functions. We generated CD19- and CD38-specific 28ζ1XX CARs. Using $CD19^{high}$/$CD38^{+}$, $CD19^{low}$/$CD38^{+}$, and $CD19^{-}$/$CD38^{+}$NALM6 lines, we found that 19-28ζ1XX CAR-T cells can only kill $CD19^{high}$ and $CD19^{low}$, but not $CD19^{-}$ cells, while 38-28ζ1XX CAR T cells can eliminate all three NALM6 lines, irrespective of the CD19 levels. T cells expressing both 19-28ζ1XX and 38-28ζ1XX CARs lysed all NALM6 lines. In vivo experiments are underway to evaluate the therapeutic efficacy, persistence and function of dual 1XX CAR T cells. Our findings provide essential guidance for improving CAR design and selecting target-adapted costimulatory and activating functions, in order to prolong functional CAR T cell persistence and mitigate the risk of antigen relapse.

361. Dual-Switch CAR-T Cells: Inducible Cell Activation and Elimination to Manage Persistence and Toxicity

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The clinical success of CAR-T cells targeting CD19-expressing B cell leukemia and lymphoma has been achieved principally with CAR platforms encoding costimulatory signaling motifs derived from 4-1BB or CD28 fused directly with the ITAMs of CD3ζ. However, adverse events due to uncontrolled CAR signaling or transient T cell persistence can limit the performance of second-generation CARs. Here, we present a dual-switch (DS) CAR platform in which expression of three engineered proteins is encoded by a single γ-retrovirus vector comprising i) a first-generation CAR directing antigen-specific cell killing, ii) inducible MyD88/CD40 (iMC) governing CAR-T cell proliferation and cytokine release under the control of the cell-permeable dimerizing ligand rimiducid (Rim), and iii) a pro-apoptotic switch, iRC9, that is regulated by dimerization with rapamycin or temsirolimus (Tem), providing enhanced safety. The dual molecular switches are rapid and orthogonally regulated by the allele specificity

of the two ligands for FKBP12 variants. CAR-T cells were prepared to express first-generation (CD19.ζ), second-generation (CD19.BB.ζ and CD19.28.ζ) or the DS CAR (iMC-iRC9-CD19.ζ). While killing of CD19-expressing Raji and Nalm6 cells by each CAR-T type was equally effective in coculture assays, robust T cell proliferation was only observed following iMC activation in Rim-treated DS CAR-T cells. Specifically, Rim-treated CD19 DS CAR-T cell expansion within one week was 540-fold greater relative to untreated DS CAR-T cells, and 132, 26 and 59-fold greater than CD19.ζ, CD19.BB.ζ, and CD19.28.ζ cells, respectively. Release of IL-2 and IFN-γ was similarly more efficient with Rim-treated DS CAR-T cells. In NSG mouse xenografts of $CD19^+$ Nalm6 or Raji tumors, Rim treatment of CD19 DS CAR-T cells significantly enhanced tumor killing and T cell expansion relative to CAR.ζ, CAR.BB.ζ and CAR.28.ζ-expressing T cells. Conversely, treatment of mice at peak T cell expansion with Tem resulted in dose-dependent elimination of CD19 DS CAR-T cells, reduction of inflammatory cytokines, and rescue of cachectic weight loss within 24 hours of drug infusion. Nalm6 tumor rechallenge in mice post-Tem treatment induced robust expansion of the remaining CD19 DS CAR T cells, suggesting that transient resolution of toxicity with the iRC9 switch is feasible while maintaining the ability to drive tumor control with the iMC switch, as necessary. Prolonged persistence and safety management are likely critical to CAR-T strategies against solid tumors. Hence, $HER2^+$ solid tumors were challenged with HER2 DS CAR-T cells, leading to effective Rim-dependent tumor elimination with concomitant T cell expansion, while expansion was modest with first and second-generation CAR-T cells. This novel platform comprising a CAR combined with regulated costimulation and pro-apoptotic signaling provides user-controlled management of persistence and safety of antigen-specific CAR-T cells against hematological and solid tumors.

362. Toward Durable Multiple Myeloma Regressions with Anti-BCMA CAR T Cells with PI3K Inhibition and Reduced Time of Culture

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B-cell maturation antigen (BCMA) is an attractive target for chimeric antigen receptor (CAR) T cell approaches to treat multiple myeloma (MM). An 85% overall response rate has been reported in relapsed/refractory MM patients treated with bb2121, an investigational anti-BCMA CAR T cell therapy in a phase 1 clinical study (JCO 36, no. 15 suppl (May 2018) 8007). While most patients demonstrated clinical regressions, many patients eventually relapsed, with a reported 11.8-month median progression-free survival. To extend the durability of the response we optimized the manufacturing of BCMA-directed CAR T cells. We previously demonstrated that T cell manufacture in the presence of a PI3Kinhibitor enriched for anti-BCMA CAR T cells with a memory-like phenotype and improved functional persistence in a stringent preclinical model of durable tumor responses. The memory composition achieved with addition of PI3K inhibitor could not be achieved simply by shortening the CAR T cell culture time. Longer T cell culture periods have been shown to reduce memory T cells. Here, we explored if anti-BCMA CAR T cells manufactured with PI3K inhibition would exhibit further improved anti-tumor activity if cultured for a shorter length of time. Anti-BCMA CAR T cells were manufactured from healthy donors with PI3K inhibition for either seven or ten days. CAR T cells expanded an additional 2.9-fold on average during the longer culture time. CD62L expression, associated with memory T cells, was significantly higher on the anti-BCMA CAR T cells cultured for seven days compared to those cultured for ten. These anti-BCMA CAR T cells were further tested using a preclinical "stress test" model. NSG mice were injected intravenously with 2×10^6 Daudi tumor cells, which endogenously express low levels of BCMA (~1000 molecules per cell). The mice were allowed to accumulate a large tumor burden to model late-stage disease. In this advanced disease model, anti-BCMA CAR T cells cultured for seven days showed greater anti-tumor efficacy at lower doses of CAR T cells than donor-matched anti-BCMA CAR T cells cultured for the longer ten-day period. We next evaluated anti-BCMA CAR T cells manufactured from 5 multiple myeloma donors for either seven or ten days both in the presence or absence of PI3K inhibition. Flow cytometry and CyTOF were used to measure the resulting cellular immunophenotypes. PI3K inhibition during culture led to enrichment of memory-like anti-BCMA CAR T cells. Culture for seven days resulted in a further increase in the composition of anti-BCMA CAR T cells expressing CD27, CD62L and CCR7 compared to ten-day culture. This result was corroborated with Nanostring gene expression data which revealed an enrichment of genes associated with memory-like T cells in the seven-day PI3K inhibitor manufacturing condition. Together, these data demonstrate that PI3K inhibition has a greater impact than culture time on the phenotype of the cell product, while the combination of PI3K inhibition and shorter culture times further enhances the frequency of cells with the desired memory-like phenotype. Finally, to begin to test the potential for a CAR T cell product enriched for memory-like T cell composition to improve the durability of the antitumor response a phase I clinical study (NCT03274219) has been initiated to test the safety and efficacy of CAR T cells made using these refined T cell manufacturing processes (both PI3K inhibition and shorter culture times) in patients with relapsed/refractory MM. Biomarkers characterizing the drug products of enrolled patients have been analyzed to assess the enrichment of memory-like anti-BCMA CAR T cells and will be discussed.

363. Developing Ligand-Based Chimeric Antigen Receptors to Target Leukemic and Bone Marrow Stem Cells

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The thrombopoietin (TPO) receptor, MPL, is a critical survival signal for hematopoietic stem cells (HSCs) and leukemia stem cells (LSCs). We are developing ligand-based chimeric antigen receptors (CARs) against MPL using a fragment of TPO as compared to an antibody-based scFv.

A GFP-P2A-TPO-CD28-CD3 CAR was generated and primary human T cells were transduced (txd) using a VSV-G pseudotyped lentiviral vector. Txd cells were cultured with MPL+ cell lines, which showed the GFP+ cells had significant increases in CD69 (74±4.4 vs 16.7±4.4, p=0.0003), CD38 (76.3±6.6 vs 17.3± 2.5, p=0.001), and CD107a (21.3±2.9 vs 5±0.6, p=0.005) compared to non-transduced (non-txd) cells. In addition, there was a significant increase in cell death of TPO responsive Mo7e megakaryoblastic leukemia cell lines compared to non-txd cells (79.7 ± 4.6 vs 11.9 ± 3.8, p=0.003), with similar findings in the MPL expressing human erythropoietic leukemia (HEL) leukemia cell line. NSG mice were administered a luciferase+ HEL clone and 7 days later treated with non-txd or TPO-CAR txd T cells, a significant survival advantage (p=0.0014) from onset of leukemia was observed in TPO-CAR treated mice compared to animals treated with non-txd cells. We also show that human TPO-CAR cross reacts with mouse MPL. Therefore, to further our understanding of the TPO-CAR's ability to target stem-like MPL+ populations, we tested primary human T cells txd with the TPO-CAR *in vivo* as a conditioning agent for bone marrow transplantation (BMT). NSG mice were first treated with a low dose of irradiation (50 Gy) prior to infusion of either naïve or TPO-CAR txd human primary T cells. After T cell stimulation, we consistently observe 30-50% transduction efficiency. Fourteen days after the injection of TPO-CAR T cells, 8% of human CD45 was detected in the BM and 75% of the human cells were GFP+, suggesting a selective expansion of txd cells in the presence of the MPL antigen. The percent reduction of BM cells per femur in mice that received non-txd compared to TPO-CAR T cells in the LK (lineage-, c-kit+) compartment was 80.1% and LSK (LK, Sca-1+) was 62.0%. Using a methocellulose-based assay, the total number of colonies formed per 80,000 BM cells treated with non-txd T cells vs TPO-CAR T cells were 70 vs 12, respectively. To eliminate CAR+ cells, mice were treated with a CARlytic (low dose fludarabine, cyclophosphamide, and alemtuzumab), and 3 days later the BM compartment was analyzed and showed a 96.6% reduction of LK cells and 98.0% reduction of cells in the LSK compartment. All mice were transplanted with BM from a GFP+ transgenic mouse and at week 2 post-transplant there was a significant difference in GFP+ derived granulocyte engraftment between the non-txd, 8.72% vs the TPO-CAR txd treated mice, 72.52%. We repeated this experiment with no pre-conditioning prior to infusion of the T cells and transplanted mice with RAG1$^{-/-}\gamma c^{-/-}$ BM. Twenty weeks post-transplantation, engraftment of donor cells was evaluated. The mean engraftment of non-txd T cell treated mice vs TPO-CAR T cell treated mice were compared and showed that the LK compartment was 28.1±9.4 vs 40.18±15.7 (p=0.22), LSK compartment was 6.6±1.6 vs 13.0±5.3 (p=0.09), and LSK CD48- compartment was 3.1±0.8 vs 8.4±3.5 (p=0.049), respectively. Collectively, our data show that TPO-CAR, i) targets MPL+ leukemia cell lines, ii) clears MPL+ leukemia cells *in vivo,* iii) selectively targets MPL+ HSCs, and iv) clears BM HSC *in vivo* allowing for engraftment of donor HSCs. Further studies are warranted to determine the usefulness of the MPL-targeted ligand-based TPO-CAR in the treatment of leukemia and conditioning for BMT.

364. Nonhuman Primate Stem Cell-Derived CAR T-Cells Traffic to Lymph Node Follicles and Engraft Following Reduced-Intensity Conditioning

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Introduction: Although chimeric antigen receptor T-cell (CAR-T) therapies have revolutionized the treatment of hematological malignancies, several barriers limit their broader application to solid tumors and infectious diseases. First, the ability of CAR-T effectors to persist and function many years after infusion, when malignant/infected target cells may reappear, remains unclear. Second, target cells may persist in secondary tissues where CAR-T trafficking is limited. We have modified hematopoietic stem and progenitor cells with CAR molecules (CAR-HSPC), offering a lifelong source of antigen specific immunity. To reduce the toxicity often associated with HSPC transplantation, we evaluated reduced intensity conditioning regimens in nonhuman primates (NHPs), and asked whether CAR-HSPC-derived CAR-T may overcome trafficking limitations inherent to adoptively transferred T-cells. **Methods**: As proof-of-principle, autologous NHP HSPCs were modified with CAR molecules specific for human immunodeficiency virus (HIV-1) envelope (CD4CAR). This enabled *in vivo* titration of antigen levels by i) infection with HIV-like viruses, and ii) administration/withdrawal of antiretroviral therapy (ART). Prior to CAR-HSPC transplantation, animals received a conditioning regimen consisting of either myeloablative total body irradiation (TBI) or reduced intensity chemotherapy regimens, including Melphalan or Busulfan. CAR-HSPC engraftment was tracked by flow cytometry and droplet digital PCR. Immunohistochemical (IHC) methods were used to define the localization of CD4CAR-expressing cells in tissues, including B-cell follicles in lymph nodes. **Results**: We have previously shown that CAR-HSPCs enhance target-specific immunity, and that their abundance correlates with circulating antigen levels. Here, we found that Busulfan- but not Melphalan-based conditioning regimens facilitate CAR engraftment at significantly lower toxicity, relative to myeloablative TBI. IHC experiments revealed CAR-expressing cells' localization to germinal centers within central and peripheral lymphoid tissues, and in central nervous system sections. **Conclusions**: CAR-HSPC engraft following reduced-intensity conditioning, and CAR-HSPC-derived CAR-T traffic to multiple secondary tissue sites. These data show that CAR-HSPC approaches can be combined with safer conditioning regimens, a promising development for a variety of infectious and malignant diseases. We observe efficient trafficking of HSPC-derived CAR-T to tissue sites, which could enhance CAR-based targeting of a wide variety of solid tumors. Importantly, our vectorization methodology allows for enhancement of activity at these sites, and/or regulation by well-characterized kill switches. The use of cell type-specific promoters in CAR-HSPC adds the intriguing possibility to combine CAR-T approaches with other therapeutic CAR subsets, including natural killer cells, in a single product.

365. Pharmacological Control of In Vivo Tumor Regression by T Cells Engineered with CD19-Car Regulated with PDE5 Derived Destabilizing Domains

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Adoptive cell therapy with chimeric antigen receptor (CAR)-engineered T cells has demonstrated clinical success in B cell malignancies. However, broad clinical application of CAR T cell therapy is impeded by underwhelming *in vivo* expansion and persistence, increased tonic signaling and exhaustion, and the potential for on-target off-tumor toxicity. Precise tuning of CAR or immunomodulatory molecules co-expressed in CAR T cells has the potential to enhance safety and therapeutic efficacy for the treatment of leukemias and solid tumors. Destabilizing domains (DDs) are small protein domains that are misfolded and inherently unstable in the cell, but which can be reversibly stabilized by the binding of a small molecule ligand. This conditional stability of DDs can be readily conferred to any protein of interest (POI) by fusing it to the DD. In the absence of ligand, the POI-DD fusion protein is degraded by the proteasome. However, addition of ligand stabilizes the fusion protein in a dose-dependent manner. Our DD discovery platform utilizes a combination of structure-guided mutagenesis and screening of diverse variant libraries to identify novel DDs with a range of properties. Human phosphodiesterase 5 (PDE5) inhibitors such as Vardenafil, Sildenafil and Tadalafil are widely used clinical agents with a long clinical record of safety and a breadth of pharmacokinetic properties. To design DDs reactive to PDE5 inhibitors, we mutagenized the catalytic domain of PDE5 using variant libraries as well as site specific mutagenesis based on structural considerations. The mutants were fused to green fluorescence protein and screened for conditional stabilization behavior using fluorescence activated cell sorting. Selected DD variants were characterized by a high throughput plate assay and exhibited a range of sensitivity to PDE5 ligands as well as a range of basal and maximum expression. To confer regulated behavior to CD19-targeting CAR, we selected PDE5 DDs with low basal expression, high maximal expression with strong reactivity to Tadalafil. We fused PDE5 DD to the C-terminus of CD19-targeting CAR, packaged this expression cassette in lentivirus and transduced human T cells. In the absence of ligand, *in vitro* tests showed minimal CAR expression, low antigen-dependent cytokine production, and low cytotoxic activity against CD19 expressing target cells. A 24-hour exposure to ligand increased CAR expression 10-fold, increased anti-tumor activity similar to that of constitutively expressed CAR with an induction of pro-inflammatory cytokines in the presence of target-bearing tumor cells. CD19 CAR-DD transduced T cells were also tested *in vivo* for tumor regression in a Nalm6 disseminated tumor model in NSG mice (n=8 mice per group tested across multiple experiments). Vehicle dosed animals showed minimal regression of tumors while Tadalafil dosing resulted in tumor regression in a ligand dose-dependent manner. Recipients of regulated CAR T cell therapy showed: (1) a dose-dependent delay in tumor progression, (2) significant tumor growth inhibition across ligand doses, and (3) a significant survival advantage compared to recipients of unmodified T cells after Tadalafil treatment ($P<0.005$). These data suggest that DD regulation can be a valuable approach for regulating CAR and other cargo proteins to enable safe and effective cellular therapies.

Immunotherapy I

366. Modeling BCMA-Specific Regulated CAR T Cells to Target Long-Lived Plasma Cells or Multiple Myeloma

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Chimeric Antigen Receptor T cell (CAR T) immunotherapies have been successful in the treatment of B-cell malignancies. We hypothesized that CAR T cells could be used to target pathogenic B cell populations in other conditions including organ transplant rejection and autoimmune diseases, applications necessitating the modification of the CAR architecture to allow regulated killing. We previously described a method to achieve "switchable" assembly of a CAR on the T cell surface. This system, termed DARIC (dimerizing agent-regulated immune-receptor complex), uses rapamycin-driven heterodimerization of the scFV and intracellular signaling domains, permitting signaling only in the presence of rapamycin and target antigen. Here, we tested the function of B-cell maturation antigen (BCMA)-specific DARIC T cells to achieve regulated targeting of plasma B cells (PCs) *in vitro* and *in vivo*. BCMA is uniquely expressed on PCs, cells responsible for sustained production of autoantibodies. CAR T cells targeting BCMA have shown early success in the clinic for treatment of multiple myeloma, a plasma cell-derived cancer. We used lentiviruses to co-express the two components of a BCMA-directed DARIC system in primary human T cells: one containing a BCMA-specific scFV fused to FKBP12 (the 12-kDa FK506-binding protein), and the other having an extracellular FRB (FKBP *rapamycin* binding) fused to transmembrane and intracellular 4-1BB and CD3zeta signaling domains. The resulting BCMA DARIC T cells upregulated activation markers when cultured with BCMA-expressing cells, but only in the presence of the dimerization agent rapamycin. BCMA DARIC T cells also showed high cytolytic activity towards BCMA-expressing cells contingent on the presence of rapamycin. In contrast, conventional BCMA CAR T cells killed BCMA-expressing cells in both settings, with or without rapamycin. We next compared the activity of the BCMA DARIC T cells vs. BCMA CAR T cells in tumor xenograft models. Immunodeficient mice were implanted with either: i) a B cell line stably over-expressing human BCMA and luciferase transgenes; or ii) U266 myeloma cells expressing endogenous BCMA and a luciferase transgene. Control mice, or mice that received BCMA DARIC T cells but no rapamycin, exhibited tumor progression and required euthanasia. In contrast, rapamycin dosing in recipients of BCMA DARIC T cells blocked progression of both tumor types. Tumor targeting by rapamycin-treated BCMA DARIC T cells was comparable to conventional BCMA CAR T cells. Importantly, discontinuing rapamycin resulted in tumor relapse in mice receiving BCMA DARIC T cells, consistent with successful drug regulated T cell killing. To address the applicability of this platform to autoimmune

indications, we assessed the ability to target primary, non-malignant, human plasma cells *in vitro*. BCMA DARIC T cells efficiently depleted *in vitro* differentiated, autologous plasma B cells in a drug-regulated manner. Based on these findings, we are evaluating the potential of BCMA DARIC T cells as an intervention in autoimmune disease models including NSG mice engrafted with long-lived human PCs and in a novel murine model expressing human BCMA. Together, these results demonstrate regulatable targeting of BCMA-expressing cells with a range of BCMA surface densities *in vivo* using the BCMA DARIC T cell platform. Thus, BCMA DARIC T cells exhibit potential for targeting PCs in autoimmune conditions and may also provide benefits in treating multiple myeloma by limiting T cell exhaustion.

367. NKT Cells Co-Expressing a GD2-Specific Chimeric Antigen Receptor and IL-15 Show Enhancedin Vivopersistence and Antitumor Activity Against Neuroblastoma

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Background: Vα24-invariant natural killer T cells (NKTs) are attractive carriers of chimeric antigen receptors (CARs) due to their inherent antitumor properties and preferential localization to tumor sites. However, limited persistence of CAR-NKTs in tumor-bearing mice is associated with tumor recurrence. Here, we evaluated whether co-expression of primary NKT homeostatic cytokine, IL-15, with a CAR construct enhances the *in vivo* persistence and therapeutic efficacy of CAR-NKTs. **Design:** Human primary NKTs were *ex vivo* expanded and transduced with CAR constructs containing an optimized GD2-specific single-chain variable fragment and either the CD28 or 4-1BB costimulatory endodomain, each with or without IL-15 (GD2.CAR or GD2.CAR.15). Constructs that mediated robust CAR-NKT cell expansion were selected for further functional evaluation *in vitro* and in a xenogeneic mouse model of neuroblastoma (NB). **Results:** Regardless of IL-15 co-expression, constructs containing 4-1BB exacerbated CAR-induced tonic signaling, increased the rate of apoptosis, and reduced numeric expansion of transduced NKTs compared with respective CD28-based constructs (GD2.28z and GD2.28z.15). Co-expression of IL-15 with both costimulatory domains increased CAR-NKT absolute numbers, and with the CD28 domain led to reduced TIM-3 expression levels and increased multi-round tumor cell killing *in vitro*. Following transfer into mice bearing NB xenografts, GD2.CAR.15 NKTs demonstrated enhanced *in vivo* persistence and increased localization to tumor sites. GD2.28.15 NKTs had superior tumor control compared with GD2.28z NKTs (Fig 1). Importantly, GD2.28.15 NKTs did not produce significant toxicity as determined by histopathological analysis. **Conclusions:** Our results informed selection of the CD28-based GD2.28.15 construct for clinical testing and led to initiation of a first-in-human CAR-NKT cell clinical trial (NCT03294954).

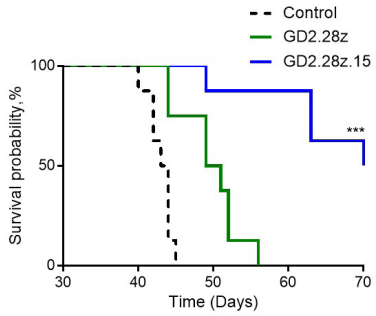

Figure 1. GD2.28z.15 enables superior *in vivo* therapeutic activity of GD2.CAR NKTs. NSG mice were injected intravenously with $1x10^6$ luciferase-labeled CHLA255 neuroblastoma cells followed seven days later by $2x10^6$ of GD2.28z or GD2.28z.15 NKTs. Control mice received no treatment.. Kaplan-Meier curve demonstrates survival probability. ***p < 0.001, Log-rank test, 8 mice per group.

368. CIS Checkpoint Deletion in Cord Blood Derived iC9/CAR19/IL-15 NK Cells for the Treatment of B-Cell Hematologic Malignancies

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Exciting clinical results using CAR-T cells against CD19 have led to recent FDA approval for relapsed/refractory acute lymphoblastic leukemia and non-Hodgkin lymphoma. However, the limitations of generating an autologous cellular product and the challenges of toxicity and disease relapse following CAR-T cells underscore the need to develop novel cell therapy products that are universal, safe and more potent. Natural killer (NK) cells are attractive contenders since they exert potent cytotoxicity against tumors and unlike T cells, lack the potential to cause graft-versus-host disease in the allogeneic setting. Our group has developed a novel strategy to genetically modify cord blood (CB) derived NK cells to express a CAR, ectopically produce IL-15 to support NK cell proliferation and persistence, and express inducible caspase 9 (iC9) as suicide gene to address potential safety concerns. We have initiated a first-in-human, phase I/II clinical trial of iC9/CAR19/IL15 NK cell therapy in patients with relapsed/refractory B cell lymphoid malignancies. We now propose to build on this platform to further enhance the potency of CAR engineered NK cells by blocking their intrinsic checkpoint molecules. The suppressor of cytokine signaling (SOCS) family of proteins plays an important role in NK cell biology by attenuating cytokine signaling and effector function against cancer. One of its members, cytokine inducible SH2 containing protein (CIS), encoded by the *CISH* gene, is as an important checkpoint molecule in NK cells and is upregulated in response to IL-15. We hypothesized that CIS may act as a potent checkpoint in

our iC9/CAR19/IL15 NK cells given the fact that they continuously produce IL-15, and that targeting this pathway would enhance their potency at lower doses. In a series of in vitro studies, we showed that *CISH* is induced in iC9/CAR19/IL15 NK cells in a time dependent manner. To examine the functional consequences of *CISH* deletion in our CAR-NK cells, we developed a protocol for combined Cas9 ribonucleoprotein (Cas9 RNP)-mediated gene editing to silence *CISH* and retroviral transduction with the iC9/CAR19/IL15 construct. On day 7 we nucleofected the CAR transduced NK cells with Cas9 alone (Cas9 control) or Cas9 pre-loaded with crRNA:tracrRNA duplex targeting *CISH* exon 4. Gene editing efficiency was >90% as quantified by PCR and western blot. *CISH* knockout resulted in significantly enhanced function of iC9/CAR19/IL15 NK cells against Raji lymphoma evident by increased cytokine production (TNFa p=0.007, IFNg p=0.033) and degranulation (CD107a p=0.003) compared to Cas9 control cells. Moreover, *CISH* KO iC9/CAR.19-IL15 NK cells killed Raji lymphoma more efficiently than Cas9 control cells and formed a stronger immunologic synapse (p=0.037). RNA sequencing with gene set enrichment analysis (GSEA) confirmed enrichment of JAK/STAT signaling, TNFα and IFN-γ inflammatory response in *CISH* KO iC9/CAR19/IL15 NK cells compared to Cas9 control counterparts, providing a molecular mechanism for their enhanced effector function. Moreover, in an in-vivo NSG mouse model of Raji lymphoma, the antitumor activity of a single dose of *CISH* KO iC9/CAR19/IL15 transduced CB NK cells was significantly better than that of Cas9 control cells; (p=0.003). These data indicate the presence of a novel interaction that regulates the functional activity of CARNK cells that could be targeted to improve adoptive NK cell therapy. We are in the process of scaling up this approach in our GMP facility for translation to the clinic.

369. Cytomegalovirus-Specific T Cells Expressing Anti-HIV CAR and CMV Vaccine Boost as Immunotherapy for HIV/AIDS: Pre-Clinical Data

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Chimeric antigen receptor (CAR)-based T cell immunotherapy was first developed 25 years ago to target and eliminate cells expressing HIV antigens in HIV/AIDS subjects. Although these anti-HIV CAR T cells have been shown to persist at low level, they did not achieve clinical benefits in subjects on anti-retroviral therapy (ART). A likely hurdle in the success of any T cell-based immunotherapy for HIV/AIDS is the lack of activation of T cells in the absence of antigen, a particular problem during ART. To overcome this, we developed CAR T cells that respond to both HIV and cytomegalovirus (CMV) antigen stimulations. Our overall hypothesis is that CMV stimulation using a CMV vaccine will maintain high levels of these bispecific CMV-HIV CAR T cells during periods of minimal HIV viremia, making them available for suppression of HIV reactivation during ART withdrawal. HIV-CAR T cells were first made by expressing the single-chain variable fragment (scFv) of several broadly neutralizing monoclonal antibodies (bNAb) targeting HIV gp120, and an scFv from bNAb N6, which neutralizes 98% of HIV strains, was selected to make CAR T cells from healthy donors. A lentivirus vector encoding the N6 scFv sequence was fused with the 4-1BB costimulatory domain, optimized IgG4 linkers, and a truncated human epidermal growth factor receptor (EGFRt) to serve for tracking and, if needed, as a suicide element. After transduction and *in vitro* expansion for ~20 days, the average HIV-CAR expression was 30-60%. The generated CAR T cell products demonstrated anti-gp120 activity, as determined by cytotoxicity and CD107α degranulation assays against HIV gp120-positive cells, and showed control of HIV replication when co-cultured with HIV NL4-3 chronically infected Jurkat cells. Next, we performed large-scale manufacturing of CMV-HIV CAR T cells from healthy and from HIV donors. CMV-specific T cells were enriched using the CliniMACS Prodigy® platform, transduced with the lentivirus vector encoding the N6 CAR construct and expanded *in vitro* for up to 20 days. The CMV-HIV CAR T cell products expressed memory T cells markers (CD62L, CD28, and CD27) with limited expression of exhaustion makers (PD-1, Tim3, and LAG3). These CMV-HIV CAR T cells exhibited IFNγ secretion, CD107α degranulation and proliferation upon CMVpp65 antigen stimulation, confirming CMV reactivity. Moreover, the CMV-HIV CAR T cells showed specific cytotoxic activity against gp120-expressing cells and control of HIV replication and in vitro reactivation. Interestingly, CMV-HIV CAR T cells derived from HIV donors - but not healthy donors - were mostly CD8+ (>95%), conferring protection to the CMV-HIV CAR T cells against HIV infection after infusion, thus minimizing the risk of potential in vivo infection of CAR T cells by HIV. Further, proof-of-concept studies in HIV mouse models are ongoing to test the anti-gp120 activity, anti-HIV infection properties, and response to CMV vaccine stimulation. If successful, this strategy could significantly improve the antiviral effect and persistence of anti-HIV CAR T cells in people living with HIV/AIDS, and potentially provide a strategy for life-long viral suppression in the absence of ART.

370. Cord Blood Derived CAR-T Cells Targeting gp350 Containing CD28/CD3ζ or 4-1BB/CD3ζ Signaling Domains Directly React Against Cells Infected with EBV Bypassing the Need of HLA-Matched Memory T Cells

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Objectives: Epstein-Barr virus (EBV) is associated with lymphoproliferative disease and occurrence of B-cell lymphomas and carcinomas in immunocompromised hosts. Patients undergoing

hematopoietic stem cell transplantation (HSCT) have substantial risks and approximately 30% do respond to standard-of-care treatment with rituximab. Adoptive immune therapy with virus-specific T cells (VSTs) is a salvage approach, but relies on the presentation of antigens through the HLA. A limitation for generation of VSTs is seen in risk groups, i.e. when the HSCT donor is EBV seronegative or cord blood is used because memory T cells are lacking. Hence, we generated T cells expressing EBV-specific chimeric antigen receptors (CARs) using cord blood T cells to bypass the need of HLA-matched EBV-reactive memory cells. We explored the EBV glycoprotein 350 (gp350) expressed during lytic EBV replication and on EBV-immortalized cells. Methods: gp350-CARs were constructed by fusion of single-chain variable fragments of two high affinity gp350-specific human mABs (7A1 and 6G4) to CAR-backbones containing the CD28/CD3ζ or 4-1BB/CD3ζ domains. γ-retroviral vectors expressing gp350-CARs were used to transduce PBMC or cord blood activated T cells. After confirming recognition of the gp350 target on 293T/gp350 cell lines, gp350-CAR-T cells were compared *in vitro* regarding recognition of autologous EBV-immortalized cells. The best modality was tested *in vivo* in mice humanized with human cord blood stem cells and infected with EBV. Results: Higher CAR expression levels were observed for T cells containing the CD28/CD3ζ than 4-1BB/CD3ζ domains and higher for 7A1-gp350-CAR than 6G4-gp350-CAR. Both 7A1-gp350-CAR and 6G4-gp350-CAR-T cells were activated, produced IFN-γ and proliferated in the presence of $gp350^+$ cells latently infected with EBV (B95.8 cell line or human B cells immortalized with the EBV laboratory strain M81). Nevertheless, killing of autologous EBV-immortalized B cells by gp350-CAR-T cells *in vitro* was overall modest, possibly due to EBV immune suppressive effects. A model consisting of Nod. Rag mice transplanted with human cord-blood (CB) stem cells and infected with an EBV/fLUC strain was used to test the antiviral effects of $CD8^+$ or $CD8^+/CD4^+$ sorted 7A1-gp350-CD28/CD3ζ-CAR-T cells. All control EBV-infected mice (n=3) showed detectable EBV genomic copies in spleen. One out of four mice (25%) administered with $CD8^+/CD4^+$ gp350-CAR-T cells and two out of three mice administered with $CD8^+$ gp350-CAR-T cell (66%) were protected and showed no detectable EBV copies in spleen. This antiviral effect was correlated with lower bioluminescence signal detectable by optical imaging and lower EBER staining in spleen of infected mice administered with gp350-CAR-T cells. Conclusions: We showed that gp350-CAR-T cells generated with cord blood reacted against EBV infected cells *in vitro* and *in vivo*. We are currently developing gene editing approaches in order to knock-out molecules involved with mechanisms of EBV immune suppression in order to maximize the cytotoxicity potential of $CD8^+$ gp350-CAR-T cells.

371. 4-1BB CAR Costimulation Drives Non-Canonical NF-kB Signaling, Thereby Enhancing CAR T Cell Survival and Suppressing Expression of Pro-Apoptotic Protein, Bim

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Chimeric Antigen Receptor (CAR) T cell therapy induces deep and durable responses in a large percentage of patients with B-cell malignancies. These responses often correlate with CAR T cell persistence in patients. The first two FDA-approved CAR-T cell therapies employ 2nd generation CARs that use different costimulatory domains derived from either CD28 or 4-1BB. Data from clinical trials suggest that 4-1BB CAR (BBz) T cells persist longer than CD28 CAR (28z) T cells, which pre-clinical studies have modeled *in vitro* by CAR-T cell expansion. We aimed to further understand the mechanisms by which BBz enhances CAR T cell expansion *in vitro*. Using primary human T cells isolated from the apheresis products of anonymous healthy donors, we generated anti-CD19 28z or BBz T cells by lentiviral transduction following activation by CD3 and CD28. We then expanded these CAR T cells using irradiated Nalm6 acute lymphoblastic leukemia cells in a well-established serial stimulation assay, measuring expansion by flow cytometry bead-based counting. Similar to previous reports, we found that the BBz CAR T cells expanded significantly more than the 28z CAR T cells. Though previous reports have demonstrated that 28z T cells proliferate as fast, if not faster than BBz T cells, we found that BBz, relative to 28z, significantly enhances CAR T cell survival. One signal associated with cellular survival and activated by endogenous 4-1BB, but not CD28, is the non-canonical NF-kB (ncNF-kB) pathway. NcNF-kB signaling is required for memory T cell persistence and enhances tumor cell survival by suppressing pro-apoptotic gene transcription. Therefore, we hypothesize that BBz, but not 28z, activates ncNF-kB signaling, which promotes CAR T cell survival by suppressing pro-apoptotic gene expression. To focus on CAR signaling, we employed an antigen-coated bead-based stimulation model. We observed expansion and survival advantages for BBz T cells relative to 28z T cells in this model as we did in the Nalm6 model. Similar to previously published studies, we also observed the activation of multiple downstream pathways upon bead-based CAR T cell activation in both 28z and BBz CAR T cells. However, following CAR activation, ncNF-kB signaling, assessed by western blot, was significantly upregulated in BBz relative to 28z T cell lysates. We investigated the function of ncNF-kB signaling by concomitant expression of a dominant-negative mutant of NF-kB-inducing Kinase (dnNIK) to block the ncNF-kB pathway. To explore mechanisms affecting survival, pro- and anti-apoptotic protein expression was analyzed by western blot over three weeks following restimulation through the CAR. Following bead-based CAR activation, control vector co-transduced BBz T cells expanded approximately 10 fold more than BBz T cells co-expressing dnNIK. DnNIK-expressing BBz T cells exhibited higher rates of cell death contributing to the

observed differences apparent in *ex vivo* expansion. In contrast, dnNIK 28z T cells had no appreciable difference in proliferation or survival compared with control. DnNIK-expressing BBz T cell death was associated with a nearly 3 fold increase in the expression of pro-apoptotic protein, Bim, relative to control. Therefore, BBz activation drives ncNF-kB signaling, which protects BBz T cells from cell death likely by restricting the expression of pro-apoptotic protein, Bim.

372. First-In-Human CAR T for Solid Tumors Targets the MUC1 Transmembrane Cleavage Product

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We developed a MUC1* targeting CAR T that is scheduled for a 1st-in-human clinical trial for metastatic breast cancers at the Fred Hutchinson Cancer Research Center in Q1 2019. huMNC2-CAR44 targets MUC1*, which is the transmembrane cleavage product of MUC1. MUC1* is a growth factor receptor that is activated when onco-embryonic growth factor $NME7_{AB}$ dimerizes its truncated extra cellular domain. The binding site for $NME7_{AB}$ is masked in full-length MUC1, as is the binding site for the huMNC2 antibody. Thus, huMNC2 does not bind to full-length MUC1, which is expressed on healthy epithelium. Previous attempts at a MUC1 targeting therapeutic all failed, presumably because they targeted the tandem repeat domain of full-length MUC1, which is shed after cleavage to MUC1*. huMNC2-CAR44 T cells are highly selective for cancerous tissues: first, because they target MUC1*, not the healthy full-length form. Secondly, the huMNC2 antibody recognizes a specific conformation of the MUC1* extra cellular domain that is created when MUC1 is cleaved by an enzyme that is overexpressed in many cancers, especially breast cancers. IHC studies of thousands of normal vs. cancerous human tissue specimens show that huMNC2-scFv almost exclusively binds to tumor tissues, hitting over 90% of breast, 83% ovarian, 78% pancreatic and 71% of lung cancers. Recognition of breast cancer specimens appears not to be limited by cancer sub-type. In vivo experiments of human tumors in NSG mice (n>300), show that huMNC2-CAR44 T cells robustly inhibited MUC1* positive solid tumors. A single injection of huMNC2-CAR44 T cells eliminated tumors in NSG mice which remained tumor free to Day 100, compared to control animals that had to be sacrificed at Day 20 due to excess tumor volume. Other animal experiments showed that huMNC2 CAR44 T cell efficacy increased with increasing antigen density. Dual tumor experiments of a high antigen density and low antigen density tumor in the same animal showed that adequate MUC1* density is required for a CAR T response, further supporting the idea that huMNC2-CAR44 T cells will selectively kill MUC1* positive tumors, while sparing normal tissues. The generation of an anti-MUC1* CAR that recognizes a conformational epitope created when MUC1 is cleaved to MUC1* by a specific cleavage enzyme, argues that CARs could be made patient specific, based on which cleavage enzymes their tumors express.

373. Pre-Clinical Investigations of CAR T Cells Directed Against the Tumour Antigen 5T4 (OXB-302) in Solid Tumor Models

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Background: A new era in cancer immunotherapy has emerged following the approval of T cells engineered to express chimeric antigen receptors (CARs) in patients with relapsed or refractory haematological malignancies. Comparable results have yet to be achieved in solid malignancies, but significant efforts are being made to translate the promise of CAR-T cells into clinical benefit across a broader range of indications. 5T4 is a tumor associated antigen which is a surface-expressed protein found on many solid tumors including ovarian, mesothelioma, breast, prostate and colorectal cancer. We have demonstrated that 5T4 is expressed on cancer stem cells (CSC) in multiple tumour types, thus enabling the potential to eradicate both bulk tumour cells and rare CSCs. Thus 5T4 is an attractive cancer target. To that end second generation CAR-T cells targeting 5T4 (OXB-302) are currently under investigation for the treatment of solid tumors including ovarian cancer. **Methods:** For ovarian cancer, matched blood and tumour biopsies were collected from patients with epithelial ovarian cancer. The expression of the 5T4 tumour antigen was determined by immunohistochemistry. Tumours were disaggregated into a single cell suspension using GentleMACS, and expression of 5T4 and EPCAM determined by flow cytometry. T cells recovered from patients with ovarian cancer were transduced with 5T4-CAR constructs and co-cultured with the autologous tumour cells. Response to the tumour was determined by quantification of IFN-gamma and IL-2 secretion. Co-expression of 5T4 with known markers of CSCs was assessed on patient-derived samples or tumour cell lines by flow cytometry. **Results:** For ovarian cancer, matched blood and tumour samples were collected from 12 patients; all tumors were positive for 5T4 expression by immunohistochemistry. Patient PBMCs were transduced with the CAR-5T4 construct and the resulting CAR-T cells were co-cultured with matched autologous tumour disaggregates; antigen-specific secretion of IFN-gamma was detected. The investigation of 5T4 being a putative marker of CSCs used a sphere-forming assay to enrich for CSCs in lung, prostate and ovarian cancer cell lines and results showed that 5T4 was enriched on CSCs. Furthermore, co-expression studies demonstrated that 5T4 was expressed with CSC markers CD133 and CD117 in ovarian cancer. **Conclusion:** These results demonstrate proof of principle that 5T4

is an attractive target for therapeutic intervention in ovarian cancer and that patient T cells engineered to express a 5T4-CAR (OXB-302) can recognise and respond to autologous tumour cells. In addition, targeting of 5T4 by OXB-302 may enable the de-bulking of tumours and the eradication of the cancer stem cells which are responsible for metastasis and relapse.

Gene Therapy for Musculo-Skeletal Diseases

374. Optimizing Dystrophin Gene Therapy in Severe DMD Murine Model

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Introduction: Duchenne muscular dystrophy (DMD) is a lethal X-linked inherited disease due to dystrophin gene mutation. The *heart failure* is the leading cause death in DMD *patients*. Gene therapy-based genetic replacement is a potential treatment for DMD. Chronic inflammation, caused by the up-regulated NF-kappaB (NF-κB) signaling, challenges DMD gene therapy. We have demonstrated that reduction of NF-κB via shRNA technology ameliorates such pathological process in *mdx* mice. Our preliminary results also showed that when two recombinant adeno-associated viral (AAV) vectors respectively carrying mini-dystrophin and NF-κB/p65-shRNA were simultaneously injected into DMD mouse model, expression of mini-dystrophin increased remarkably via reduction of NF-κB. Since the injection of two kinds of AAV is too complex to apply in clinic, therefore, it is urged to develop a dual-cassette gene vehicle containing both the mini-dystrophin gene and NF-κB/p65-shRNA that can ensure muscle-specific dystrophin expression in both skeletal and cardiac muscles and also inhibit the activation of NF-κB in both immune cells and muscle cells. **Methods:** All strains of mice approved by IACUC protocol include wild-type (WT, C57/BL10), dystrophin/utrophin double knockout (dys-/-:utro-/-, dKO-homo) mice. As shown in Figure 1A, a computer-codon optimized mini-dystrophin gene, including N-terminus, 5 Rods (1,2, 22, 23, 24), 3 Hinges (1, 3, 4), and CR terminus, was cloned into a single string AAV vector and driven by a MCK modified enhancer and muscle synthetic promoter (single cassette). We designed a mouse p65/shRNA driven by a U6 promoter that were respectively cloned into the single cassette we described before (dual cassette). A single I.P. injection with 50μl ($1x10^{11}$ viral genome particles) virus was performed to 5-day-old dKO-homo pups. After 8 weeks, human dystrophin (Rods 1 & 2), phosphorylated NF-κB/p65, CD4, CD8, CD68, ColIV were histologically analyzed. Images were taken at 200X magnification. **Results:** As shown in Figure 1B, efficient expressions of full-length dystrophin (450 kDa) in WT mice and mini-dystrophin (150 kDa) in dKO-homo mice treated with either single or dual-cassette vector were observed. IF staining of dystrophin also confirmed the specificity of muscle-targeted gene delivery in heart, as demonstrated by no expression of dystrophin in liver tissue (Figure 1C). Gastrocnemius muscles (GAS) were IF stained by p-P65 to determine the level of active NF-κB, CD4 and CD8 to evaluate immune cell infiltration, and CD68 to detect inflammatory macrophage. As shown in Figure 2, GAS muscle treated with dual-cassette AAV represented the less inflammatory markers such as CD4, CD8 and CD68 via the reduction of NF-κB, comparing to the treatment with a single-cassette AAV. WT GAS did not show inflammation in muscle. However, the non-treated GAS of dKO-homo mice had remarkable inflammation. For analysis of improved muscle physiology, the skeletal muscle force experiment is ongoing.

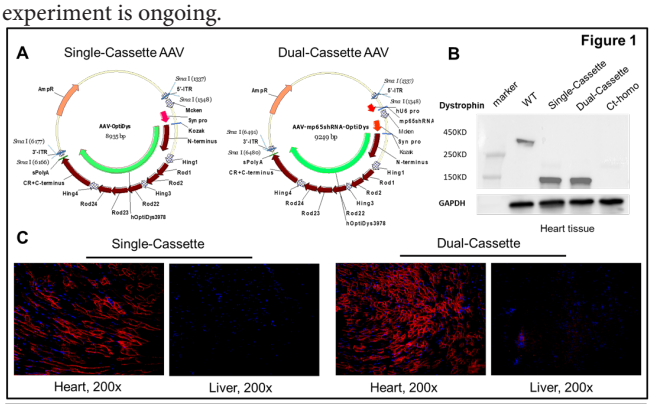

Figure 1. A. Schematic diagram of single- and dual-cassette AAV constructs. **B.** Western blot of dystrophin tested in cardiac tissues. **C.** IF staining of dystrophin in cardiac and liver tissues.

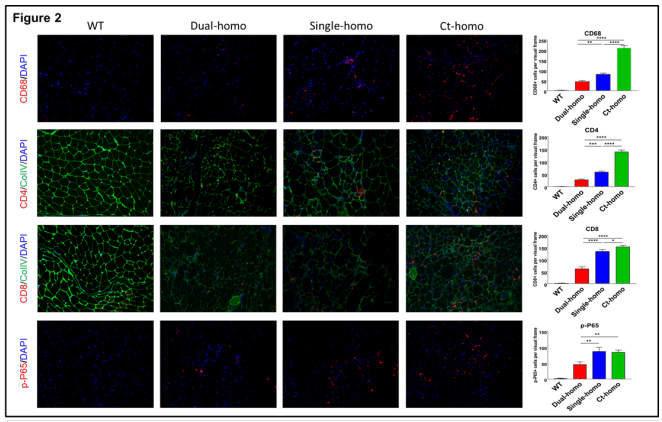

Figure 2. IF staining of CD68, CD4, CD8 and p-P65 in gastrocnemius muscles. WT: wild-type, Dual-homo: Dual-cassette AAV treated dKO-homo, Single-homo: Single-cassette AAV treated dKO-homo, Ct-homo: Untreated dKO-homo. Magnification: 200x.*(*means p<0.05, **means p<0.01, ***means p<0.001, ****means p<0.0001)*

375. rAAV9- Mediated NDUFS3 Replacement Reverts the Myopathy Phenotype in Pre- And Post-Symptomatic Muscle Ndufs3 KO Mice

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Mitochondrial disorders can be caused by mutations in nuclear genes, such as *Ndufs*3. This gene encodes one of the iron-sulfur protein components of complex I and is believed to be required for catalysis. We have created and characterized a conditional muscle *Ndufs3*-KO model which presents a severe myopathy at 2-3 months and shortened life span (7-9 months). We treated these mice by

delivering rAAV9-*Ndufs3* systemically, via retro-orbital injections. rAAV9-*Ndufs3* injections in P15-18 mice effectively restored NDUFS3 levels which was accompanied by a block in the development of the myopathy phenotype, assessed by treadmill and wire hanging time tests. Quadriceps of 6 months old mice showed normalized weight and oxidative phosphorylation protein levels. In-gel activity of Complexes I and IV showed a complete reversion of the KO mice biochemical phenotype in rAAV-*Ndufs3* injected mice. Complex I activity in muscle homogenates was similar to control animals, as was serum lactate levels. Furthermore, we tested whether it was possible to treat post-symptomatic mice (2 months). The results showed an overall significant improvement of the mitochondrial myopathy phenotype, 4 months after the injection. Our results showed a wide gene therapy window for replacing missing genes in mitochondrial myopathies.

376. Multiparameter In Vivo Screen of AncAAV Libraries in Muscle for Potency, Specificity and Impact of Disease State

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AAV-mediated gene transfer, utilizing naturally occurring capsid serotypes, is a powerful method to deliver potentially therapeutic payloads. With the advent of extensive next-generation screening tools, customized capsids can now be identified based on multiple metrics, including efficient gene transfer and transcriptional activity in specific target and off-target tissues. These approaches may allow multi-parametric optimization along a particular drug profile tailored to the indication. Here, we seek to identify improved AAV vectors that target global musculature following intravenous injection, and aim to gauge on versus off target distribution and activity, the stability of transduction, and the impact of disease state on vector performance. Specifically, several combinatorial libraries of synthetic, molecularly defined AncAAV variants were pooled with known AAV serotypes. On a 1:1 basis, each viral variant carried a unique barcode identifier in its viral genome that allowed a quantitative readout. Using an Illumina based NGS method called AAVSeq, the quantitative readout can provide information on the enrichment or reduction in each barcode's counts at the plasmid, viral vector, in vivo gene transfer (DNA) and in vivo transcriptional activity (RNA) level. The barcoded AncAAV library was injected in a mouse model for Duchenne muscular dystrophy, DMDmdx, and analyzed for an increase in biodistribution to and expression in affected tissue types over naturally occurring capsids. Initial screens have resulted in numerous capsids that appear to be more prevalent in target tissue types, and these capsids are being further characterized for not only their ability to specifically transduce multiple muscles but also manufacturability and their unique immune profile.

377. Gene Therapy for Mitochondrial Diseases

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Mutations in the mitochondrial DNA can lead to mitochondrial diseases affecting organs and tissues with high energy demand. Mutant mtDNA can coexist with wild-type mtDNA in a phenomenon called heteroplasmy. If the mutant mtDNA levels pass a threshold, disease ensues. We have been working in reducing the mutant mtDNA load both "*in vivo*" and "*in vitro*" using Transcription Activator-Like Effector Nucleases targeted to the mitochondria to cleave specific mutant sequences in the mtDNA. We have previously successfully designed and tested mitoTALENs that reduced the levels of several pathogenic mtDNA mutations in cultured human cells. To test the approach "*in vivo*", we developed specific mitoTALENs for a mouse mitochondrial gene coding for the $tRNA^{ALA}$ mutation m.5024C>T. The $tRNA^{ALA}$ mice have reduced body mass and cardiomyopathy at older ages when carrying high levels of this pathogenic mtDNA mutation. The mutation leads to decreased levels of $tRNA^{ALA.}$. We first tested this mitoTALENs in MEFs derived from the m.5024C>T mouse and found that it localized to mitochondria and reduced the levels of the pathogenic mtDNA. This reduction was associated with a recovery of oxidative phosphorylation protein levels after 2 weeks of the mitoTALENs transfection. For the "*in vivo*" experiments, intramuscular injection of the tibialis anterior (TA) with AAV2/9 particles coding for each of the mitoTALEN monomers were able to reduce the mutant levels of the pathogenic m.5024C>T mtDNA mutation, when compared to the opposite leg injected with the control AAV2/9-GFP. This was observed starting at 4 weeks till 24 weeks after injection. The biochemical marker, $tRNA^{ALA}$ levels were recovered in the TA after the mitoTALENs injections. Systemic injections in the retro-orbital sinus and intraperitoneal injections (IP) in neonates with both AAV2/9-mitoTALENs monomers coding for each mitoTALEN also showed consistent decrease of the mutant mtDNA in skeletal muscle and heart when compared to other tissues that did not express the AAV2/9-mitoTALENs such as brain and kidney. We are currently in the process of targeting different regions of the brain using a more specific serotype, AAV2/9-PHP.eb as a vector carrying the same mitoTALENs for $tRNA^{ALA}$ m.5024C>T mutation. In conclusion, our results showed that mitoTALENs, when expressed in affected tissues, could revert disease-related phenotypes in vivo, bringing mitochondrial nucleases closer to clinical trials.

378. Pre-Clinical Efficacy of Human Acid Sphingomyelinase as a Treatment for Limb Girdle Muscular Dystrophy 2B

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Background: Miyoshi Myopathy (MM) and Limb-Girdle Muscular Dystrophy 2B (LGMD2B) are autosomal recessive muscular dystrophies characterized by early adult onset of progressive skeletal

muscle weakness and wasting. Similar to a growing number of other muscle diseases, MM and LGMD2B are characterized by poor ability of injured myofibers to repair, leading to their death. Both diseases are caused by mutations in the dysferlin gene. We have previously demonstrated that dysferlin is required for enabling lysosomes in injured muscle cells to release a membrane-lipid modifying enzyme acid sphingomyelinase (ASM) that is required for repair. Yet with dsyferlin deficiency, as in MM and LGMD2B, ASM secretion is attenuated resulting in poor repair of injured myofibers. As there are currently no treatments for diseases like MM or LGMD2B that are associated with poor myofiber repair, we sought to examine the use of exogenous recombinant ASM protein as a LGMD2B therapy. **Methods:** Bacterial sphingomyelinase and recombinant human ASM (rhASM) was administered at titrated concentrations to LGMD2B patient myoblasts in-vitro, to determine dose and efficacy of this therapy for improving patient muscle cell repair. Cell repair was tracked in real-time with confocal microscopy. Subsequently, gene transfer of rhASM was tested in-vitro for its capacity to boost endogenous production and extracellular secretion of ASM. Secreted ASM was subsequently tested for its efficacy in improving LGMD2B patient myoblast cell repair as above. Lastly, in-vivo and ex-vivo effects of rhASM on dysferlin deficient muscle were determined via in vivo gene therapy in a mouse model of LGMD2B over a span of 12 weeks. Ex-vivo repair effects were determined via focal membrane injury and mechanical injury to isolated whole skeletal muscles using microscopy with pulsed laser, and eccentric contraction-induced injury respectively. In-vivo effects were assessed with histology, and muscle function assessed with grip strength. T-tests were used to assess for differences between control and rhASM-AAV-treated groups on muscle health. Repeated measures ANOVA was used to assess improvement in muscle cell membrane repair kinetics between conditions. **Results:** Exogenous rhASM improved LGMD2B patient muscle cell repair in a dose-dependent manner, with improvement only observed at concentrations exceeding 6 U/L ($p<0.05$). Gene transfer of rhASM promoted cellular secretion of therapeutically effective dose of rhASM, that subsequently improved dysferlin-deficient myoblast cell membrane repair ($p<0.05$). In vivo studies show benefits of rhASM for muscle membrane repair following focal or repeated lengthening contraction-induced injury (both $p<0.05$). The treated mice also exhibited increased myofiber survival, reduced need for myofiber regeneration, larger fiber size, reduced fibrosis, and improved limb grip strength compared to control-treated mice (all $p<0.05$). **Conclusions:** Exogenous administration of rhASM improves repair of dysferlin deficient LGMD2B patient and mouse muscle cells. Similar therapeutic benefits were observed in vivo by gene therapy to boost rhASM production. This identifies rhASM as a potential therapeutic option for MM and LGMD2B patients and further studies to test this are warranted.

379. Global Metabolic Profiling of Skeletal Muscle Reveals Profound Correction in FKRP-Deficient Mice after FKRP Gene Therapy

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Muscular dystrophy-dystroglycanopathies are a group of rare, progressive genetic disorders caused by loss-of-function mutations in a multitude of genes that disrupt the glycobiology of α-dystroglycan, thereby affecting its ability to function as a receptor for extracellular matrix proteins. Of the many genes involved, *FKRP* (OMIM 606596) plays a critical role in the functional maturation of a unique glycan on α-dystroglycan, operating as a ribitol 5-phosphate transferase and assisting in the formation of a tandem ribitol 5-phosphate moiety. Despite knowing the genetic cause of FKRP-related dystroglycanopathies, the molecular pathogenesis of disease and the metabolic response to gene replacement therapy has not been fully elucidated. To address these challenges, we utilized analytical technologies based on ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry to generate comprehensive global metabolite profiles from skeletal muscle across diseased, treated, and normal states. In all, 650 metabolites with known identity were identified and quantitated from the biosamples. In aged FKRP-deficient mice, we were able to detect significant ($p < 0.05$, $q < 0.10$) alterations in 290 metabolites (45% of total), compared to wild-type (C57BL/6) mice. Notably, the altered metabolites include changes in pentose sugars and glycolytic/gluconeogenic intermediates, markers of extracellular matrix damage/remodeling, and other energy generating pathways. More importantly, the restoration of FKRP protein activity and subsequent functional glycosylation with an intravenous injection of rAAV9-hFKRP vector led to a profound correction of numerous metabolite abnormalities, with only 120 metabolites (18% of total) being significantly altered and 124 metabolites being normalized, compared to wild-type levels. While the interconnections of the affected molecular mechanisms remain unclear, our global mass spectrometry-based metabolomics datasets demonstrate that FKRP deficiencies elicit diverse metabolic abnormalities in dystrophic mice. Furthermore, these metabolic impairments respond well to systemic rAAV9-hFKRP gene delivery, supporting the notion that global metabolic profiling could be valuable for identifying biomarkers of disease progression, distinguishing molecular markers and targets for therapeutic intervention by FKRP gene replacement therapy, and predicting long-term treatment-related side effects.

380. 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 strategies for corrective therapies for 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 deleting 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 exonic regions that are deleted from the patient's genome. Recently, AAV delivery of CRISPR/Cas9 strategies 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. We have confirmed targeted exon 52 integration in cultured cells, and current studies include assessment of HITI-mediated editing efficiencies and dystrophin protein restoration 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.

Preclinical Studies of Nonviral Gene Therapy and the Progress towards Translation

381. Development of a Reporter Mouse Model For In Vivo Evaluation of Genome Editing

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The CRISPR-Cas9 system is a widely used and popular tool for *in vitro* and *in vivo* genome editing studies. It has a great potential for correcting disease-causing mutations. Several proof-of-concept reports have been published and stated the need for improvements in animal models, methods of delivery, and editing efficacy and specificity. In this study, we generated transgenic mouse line with a defective eGFP coding sequence (ΔeGFP Tg) that can be used to compare the *in vivo* editing efficiency of CRISPR-Cas9 system. To verify the utility of ΔeGFP Tg, we used three techniques of gene delivery: 1) zygote microinjection, 2) oviducts-focused electroporation, and 3) hydrodynamic delivery in mouse liver. 1) We demonstrated 25% of eggs exhibiting eGFP fluorescence at the blastocyst stage when Cas9 mRNA, gRNA, and ssODN for DNA repair were injected into the fertilized eggs. The precise knock-in of ssODN by homology directed repair (HDR) was confirmed by sequencing. 2) Direct delivery of the same composition to the oviducts followed by an electroporation results in restoration of eGFP fluorescence in oviductal cells at the injection site. However, we observed expression of eGFP even in the lack of ssODN when gRNA sequence contains the eGFP mutation site, revealing that non-homologous end joining (NHEJ) DNA repair was sufficient to adjust the reading frame and restore eGFP fluorescence. On the other hand, we also revealed that only HDR-mediated repair was observed when gRNA has a slight shift in sequence to the eGFP mutation site. 3) Hydrodynamic delivery of CRISPR system to ΔeGFP Tg mice demonstrated successful conversion of liver cells to GFP positive cells. We found that ribonucleoprotein format (with Cas9 protein) is more efficient than vector DNA format (with Cas9 expression vector) in genome editing of liver cells.These results prove that ΔeGFP Tg mice are an excellent *in vivo* model for germline and somatic genome editing research.

382. Immunomodulation of Factor FVIII Inhibitors in Hemophilia A Mice Using Messenger RNA Lipid Nanoparticles

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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, approximately 30% of treated hemophilia A patients develop antibodies against infused FVIII that neutralize FVIII coagulation function, leading to treatment complications and significantly increased morbidity and mortality in patients. Recently we have demonstrated that delivery of FVIII variant mRNAs packaged into lipid nanoparticles (LNPs) in several different doses achieved high levels of FVIII expression following injection. In order to overcome anti-FVIII immune response, we sought transient immunosuppressive strategies that can prevent the formation of anti-FVIII inhibitory antibodies and induce long-term tolerance to FVIII. Anti-CD3 therapy has been used in clinical trials to treat immune-mediated disease such as allograft rejection or autoimmune diseases. Anti-CD3 treatment significantly depletes both CD4+ and CD8+ T cells and increases regulatory T cells (Tregs) population leading to adaptive immune tolerance. We investigated the use of LNPs encapsulated mRNA encoding anti-CD3 monoclonal antibody to modulate immune responses against FVIII. We first investigated the efficiency of depleting T lymphocytes using various anti-CD3 LNPs. Four different 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. We then examined the potential immunomodulation of anti-CD3 LNPs

for treatment of hemophilia A. Different groups of hemophilia A mice were injected with anti-CD3 LNPs and FVIII LNPs using combinations of different dosages and frequencies, respectively. After anti-CD3 LNPs treatment, hemophilia A mice displayed high percentage of Tregs (CD4+CD25+Foxp3+) population in CD4+ T cells compared to control group. Furthermore, in a multidose studies in mice of anti-CD3 LNPs combined with high dose of FVIII LNPs showed longer tolerance to FVIII up to 2 months compared to control mice which developed FVIII antibodies within two weeks. In conclusion, our study established a potential strategy of immunomodulation therapies for hemophilia A.

383. Efficient mRNA Therapy for Treating Ornithine Transcarbamylase Deficiency in Two Mouse Models

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Inborn errors of enzymes in the urea cycle can trigger hyperammonemia, which often leads to irreversible cognitive impairment, coma and death. The prevalence of urea cycle disorders is estimated to be at least 1 in 15,000. Patients with X-linked ornithine transcarbamylase (OTC) deficiency (OTCD), account for nearly half of all cases of inborn errors of urea synthesis, making it a compelling disorder for developing new therapies. Current therapies for OTCD have numerous challenges. Patients can be managed with a low-protein diet combined with medications that activate alternate nitrogen clearance pathways; however, this regimen does not prevent hyperammonemic crises. Liver transplantation can cure OTCD, but it is limited by the availability of donor livers, associated morbidity and mortality of the procedure, and immunosuppressive drugs to prevent graft rejection. Vectors based on adeno-associated virus (AAV) have shown great potential for sustained expression of therapeutic transgenes. A Phase I/II clinical trial is currently testing an AAV8.OTC vector developed by our group in adult OTCD patients. However, the prevalence of pre-existing AAV neutralizing antibodies (NAbs) in humans is a major concern for systemic delivery of AAV vectors. Re-administration of the same vector is not possible due to the NAbs generated after the initial dose. On the other hand, OTC mRNA formulated in lipid nanoparticles (LNPs) can be dosed repeatedly and with quick onset, which could benefit patients under hyperammonemic crisis. In the current study, we evaluated OTC mRNA therapy in two OTCD mouse models. We detected robust OTC protein expression in hepatocytes of both neonatal and adult spf-ash mice that persisted for at least 7 days. We then evaluated the efficacy of OTC mRNA-LNP (1) to protect and rescue adult spf-ash mice from acute crisis induced by a high-protein diet and (2) to rescue OTC-knockout (KO) pups that normally die within 24 hours after birth. In adults challenged with a high-protein diet, we achieved dose-dependent efficacies. On day 7, plasma ammonia levels in OTC mRNA-LNP- treated spf-ash mice did not differ from levels in wild-type mice and were significantly reduced compared to control spf-ash mice. Mice dosed 3 days after being on a high-protein diet induced crisis showed lower plasma ammonia levels 4 days post dosing and steady, dose-dependent weight gain. We successfully rescued OTC-KO pups for up to 7 days with a single injection of OTC mRNA-LNP. Based on these promising results, we are currently evaluating repeat administration of OTC mRNA-LNP as a chronic therapy to achieve long-term protection in adult spf-ash mice continuously fed a high-protein diet as well as long-term rescue of OTC-KO pups.

384. Monopolar Electrotransfer Enhances Gene Delivery to a Beating Heart

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A novel monopolar electroporation system and methodologies were developed for *in vivo* gene delivery intended for potential clinical translation. We hypothesize that asymmetric anode/cathode electrode applicator geometry could produce favorable electric fields for electroporation, without the typical drawbacks associated with traditional needle and parallel plate geometries. Monopolar electrode prototypes were initially tested for gene delivery of reporter genes to the skin in a guinea pig model with favorable results in terms of kinetics as well as gene expression distribution. In the current study monopolar electrodes were utilized for gene delivery directly to the beating heart in a rat model, compared to plasmid DNA injection without electroporation as well as bipolar 4-needle electrodes previously established for delivery in a rat and swine model. Gene expression was evaluated in terms of kinetics over time with bioluminescence imaging and distribution within tissue with immunofluorescence imaging. Electrocardiograms (ECG) were continuously recorded to evaluate any arrhythmias induced by electroporation. Masson's Trichrome analysis was performed to evaluate any damage from the treatments. For shorter pulse durations of 20ms, there was no significant enhancement of gene expression with monopolar GET over plasmid DNA injection without pulses, however longer pulse duration of 100ms resulted in significantly enhanced gene expression levels comparable to the levels achieved with 4-needle electrodes. ECG data indicates that with monopolar electrodes ventricular arrhythmias did not occur. Conversely, the 4-needle bipolar electrode did cause arrhythmias in the majority of the animals if synchronization of pulses with the absolute refractory period was imperfect. Both methods enhanced gene expression, and both methods resulted in comparable tissue damage, largely due to injections themselves. However, survival data indicates a significant difference with monopolar electrode resulting in 100% survival and no observed ventricular arrhythmias, even when pulse administration occurred outside of the absolute refractory period as measured by the ECG. Therefore, the new electrode geometry can be a safer option of gene delivery applications to sensitive/excitable tissues such as cardiac muscle and may facilitate translation of many electroporation-based clinical therapies utilizing gene electrotransfer.

385. IR-Laser Assisted Gene Electrotransfer to the Skin for Non-Invasive DNA Vaccination

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Efficient delivery of plasmid DNA to the skin can be an effective minimally invasive technique for vaccine administration. One of the challenges facing gene electrotransfer (GET) to the skin is penetration of the stratum corneum. This generally requires the application of tissue-damaging high voltage electric fields, with the expression being short-lived and typically confined to the epithelium. We previously established that moderate pre-heating to 43°C by an infrared (IR) laser enhances GET both *in vitro* and *in vivo,* allowing for the use of milder pulsing parameters. In the current work, we improved upon our electrode design by incorporating nine optical fibers within our multi-electrode array (MEA) to better distribute the exogenous heat across the target. We tested a variety of GET conditions in a guinea pig model with and without preheating the skin to 43°C, focusing on reducing either the applied electric field or pulse number. We also varied the plasmid DNA concentration and volume to determine optimal expression levels. Reporter gene expression was evaluated kinetically using bioluminescence imaging as well as immunohistochemically to determine distribution. Our results indicate that the pulse number and applied voltage could be reduced by 50% and 23% respectively, with the addition of moderate heating to achieve the same expression levels. Immunohistochemical evaluation demonstrated that expression could be observed in the dermis and into the muscle layer of the tissue when GET was combined with moderate heat, suggesting a long-lasting effect. We further evaluated the combination of moderate heating and GET for delivering a DNA vaccine against Hepatitis B Virus in a minimally invasive manner. Guinea pigs were injected intradermally with a plasmid encoding Hepatitis B virus surface antigen followed by GET parameters with or without moderate heating. αHBSAg serum titers indicated a 12-fold increase in antibody levels up to 30 weeks post-vaccination in those animals receiving GET combined with moderate heating compared to those animals receiving the vaccine with GET and no heating. These results suggest moderate heating can be used as a potential adjuvant for GET with respect to DNA vaccination to the skin.

386. Novel DNA Vaccination Utilizing a Newly Developed Pyro-Drive Jet Injector (PJI) Induced Serological and Cellular Immune Responses Leading to In Vivo Suppression of Tumor Growth in a Rat Model

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Various types of vaccines for treatment of diseases such as pathogens and cancers have been developed and evaluated for their clinical efficacies. Among of them, plasmid DNA-based vaccine has been attracting much attention because of its potential effectiveness and relatively quick and easy manufacturing. Indeed, in the area of veterinary medicine, several products for treatment of pathogens have been in practical use. These advances clearly indicate that DNA vaccine is a promising treatment modality and further optimization of administration method and/or protocol would help us to establish more effective DNA vaccine. Recently, DAICEL has originally developed a pyro-drive jet injector (PJI) for the intradermal delivery of various forms of substances. In this study, we aimed to determine the potential usefulness of PJI especially for DNA cancer vaccine. For this purpose, we took advantage of an immunogenic cancer/testis antigen NY-ESO-1, which is frequently expressed in a variety of tumors, as a surrogate antigen. We immunized WKA rats four times by PJI with plasmid DNA (1 to 100 μg dose) encoding full length NY-ESO-1 at 2-weeks intervals. Serums were periodically obtained from vaccinated rats, and then we examined serological immune responses by measuring NY-ESO-1-specific IgG antibody titers by ELISA assay. As expected, we observed that the amounts of NY-ESO-1-specific IgG antibody increased in a time- and dose-dependent manner and reached a plateau after the second vaccination in the case of 50 and 100 μg DNA dose. Surprisingly, detailed analysis showed that the majority of IgG antibody was IgG2a but not to IgG1 class antibody, indicating that DNA vaccination using PJI elicited immune responses shifted toward Th1. We further investigated whether DNA vaccination using PJI can induce in vivo resistance against NY-ESO-1-expressing tumor cells. Naive WKA rats were immunized twice with plasmid DNA (100 μg dose) encoding NY-ESO-1 or a mock vector at 2-weeks intervals. One week after the last immunization, the rats were subcutaneously inoculated with NY-ESO-1-expressing W14 rat tumor cells (oncogene-transformed fibroblast cell line) or parental W14 tumor cells in their flanks. Complete protection from NY-ESO-1-expressing W14 tumor cells was observed in the rats immunized with 100 μg dose, while no protection from NY-ESO-1-expressing W14 tumor cells or parental W14 tumor cells was observed in the rats immunized with a mock vector or 100 μg dose, respectively. Furthermore, no reappearance of tumor cells in the case of complete protection was observed during the subsequent observation period. Collectively, these data indicate the potential usefulness of PJI for eliciting immune responses against tumor. We propose that a newly developed PJI could be a useful tool for development of more effective DNA cancer vaccine.

387. Polymers for Transfection of mRNA: Polymer End Groups Dramatically Affect Efficiency and Biodistribution

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mRNA has drawn broad attention as a potential therapeutic agent, but its application entailsefficient and safe delivery. Previously, we described the use ofpoly(amine-*co*-ester) (PACE) terpolymers for mRNA delivery. This biodegradable family of polymers are safe and efficient mRNA delivery vehicles, particularly after undergoinga

controlled hydrolysis process we call "actuation". [1]We observed that the increase of PACE transfection efficiency after actuation is associated with the exposure of new end groups. To understand the mechanism by which end groups affect PACE transfection efficiency, we synthesized PACE using enzyme-based catalyst with 10% ω-pentadecalactone content, and purified the polymer by hexane precipitation. Gel permeation chromatography suggests homogenous molecular weight distributions at around 5kDa. We then modified the end group of the same batch of PACE with 35 different small molecules using a facile method previously developed in our lab. [1, 2]This approach resulted in a library of 35 polymers with the same backbone chemistry but different end groups. Further, we characterized their physicochemical properties, as well as their ability to encapsulate, protect, and to transfect mRNA *in vitro*and *in vivo*. Our experiments show that all the polymers we synthesized successfully formed complexes with firefly luciferase mRNA, but demonstrated drastically different transfection efficiencies. We then performed correlation analysis with the transfection efficiency of all the polymers in this library against their physicochemical characteristics and biological features, including size, zeta potential, in-solution pKa, encapsulation efficiency, cellular uptake, ability of endosome escape, and toxicity, etc. By analyzing this dataset, we were able to identify the most relevant parameters associated with mRNA transfection efficiency, and come up with an empirical formula to predict mRNA transfection efficiency of the polymers based on these measurable parameters before transfection. Moreover, we selected the top 10 polymers from this library with the highest transfection efficiency and tested their performance *in vivo* after direct intravenous injection. Interestingly, PACE with different end groups appear to home mRNA expression to different organs *in vivo*. Most of the polymers we studied deliver mRNA to the lung, and some of them demonstrate specific mRNA expression in the spleen. Overall, our results suggest a promising way to increase mRNA transfection efficiency for polymeric materials, and a potential method to modulate the biodistribution of gene therapy agents. **References**1. Jiang, Y, Gaudin, A, Zhang, J, Agarwal, T, Song, E, Kauffman, AC, *et al.*(2018). A "top-down" approach to actuate poly(amine-co-ester) terpolymers for potent and safe mRNA delivery. *Biomaterials***176**: 122-130.2. Kauffman, AC, Piotrowski-Daspit, AS, Nakazawa, KH, Jiang, Y, Datye, A, and Saltzman, WM (2018). Tunability of Biodegradable Poly(amine- co-ester) Polymers for Customized Nucleic Acid Delivery and Other Biomedical Applications. *Biomacromolecules***19**: 3861-3873.

Rational Engineering of AAV Vectors II

388. Engineering AAV Vectors for Antibody-Mediated Retargeting to Specific Cell Types In Vitro and In Vivo

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Adeno-associated virus (AAV) is currently one of the leading viral vectors used in gene therapy to treat human diseases. AAV has a favorable safety profile and the vector provides long-term, stable transgene expression. Because of these attractive features, AAV-based therapies have been developed to treat genetic disorders such as hemophilia and lysosomal storage disorders. Although AAV has many advantages as a gene therapy vector, one of the drawbacks to its use in systemic gene delivery is the relatively broad tropism of the virus and its tendency to preferentially target the liver. For many potential gene therapy applications, limiting infection to specific tissues or cell types would be advantageous. We are therefore interested in developing a cell type-specific AAV vector. Our goal is to rationally engineer the virus to target specific cell types by genetically abolishing the natural tropism of the virus, then redirecting the virus to target cells using monoclonal antibodies. To accomplish this, we have taken two complementary antibody-based approaches. The first uses a modular protein tagging system to covalently couple antibodies to surface-exposed residues on the viral capsid proteins. The second approach utilizes bispecific antibodies; one arm of the antibody binds to an epitope tag inserted into the viral capsid, while the other mediates binding to cell-surface proteins. We have successfully used both of these approaches to retarget AAV2 to specific cell types in vitro, using the cellular target of the retargeting antibodies to drive the specificity of infection. In both modalities, the natural tropism of the virus was abolished through the introduction of point mutations or peptide insertions into sites previously identified as primary determinants of vector tropism. We did not observe an appreciable reduction in virus titer compared to the parental vector with either approach. Using our covalent and affinity-based antibody retargeting systems, we have now demonstrated efficient transduction of a variety of mouse and human cells in culture using antibodies that recognize several different cellular targets. Critically, we found that overmodification of the virus with antibodies using either approach inhibits AAV transduction. We therefore developed methods to control the number of antibodies per AAV particle. For instance, we altered the accessibility of the epitope tag on the surface of the particle by adding or removing linker amino acids flanking the tag. We also modulated the number of antibodies that could successfully interact with the virus by creating mosaic AAV particles comprised of a mixture of tagged and native capsid proteins. For both covalent and affinity-based retargeting modalities, we found that optimal AAV retargeting occurs when very few antibodies are bound to the virus. Although we initially optimized the retargeting systems for AAV2, we have now successfully applied both techniques to additional AAV serotypes, and believe our systems can be capable of retargeting any AAV serotype. We then tested our retargeting systems in vivo by injecting our retargeted viruses and wildtype controls systemically into transgenic mice engineered to express the human protein recognized by the retargeting antibody. Infection was monitored by in vivo bioluminescent imaging and by sectioning and staining infected tissues. We found that both retargeting modalities were able to specifically transduce the intended target tissue in transgenic mice expressing the human protein, while the same virus showed very little off-target transduction in control animals that did not express the antibody target. Currently our efforts focus on applying these AAV retargeting approaches in vivo to non-human primates and additional cell types and tissues in mice.

389. Development and Characterization of Caspase-Activatable Adeno-Associated Virus Vectors for Targeted Gene Delivery Following Systemic Injection

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Adeno-associated virus (AAV) is a promising vector for gene therapy, but its broad tropism can detrimental if the transgene being delivered is harmful when expressed in non-target tissues. Delivering the transgene of interest to target cells at levels high enough to be effective while maintaining safety by minimizing delivery to off target cells is a prevalent challenge in the field of gene therapy. One method to combat this problem is to inject directly at the disease site, which can be invasive and impracticable in cases where there are multiple sites, or the site is not known or is difficult to access. Our lab has developed a protease activatable vector (provector) platform based on AAV9 that can be injected systemically and deliver genes site-specifically to diseased cells by responding to extracellular proteases present at the disease site. The provector platform consists of a peptide insertion into the virus capsid which disrupts the virus' ability to bind to cell surface receptors. This peptide consists of a blocking motif (aspartic acid residues) flanked on either side by cleavage sequences that are recognized by certain proteases. Exposure to proteases cleaves the peptides off the capsid, activating or "switching ON" the provector. In response to the activation, the provectors regain their ability to bind and transduce cells. The first iteration of the provector platform was activatable by metalloproteases (MMPs) and exhibited a 5.4-fold increase between its OFF and ON states *in vitro*, as well as an 8.1-fold reduction in transduction in the OFF state compared to AAV9. This vector was characterized in an *in vivo* murine model of heart failure (HF) where targeted delivery to the damaged part of the heart was observed via fluorescence microscopy and reduced delivery to off-target organs was quantified with qPCR. We have now designed a provector platform that is activated by cysteine aspartic proteases (caspases), which have roles in inflammation and apoptosis and thus are elevated at sites of diseases such as HF, neurodegenerative diseases, and ischemic stroke. These caspase-activatable provectors demonstrate up to 200-fold reduction in transduction ability in the OFF state compared to AAV9, reducing the virus' ability to transduce health tissue. Following proteolysis by caspase-3, the provector shows a 95-fold increase in transduction compared to the OFF state. These improvements in performance metrics compared to the initial provector design should allow for higher dosages to be used *in vivo* for therapeutic studies with transgenes that need to be expressed at high levels while maintaining lower delivery to off-target organs. Ongoing studies are confirming that the switchable transduction behavior is a result of the peptide insertion ablating the ability of the virus to bind to cells, determining the extent to which these engineered capsids can protect their genome, and visualizing the structure of the intact and proteolyzed capsids via cryo-electron microscopy with reconstruction. Further development of this provector platform has produced capsids with a dual-input AND logic gate requiring the presence of both MMPs and caspases to activate the virus. *In vivo* studies are underway characterizing the biodistribution, blood circulation time, neutralizing antibody (nAB) formation, and targeted delivery ability of the caspase-activatable provector in healthy and HF mice, followed by determining the therapeutic efficacy of the provector by delivering a clinically relevant transgene.

390. Optimization of MMP Activatable Provector for Targeting of Pancreatic Cancer

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Metastatic cancer remains a major public health concern, particularly with pancreatic cancer. As of 2015, pancreatic ductal carcinoma (PDAC) has a 5-year survival rate of 8.5%. The low survival rate is attributed to the fact that most cases are diagnosed after the cancer has metastasized. In epithelial cancers, such as PDAC, metastasis results in an increase in expression of certain membrane bound proteins including MT1-MMP (MMP-14). MMP-14 is a member of the matrix metalloproteinase (MMP) family of enzymes that have been directly correlated with tumor progression because of their roles in inflammation and extracellular remodeling. For this reason, our lab is developing protease responsive adeno-associated virus (AAV) vectors, known as provectors, to target sites of MMP overexpression such as metastatic cancer sites. Previous work from our lab has demonstrated switchable transduction of provectors for AAV2 and AAV9 (PAV1 and 453-L001, respectively) against MMPs -2, -7, and -9. Here we have developed an AAV serotype 9 mutant capable of sensing the presence of upregulated MMP-14, referred to as 453-L050. Protease-switchability is accomplished by blocking the receptor binding site on the virus capsid with a highly negatively-charged motif flanked by protease cleavage sequences. At sites of high protease concentration, the negative motif is cleaved from the virus capsid, "un-locking" the binding site for transduction. Silver-stain cleavage assays show 453-L050 is cleavable, or can be switched ON, by MMP-14. After switching ON, 453-L050 shows *in vitro* transduction efficiency that is 35% of the unmodified AAV9. We believe the provector is unable to reach the same level of transduction as AAV9 due to the residual amino acid "scars" left behind on the capsid surface after MMP cleavage. In order to increase the ON level of transgene expression, we have developed a provector library of different scar residues. Preliminary data has shown that inclusion on the N-terminus or C-terminus results in different transduction data, so a library was designed included each of the 20 amino acids on each direction of the cleavage sequences. This library is currently being screened to determine the impact of scar identity on provector performance. Optimal variants will demonstrate low OFF and high ON transduction behaviors. Generalization of optimized scars will be tested through inclusion to the scar of 453-L050 which is of same length but differs in charge from 453-L001. A provector that is optimized to be as efficient in the ON state as AAV9 would be very beneficial to the field of gene therapy. A viral vector optimized to target metastatic sites through their upregulation of MMP-14 and express as effectively as AAV9 is a promising avenue for non-invasive treatment.

391. Customized Blood-Brain Barrier Shuttle Peptide to Increase AAV9 Vector Crossing the BBB and Enhance Transduction in the Brain

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Recombinant adeno-associated viruses (rAAVs) have been widely used as favored delivery vehicles for treatment of multiple diseases in preclinical and clinical trials, including central nervous system (CNS) diseases. Yet, the delivery of rAAV to the brain is severely hindered by the blood-brain barrier (BBB). Although several AAV variants with genetic modifications of rAAV capsids are able to enhance brain neuron transduction in mouse models by intravenous administration, recent studies have demonstrated that these observations from mice with modified rAAV vectors are not always predictive of a desirable outcome in other mouse models and nonhuman primates (*Matsuzaki, Y., Neurosci Letter, 2017; Hordeaux, J., Molecular Therapy, 2018*). Moreover, the engineered rAAVs may present potential unknown and unpredicted structural changes and tissue tropism. These concerns highlight the need to explore novel strategies to increase the ability of AAV to cross the BBB for enhanced brain transduction. Our recent research has showed that the BBB shuttle peptide THR could effectively increase AAV8 transduction in mouse brain by systemic administration (*Zhang X. et al. Biomaterials, 2018*). The THR peptide could bind with AAV8 directly and enhance its transduction by various mechanisms. However, the THR peptide failed to increase the ability of AAV vectors from other serotypes to cross the BBB and enhance brain transduction. To explore novel BBB shuttle peptides able to specifically bind to other AAV serotypes and enhance the specificity of transduction after systemic administration, we performed combinatorial *in vivo* phage display library-based and *in vitro* AAV9-based screenings and identified a novel peptide named PB5-3. The PB5-3 peptide specifically bound to AAV9 virions without competition with the AAV9 primary receptor galactose and enhanced AAV9 transduction in mouse brain after systemic administration. Mechanism studies showed that the PB5-3 effectively increased the AAV9 trafficking ability and the transcytosis efficiency in the human BBB model hCMEC/D3 endothelial cell line. Additionally, it slowed down AAV9 blood clearance without hepatic toxicity. This study highlights the potential of this combinatorial approach for the isolation of peptides that interact with specific AAV vectors for enhanced and targeted AAV transduction. It will open new combined therapeutic avenues and shed light on the potential applications of peptides for the treatment of human diseases in future clinical trials with AAV vectors.

392. A Barcode System for Evaluating Adeno-Associated Virus Capsids with Low Immunogenicity in Nonhuman Primates

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The barcode method is useful for evaluating the performance of adeno-associated virus (AAV) capsids. We have developed a barcode system that works in nonhuman primates (NHPs). We chose the self-complementary AAV (scAAV) vector to reduce the chance of vector-genome annealing. The transgene cassette was an enhanced green fluorescent protein (eGFP) reporter gene driven by a cytomegalovirus enhancer/beta-actin promoter, flanked by a SV40 intron at its upstream and SV40 polyadenylation (polyA) signal at its downstream. Within the eGFP region, we eliminated the ATGs of all potential open reading frames (ORFs) that were longer than 50 bp to reduce transgene-protein-induced immunogenicity. We inserted primer binding sites and a 6-bp barcode right after the stop codon of the mutated eGFP ORF and before the polyA signal. The location of the barcode conferred three advantages: 1) given its proximity to the polyA signal, the barcode can be easily picked up by oligo dT-primed reverse transcription; 2) as the barcode is not too close to the polyA signal, we can avoid signal loss during mRNA processing due to potential cleavages upstream of the cleavage site; 3) the size of real-time PCR products fits downstream processing, including next-generation sequencing (NGS). The codes are only 6-bp long to minimize their impact on transcription efficiency, RNA stability, and RNA processing. We used 12 codes labeled barcode (BC) 01 through BC12. Transient transfection on HEK293 cells showed faithful transmission by the codes of abundance information between the input and the cDNA output (except BC06) and no eGFP fluorescence. We then individually packaged the 12 barcoded vectors into a clade E AAV variant. We pooled the vectors together in a geometrically progressive manner (i.e., in the pool, the percentage of the highest-abundance code was 100 times the percentage of the lowest-abundance code). We evaluated the barcode system in three mice that received intravenous administrations of the vectors at a dose of 2e12 genome copies (GC)/animal. Two weeks later, we retrieved cDNAs and tissue DNAs from liver, heart, and muscle for NGS analysis. The theoretical and actual abundances of the injection mix were well correlated. For tissue DNA, the abundance information transmission between the input and output was good in liver, heart, and muscle. The inter-animal variations were small. Specifically, cv% of the abundances of the 12 codes among the three animals were: liver 0.4% - 8.4%, heart 3.5% - 50.9%, and muscle 4.4% - 86.5%. For cDNA, the transmission quality was similar except for BC06, which showed clear transcription enhancement in liver, heart, and, to a smaller extent, muscle in all three animals. Further analysis revealed that BC06 contains a transcription factor binding site called the CAAT box. We used the remaining 11 codes to tag several sets of AAV variants and their controls and intravenously injected the vectors into three monkeys at doses of 2.9e13, 2e13, or 2e13 GC/kg. The first animal showed ALT levels below 30 U/L on day 0, 14, and 27 after vector administration; these levels were much lower than a historical control with eGFP-expressing vector. The second animal had an ALT elevation from 60 U/L on day 0 to 296 U/L by day 7. This animal had an ALT elevation event one year prior for

unknown reasons. The third animal, which was approximately nine years old, had a mild ALT elevation (38 U/L on day 0 to 52 U/L by day 7). Taken together, our barcode system demonstrates very small animal-to-animal variations over a 2-log dose range in mice and low immunogenicity in NHPs with intravenous vector administration.

393. Human Polyclonal Anti-AAV Neutralizing Antibody Epitope Mapping by NGS Identifies Common Epitopes and Enables the Design of Stealth Mutants

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Despite the proven ability of AAV as a gene therapy vector, significant hurdles in therapy optimization still remain. One of these obstacles is overcoming the high prevalence of anti-AAV neutralizing antibodies (NAbs) in humans that limits the utility and efficacy of AAV gene therapy. To cope with this issue, genetically engineered stealth AAV capsids have been actively sought, showing some success; however, better rational design of stealth vectors will require comprehensive knowledge of the NAb epitopes that could vary from person to person. We have previously reported IP-Seq (Immunoprecipitation-Seq), a next-generation sequencing (NGS)-based high-throughput method to identify conformational epitopes of both polyclonal and monoclonal anti-AAV capsid antibodies in a large number of serum samples and its successful application to murine antibodies. Although very effective, this approach is unable to differentiate between epitopes of NAbs and epitopes without neutralizing ability. Here, we apply the IP-Seq-based epitope mapping method to 34 human serum samples positive for anti-AAV2 antibodies and successfully draw a comprehensive map of conformational epitopes of human polyclonal anti-AAV2 antibodies, enabling the rational design of stealth AAV2 capsids by targeted mutagenesis. We also report here PK-Seq (Pharmacokinetic-Seq), a novel NGS-based approach that overcomes the limitations of IP-Seq, and demonstrate its successful application to the identification of neutralizing epitopes of human polyclonal anti-AAV2 NAbs. For IP-Seq epitope mapping, we created two DNA-barcoded AAV libraries containing AAV9 capsid mutants with hexapeptide (HP) and/or dodecapeptide (DP) replacements from the AAV2 capsid. The HPs were designed to scan the entire region of the AAV2 capsid protein (153 HP mutants) while the DPs (19 DP mutants) were created to target the common epitopes that had been identified by a preliminary IP-Seq experiment. The principle of IP-Seq is to immunoprecipitate AAV9HP or AAV9DP mutants that contain anti-AAV2 antibody epitope-derived peptides using human immunoglobulins as bait, followed by identification of the mutants using AAV Barcode-Seq. The IP-Seq analysis of human serum samples revealed that 9 epitopes are shared with at least two out of the 34 individuals, and among the 9 shared epitopes, 4 epitopes (Ep1, 2, 3 and 9) are identified in many individuals. For PK-Seq, we reacted the AAV9HP or DP libraries ex vivo with individual human sera, then injected this mixture into mice. The in vivo blood clearance rate of each HP or DP mutant was determined by AAV Barcode-Seq, where the clearance rate serves as a measure of each epitope's neutralizing ability. The PK-Seq analysis of 6 human samples (3 positive and 3 negative for anti-AAV2 antibodies) confirmed that at least 5 out of the 9 shared epitopes are neutralizing epitopes. To demonstrate the stealth vector application of our epitope-mapping data, we randomly mutagenized the most commonly identified epitopes, Ep1, 2 and 3, and performed a preliminary directed evolution using HEK293 cells under a pressure given by IVIG. One of the selected mutants, AAV2Ep123mt1, has shown 3 to 10-fold greater NAb-escaping ability than the wild-type AAV2. In summary, we demonstrate that IP-Seq combined with PK-Seq is an effective non-traditional method to identify conformational epitopes of polyclonal anti-AAV NAbs and allows the rational design of stealth vectors. This method involves only standard molecular biology techniques and can be adopted by a broad range of labs and utilized in theory for any serotypes and variants besides AAV2.

394. Evaluation of Tropism and Transduction Rfficiency of AAV Variants in the CNS of NHP Using DNA/RNA Barcode-Seq Technology

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Gene therapy with adeno-associated viral (AAV) vectors has shown great potential in recent years for use in the treatment of neurological disorders. Direct delivery of transgenes with AAV vectors into the brain has resulted in safe and long-term expression with therapeutic benefit; however, limited biodistribution has restricted the broad application of AAV vectors in central nervous system (CNS) diseases. Although there has been tremendous effort to achieve widespread gene delivery throughout the CNS after systemic administration, this is still a major challenge in large mammals, due to the limited ability of naturally occurring AAV serotypes to cross the blood-brain barrier (BBB). Since the brain contains a vast blood vessel network with a high density of capillary vessels, delivery of AAV vectors via intravenous (IV) dosing is a promising route of administration for broad CNS coverage, if efficient crossing of the BBB can be achieved. To identify capsids that have improved ability to cross the BBB, we characterized a library of selected novel AAV variants using a DNA/RNA barcode-seq approach in mice. The data revealed that there were many AAV variants showing greater than 100-fold increases in CNS gene delivery relative to their parental capsid in terms of biodistribution manifested by vector genome DNA levels and transduction efficiency by RNA transcript levels, while their peripheral tissue biodistributions were similar to or lower than the parental capsid.* These results suggested that AAV variants can be engineered to effectively cross the BBB to achieve broad biodistribution in the brain after IV dosing and identified, using this approach. To identify AAV variants with enhanced ability to cross the BBB with improved CNS tropism and transduction in a large mammal, we next screened the same AAV variant library in NHP following systemic administration. We found that a subset of capsid variants exhibited enhanced biodistribution and increased transduction in specific areas of the NHP brain. As observed in mice, the peripheral tissue distribution of these variants was comparable to or lower than the parental capsid. Taken together, our results indicate that the ability of an AAV variant to cross the BBB can differ

dramatically from one species to another. Ideally, evaluation of the BBB penetrating ability of AAV capsids should be done in multiple large mammalian species to augment the translatability to human for application to neurodegenerative diseases and neurological disorders. Our work shows that AAV capsids with improved ability to cross the BBB in large mammals can be identified using appropriate approaches.

*Please also see presentation authored by Giri Murlidharan *et al.*, Voyager Therapeutics, Cambridge, MA 02139

Gene Replacement for Neurologic Diseases

222. AXO-Lenti-PD: A Second-Generation Lentiviral Gene Therapy for the Treatment of Parkinson's Disease

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Parkinson's disease (PD) is caused by the progressive degeneration of dopaminergic neurons. The primary standard of care for PD is oral dopaminergic-based therapies; these are highly efficacious but their long-term use is complicated by motor fluctuations from intermittent stimulation of dopamine receptors and off-target effects. Therefore, a therapy that provides a localized and continuous supply of dopamine to the area of the putamen offers the potential for reduced motor fluctuations and off-target effects for these patients. AXO-Lenti-PD is a novel gene therapy product that utilizes a lentiviral vector to transfer three genes that are critical for *de novo* dopamine biosynthesis from endogenous tyrosine in the striatum, the area of the brain that is depleted of dopamine in PD. The first-generation construct of this product (known as ProSavin®) has been investigated in a 15-patient open-label dose-ranging study with a subsequent long-term follow-up study. ProSavin® was found to be well-tolerated throughout administration and long-term follow up and while the study was not designed to demonstrate efficacy, all patients displayed numerical improvement in the UPDRS part III OFF score over baseline at both 6 and 12 months, which were sustained in some patients up to six years. While these results were encouraging, the size of the observed effect was deemed clinically insufficient to justify continued treatment in more patients. Therefore, to further increase potency, the second-generation product AXO-Lenti-PD, was developed utilizing the same genes as the first-generation construct but with 5-10-fold increased dopamine production per genetically modified cell. AXO-Lenti-PD has been tested in primary human neurons and non-human primates, which confirm both increased dopamine production and related symptom improvement in the MPTP NHP model. AXO-Lenti-PD is being further investigated in a two-part clinical study: a dose-ranging study to confirm the therapeutic dose (building off data from the study of the first-generation product), followed by a sham control study to establish the efficacy of the confirmed dose. The study commenced in October 2018, and to date two patients have been dosed in the lowest of three escalating dose cohorts. An update will be presented on data from the first patients in the dose-ranging part of the study.

396. Efficacy and Safety in Mice and Non-Human Primates of CSF Delivered AVXS-201 for the Treatment of Rett Syndrome

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Rett syndrome is an X-linked, progressive neurodevelopmental disorder affecting approximately 1 in 10,000 girls. Patients typically experience a loss of achieved developmental milestones beginning at 6-18 months of age followed by progressive loss of motor function. By adulthood, patients require 24/7 care and typically die in middle age. 90% of cases of Rett syndrome are caused by loss of function mutations in the gene encoding MeCP2. Published work shows that Rett syndrome is reversible in mice via re-expression of MeCP2 in the central nervous system by transgene or gene therapy. For clinical translation, we developed AVXS-201 to provide sustained, physiological expression of MECP2. Dose ranging efficacy studies in Mecp2 deficient male mice show amelioration of behavioral phenotypes and up to a >200% increase in median survival. Scale up of therapeutic doses to non-human primates (NHP) show that AVXS-201 is safe and well tolerated with no aberrant behavior, clinical or anatomical pathology. Further AVXS-201 maintains mRNA expression in the NHP CNS through 18 months post injection with protein expression at physiological levels. Dose escalation studies in NHP show dose correlation between mouse and NHP delivery into the cerebrospinal fluid via digital droplet PCR mediated detection of vector genomes. Finally, toxicity studies in mice show AVXS-201 is safe and well tolerated. Together these data show that AVXS-201 is suitable for first in human studies.

397. CNS-Directed AAV9 Gene Therapy for the Treatment of Canine Globoid Cell Leukodystrophy (Krabbe Disease)

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Globoid cell leukodystrophy (GLD, Krabbe disease) is caused by a deficiency in the hydrolytic enzyme galactosylceramidase (GALC), which degrades the myelin lipids galactosylceramides and galactosylsphingosine (psychosine). Toxic accumulation of cytotoxic psychosine results in widespread central and peripheral nervous system (CNS, PNS) demyelination. Neurologic dysfunction in patients is apparent in the first year of life and death often occurs by two years of age. Notably, GLD is a naturally occurring hereditary disease in dogs and disease progression closely recapitulates clinical, pathological, and biochemical abnormalities of human disease. The predictable disease progression and lifespan (15.7 ± 4.8 weeks of age) allow for timely identification of pathological changes and evaluation of therapeutic interventions. Gene therapy has been evaluated in GLD dogs using AAV9 encoding canine *GALC* (AAV9-cGALC). Ten 2 week-old GLD dogs received AAV9-cGALC by intrathecal delivery (IT) at the cerebellomedullary cistern at a dose of 1E14 vector genomes (vg). Four treated dogs were euthanized at 16 weeks of age (short-term), comparable to the endpoint of untreated GLD dogs, while 6 dogs are being followed long-term. Additionally, 4 2-week-old GLD dogs were treated with a reduced dose, 2E13 vg, to evaluate dose response, 4 GLD dogs received delayed treatment of AAV9-cGALC at 1E14 vg at 6 weeks of age, the age of onset of clinical signs, to evaluate the timing of intervention, and 4 GLD dogs received both delayed administration (6 weeks) and reduced dose (2E13 vg). GLD dogs in the short-term IT group had no clinical evidence of disease, normal nerve conduction velocities, and no indication of brain atrophy on MRI, although there were localized areas of white matter hyperintensity found on MRI. Significant increase in GALC enzyme activity and significant reduction of psychosine levels were seen in the CNS and PNS. Histological analysis demonstrated improved myelination, reduction in storage material, and attenuation of neuroinflammation. At time of abstract submission, high dose IT treatment has significantly extended lifespan of GLD dogs ($p < 0.000001$), which are currently beyond 65 weeks of age and have no clinical evidence of disease, improved nerve conduction velocity, stabilization of MRI comparable to scans at 16 weeks of age, and significantly reduced CSF psychosine levels. GLD dogs treated with reduced dose, 2E13 vg, reached humane endpoint at approximately 30 weeks of age, demonstrating a clear dose response. IT delivery of AAV9-cGALC is having substantial therapeutic effect in canine GLD; however, narrow dosing and timing windows have been identified and should guide translation of this promising therapy in to children affected with Krabbe.

398. Intrathecal Administration of AAV9-SOD1 for Amyotrophic Lateral Sclerosis: Survival Extension and SOD1 Reduction in Mice and Nonhuman Primates

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ALS is a neurodegenerative disease affecting motor neurons, leading to death within 3-5 years of diagnosis. There is no known cure. Twenty percent of genetically-associated ALS cases are linked to mutations in the superoxide dismutase-1 (SOD1) gene. Previously, AAV9-mediated delivery of a small hairpin RNA (shRNA) targeting human SOD1 resulted in efficient downregulation in mice and non-human primates (NHPs). In ALS mice we achieved significant extension in survival with a single administration of an experimental recombinant vector, AAV9-GFP-shRNA-SOD1, in newborn animals and also at later stages after disease onset. These highly promising results set the stage for developing this approach further towards translation to clinical trials. To move forward to clinical trials, we modified our experimental vector using an expression cassette for the delivery of shRNA against human SOD1 devoid of any foreign transgenes (GFP) but maintaining the same cassette size. This was efficiently packaged into an AAV9 viral capsid, creating a new AAV9-SOD1-shRNA ("AAV9-SOD1" herein) translatable to the clinic. Intracerebroventricular delivery (ICV) of AAV9-SOD1 directly into the cerebrospinal fluid (CSF) was performed in the $SOD1^{G93A}$ ALS mouse model and intrathecal lumbar delivery in NHPs was performed to show significant survival extension and reduction of SOD1 throughout the CNS. We found that a one-time ICV administration of AAV9-SOD1 lead to significantly improved motor function and prolonged survival in mice overexpressing human mutated SOD1 using three independent lots of AAV9-SOD1 material. Analysis of CNS tissue following ICV injection revealed a significant, dose-dependent knockdown of SOD1 at both the RNA transcript and protein levels in the brain and throughout the spinal cord, indicating that the CNS was successfully targeted. We also tested intrathecal administration of AAV9-SOD1 in 3-4 year-old cynomolgus macaques, a larger animal model, at several doses. With a single lumbar intrathecal administration of AAV9-SOD1 engineering lot material, we achieved efficient transduction and SOD1 downregulation throughout the entire CNS and showed a direct relationship between dose and identified vector genomes in CNS tissue. Furthermore, we observed up to a striking 93% reduction in SOD1 RNA transcript in spinal motor neurons. Importantly, administration of AAV9-SOD1 was safe and well tolerated in both mice and NHPs. Together, these results represent an important advancement towards clinical trials for ALS patients.

399. Review of Safety and Interim Analysis of Efficacy of a First-in-Human Intrathecal Gene Transfer Study 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. 11 GAN patients have been dosed thus far at three dose levels (ranging from $3.5x10^{13}$vg via $1.2x\ 10^{14}$vg to $1.8x10^{14}$vg) 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), myometry, grip and pinch strength, timed testing, electrophysiology, and neuroimaging. We highlight the overall safety and feasibility of an intrathecal route of AAV9 based gene transfer. The efficacy interim analysis highlights several key aspects: 1) feasibility for adequately targeting the nervous system, 2) characterization of an 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. We show that the disease progression compared to natural history is slowed in a dose dependent manner. The posterior probability that post-transfer change is better than this -8 MFM32 points/year (any slowing of progression) was determined. The decline appeared to be unchanged at the $3.5x10^{13}$vg does, while it was slowed to about -4 in the middle dose of $1.2x\ 10^{14}$vg. Data for the highest dose group of $1.8x10^{14}$vg 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.

400. GDNF Gene Therapy for Advanced Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic (DA) neurons of the substantia nigra (SN) pars compacta and DA denervation of caudate nucleus and putamen. Research with neurotrophic factors provides evidence of their utility in delaying or preventing DA neuronal loss and mitigating or arresting the clinical deficits in animal models of PD. We have shown that MRI-guided convection-enhanced delivery (CED) of the AAV2-GDNF vector within the putamen was able to reverse PD signs in MPTP non-human primates (NHP) after 6 months, with beneficial effects persisting up to 2 years (longest observation period). The delivered AAV2-GDNF utilized anterograde axonal transport from putamen to SN, via direct and indirect striato-nigral projections that are resistant to both MPTP and idiopathic PD. Based on the safety and efficacy shown for AAV2-GDNF vector delivery in multiple NHP studies, we launched a Phase I clinical trial on advanced PD patients at NINDS, to investigate the safety, tolerability, and early clinical efficacy of escalating doses of AAV2-GDNF delivered into the bilateral putamen. Thirteen (13) adult subjects with advanced PD received co-infusions of AAV2-GDNF and the MRI tracer gadoteridol, via CED (450 µL/hemisphere) into the bilateral putamen. Three (3) escalating vector genome (vg) doses were evaluated: $9x10^{10}$vg (n=6); $3x10^{11}$vg (n=6); and $9x10^{11}$vg (n=1). Intraoperative MRI (iMRI) allowed real-time monitoring of the CED. Pre-operatively, and at 6-12-month intervals post-operatively, a Unified Parkinson's Disease Rating Scale (UPDRS) score and positron emission tomography (PET) scanning with [^{18}F]FDOPA provided assessments of motor function and DA function, respectively. Putaminal CED with AAV2-GDNF was well-tolerated in humans, without short- or long-term clinical or radiographic toxicities. iMRI documented the putaminal AAV2-GDNF infusions, with an average 26% ± 10% putaminal coverage attained. Visualization of the co-infused vector within the brain, via gadoteridol, displayed extent of target and off-target coverage, including via perivascular spaces or from cannula backflow. Regardless of the vector dose delivered, UPDRS scores and levodopa equivalent daily dose remained relatively stable throughout the study. Post-operative [^{18}F]FDOPA PET uptake was increased in the volume of distribution bilaterally in 10/13 patients at 6 months (median percentage increase = 39%; and interquartile range [IQR] of 14%-60%) and in 12/13 patients at 18 months (57%; 35%-73%). Differences in PET-derived Ki values from baseline to post-operative timepoints were statistically significant for the bilateral CED sites (repeated measures one-way ANOVA, $p < 0.0002$).Three escalating doses of AAV2-GDNF delivered by CED into the bilateral putamina of humans with advanced PD appeared safe and well-tolerated up to 60 months of maximum post-

operative follow-up. Increased [18F]FDOPA PET uptake and Ki values at the infusion sites suggest a possible neurotrophic effect on residual nigrostriatal DA neurons. These encouraging preliminary results in humans support the transition to a Phase Ib trial that extends similar and higher vector doses in less advanced PD patients (<5 years from clinical diagnosis) and maximizes putaminal coverage by increasing infusion volume and utilizing a novel surgical trajectory for maximizing putaminal coverage.

401. Intravenous Delivery of AAV Gene Therapy in GM1 Gangliosidosis

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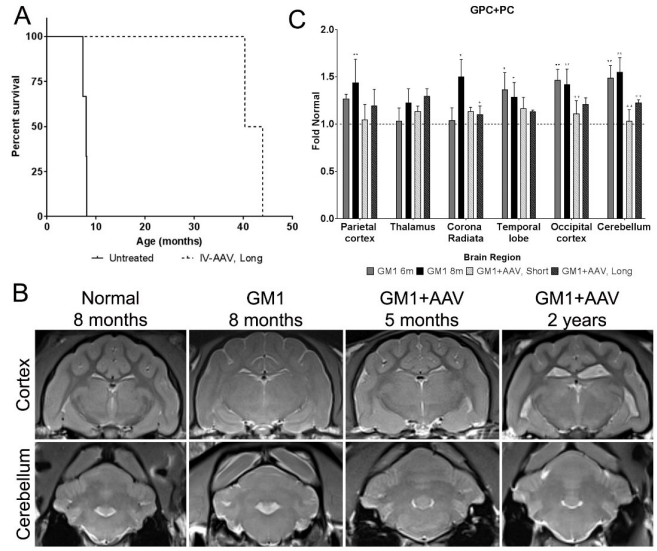
GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (β-gal). Intracranial injection of adeno-associated viral (AAV) therapy resulted in a 7.5-fold increase in lifespan of GM1 cats. In an attempt to bypass the invasiveness of brain injection and increase systemic biodistribution, AAV9 was delivered at 1.5e13 vector genomes/kg body weight via the cephalic vein into six GM1 cats at approximately one month of age. The six animals in the study were divided into two cohorts for short term and long term evaluation. Untreated GM1 animals survived 8.0±0.6 months while IV treatment increased survival to approximately 3.5 years (Fig. 1A). Clinical assessments included neurological exams, cerebrospinal fluid (CSF) and urine biomarkers, and 7T magnetic resonance imaging (MRI) and spectroscopy (MRS). Postmortem analysis included β-gal and virus distribution as well as histological analysis. Neurological abnormalities, which in untreated GM1 animals progress to the inability to stand and debilitating neurological disease by 8 months of age, were mild in treated animals. For example, treated cats had only mild hindlimb muscle atrophy and fine ear tremors, symptoms that occur early in the disease process of untreated cats. CSF biomarkers such as aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were normalized, indicating decreased central nervous system (CNS) cell damage in the treated animals. Urinary glycosaminoglycans, known to be elevated in GM1 patients, decreased to normal levels in the long term cohort. MRI revealed the preservation of brain architecture (Fig. 1B) in AAV-treated cats, and MR spectroscopy revealed correction of glycerophosphocholine and phosphocholine, a marker of demyelination (Fig. 1C, GPC+PC). β-gal activity was increased throughout the CNS, reaching normal levels in the CSF, cerebellum and spinal cord (cervical, thoracic and lumbar regions). Peripheral tissues such as heart, skeletal muscle and sciatic nerve also had normal β-gal activity in treated GM1 cats. GM1 histopathology in the CNS was largely corrected with treatment, with little to no evidence of storage lesions. There was no evidence of tumorigenesis or toxicity. This data supports the promise of IV gene therapy as an alternative for intracranial injection and as a safe, effective treatment for GM1 gangliosidosis.



Oncolytic Viruses II

402. Nectin-Elicited Cytoplasm Transfer: A New Process Extending the Tropism of an Oncolytic Virus

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Viruses are being developed as experimental cancer therapeutics. Several oncolytic viruses currently in advanced clinical trials use cell adhesion molecules as receptors, probably because - to reach tumors and metastases - they must efficiently traverse or break epithelia (*Nat. Rev. Microbiol. 12, 23-34, 2014; J. Cell Sci. 128, 431-439, 2015*). We discovered a process that enables an oncolytic virus (measles virus, MeV) to extend its tropism. This process depends on intercellular contacts established by nectins, adherens junction proteins that also function as viral receptors. We show that *A431D* epithelial cells expressing nectin-1 internalize plasma membrane of *A431D* cells expressing nectin-4, the MeV epithelial receptor. The nectin-1 cytoplasmic tail controls transfer: its deletion prevents it, while its exchange with the nectin-4 cytoplasmic tail reverses transfer direction. We also show that the internalization process depends on the nectin

adhesive interface, and that cytoplasmic proteins are transferred simultaneously with nectin-4, possibly within vesicles. We name this process nectin-elicited cytoplasm transfer (NECT). To assess whether cytoplasmic materials transferred by NECT remain functional, we developed an assay based on the transfer of MeV ribonucleocapsids (RNP). To monitor functional gene expression from RNP, we generated a recombinant MeV expressing nuclear cyan fluorescent protein (nCFP). We then co-cultured nectin-1 expressing *A431D* cells with MeV-infected, nectin-4 expressing *H358* cells, a cell line expressing physiological levels of nectin-4. About 30-35% of nectin-1 expressing cells took up nectin-4, and in about a third of these NECT-receptive cells, viral RNPs expressed the nCFP reporter protein. Thus, NECT supports functional transfer of viral RNP between cells. We then asked whether NECT can extend MeV tropism: nectin-1 is expressed on neurons, which are not infectible by MeV particles because they lack a specific receptor. To assess whether NECT can transfer MeV infections to neurons, and whether transfer depends on nectin-4 expression, we knocked out nectin-4 expression in *H358* cells by CRISPR/Cas9 gene inactivation, generating *H358/N4null* cells. We then used lentiviral transduction to express the lymphatic MeV receptor SLAM, generating *H358/N4null/SLAM+* cells. Infected *H358* cells transferred MeV to primary mouse neurons 10-50 times more efficiently than infected *H358/N4null/SLAM+* cells. *H358/N4null* cells, which do not express a MeV receptor, did not transfer infection. Thus, NECT can extend MeV tropism, accounting for neuronal entry, the first step of brain invasion that can result in rare but always lethal disease subacute sclerosing panencephalitis. NECT-related processes may extend the tropism of other viruses that take advantage of cell junctions and the connected cytoskeletal infrastructure to spread in specific organs. Our discovery has important implications for the study of viral pathogenesis, and for the development and targeting of oncolytic vectors.

403. A Novel Chimeric Poxvirus Encoding an Anti-PD-L1 Protein Shows Safety and Anti-Tumor Activity in Breast Cancer Model

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An effective oncolytic virotherapy must successfully harvest the natural and gene-engineered ability of viruses to selectively target cancer cells while maintaining a good safety profile. While oncolytic poxviruses have shown excellent safety profiles in pre-clinical and clinical studies, there is a need to improve their therapeutic efficacy. We generated a chimeric poxvirus (CF33) through recombination amongst 9 species of orthopoxvirus including multiple strains of vaccinia virus. In a high throughput screen testing activity against the NCI-60 panel, our chimeric virus outperformed all parental viruses. Interestingly, human and murine cancer cells demonstrated increased PD-L1 expression following infection with CF33. To counter the elevated PD-L1 on cancer cells and potentially enhance immune cell-mediated tumor destruction, an anti-PD-L1 expression cassette was inserted into the virus (CF33-antiPD-L1). We confirmed that the virus express functional anti-PD-L1 both *in vitro* and *in vivo* in mouse tumors. Furthermore, we tested anti-tumor potential of the virus in a syngeneic murine tumor model 4T1. Intra-tumoral injection of the backbone virus (CF33) alone was able to significantly increase CD8+ T cells infiltration in tumors compared to PBS control. The ability of the virus to facilitate CD8+ T cells infiltration in tumors was further enhanced by the insertion of anti-PD-L1 expression cassette. The anti-PD-L1-encoding virus was able to significantly delay tumor progression resulting in significantly increased survival of mice.

404. Cargocyte Biofactories: A Novel Platform for Delivering Oncolytic Viruses to Treat Metastatic Cancer

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Metastatic disease remains the most significant life-threatening event for cancer patients. While intravenous administration of oncolytic viruses (OVs) is a promising method to target tumor cells and metastatic cancers, the immune system can rapidly neutralize intravascular circulating virus hindering overall efficacy towards disseminated tumor cells. Therefore, there is a critical need to design new viral carrier systems that can both specifically deliver OVs to metastatic sites and prevent immune recognition until OVs reach the target site. Mesenchymal stromal cells (MSCs) are attractive delivery vehicles because they have innate tumor trophic (homing) properties and can shield the virus from immune recognition. However, MSC homing efficiency needs to be improved for clinical use, and research has shown that MSCs can engraft in tumors and contribute to tumor growth and metastasis. While genetic and bioengineering strategies can overcome many of these limitations, modification of genomic DNA or introduction of new genetic material into therapeutic cells raises substantial safety concerns, ultimately making FDA approval difficult. To address these issues, we developed a novel, safe, cell-based platform whereby genetically engineered MSCs-derived vehicles can home to metastatic tumors and deliver OVs. We genetically engineered hTERT-immortalized MSCs (hTERT-MSCs) to express chemokine receptors CXCR4 and CCR2, which improve cell homing towards gradients of SDF-1α and CCL2 respectively. These chemokines are commonly increased in inflamed stroma and produced by tumor cells. Additionally, the MSCs were engineered to co-express PSGL-1 and α1,3/4-fucosyltransferase to mediate hTERT-MSC adhesion to inflamed endothelial cells in the tumor vasculature. The genetically modified nuclei of these engineered cells were subsequently removed using an optimized enucleation protocol and Ficoll density centrifugation. These enucleated cells (Cargocytes™) have greatly improved safety profiles compared to nucleated MSCs because they do not permanently engraft into the body nor do they transfer any dangerous genomic DNA to the patients. Cargocytes retain many desired biological functions (endogenous and engineered), such as viability up to 72 hours, retention of cell surface markers/proteins, translation and secretion of bioactive molecules, and robust *in vitro* and *in vivo* homing. Cargocyte therapeutics are highly versatile biofactories that can be engineered to produce or to be exogenously loaded with many important biologics including secreted cytokines, RNA-based

therapeutics, peptides, small molecule drugs, and importantly, OVs. When infected with OVs, Cargocytes show dramatically increased OV production due to lack of transcription-dependent host defense mechanisms against viruses. In fact, Cargocytes harboring oncolytic vesicular stomatitis virus (oVSV) robustly propagate, package, and release functional virus capable of infecting and killing tumor cells. In *in vitro* homing assays and in a preclinical mouse model of metastatic breast cancer, Cargocytes harboring oVSV retain chemokine homing potential and their ability to transfer virus to lung metastases. Overall, our findings demonstrate that Cargocyte biofactories are a genetically tractable cell therapy platform that is scalable for clinical use, and has potential to improve systemic delivery of OVs alone or in combination with other biologics for the treatment of metastatic cancer.

405. Viral Derived Innate Immune Antagonists Sensitize Interferon Responsive Cancer Cells to Oncolytic Mengo and Vesicular Stomatitis Viral Vectors

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Oncolytic virotherapy is a cancer treatment strategy that uses replicating viruses to specifically infect and kill tumor cells. Mengovirus and Vesicular Stomatitis virus (VSV) are attractive candidates for oncolytic vectors because they can be evaluated in immune competent animal models, have demonstrated oncolytic activity against diverse cancer types, and have multiple safety mechanisms capable of restricting viral tropism. However, both of these viruses are sensitive to Type I interferon (IFN) mediated antiviral immunity and their therapeutic efficacy is reduced in IFN sensitive tumor models. In this study, we characterized the ability of virally-derived IFN antagonists to sensitize IFN responsive cancer cells to Mengovirus and VSV vectors. We demonstrated the impact of IFN responsiveness on viral susceptibly using the CT-26LacZ (IFN-resistant) cell line and its parent line CT-26WT(IFN-sensitive). Interferon pretreatment (100 U/ml mIFNα_2) delayed viral cytotoxicity and lowered viral titers by a factor of 10^5 and 10^3 for VSV and Mengovirus, respectively, in the CT-26WT cells. In contrast, IFN did not impact infection of CT-26LacZ cells. Combination of Mengovirus or VSV with vaccinia virus, known to encode multiple IFN antagonists, prevented IFN induction, enhanced cytotoxicity, and completely restored titers of both Mengovirus and VSV in IFN treated CT-26WT cells. To expand upon these results, we sought to determine if a single gene encoding a virally derived IFN antagonist could restore susceptibility to the virus and then develop a system to safely deliver the gene *in vivo.* To this end, we generated a panel of Mengovirus- or VSV-based replicons encoding 8 different viral genes known to disrupt IFN signaling. These replicon-based systems allow for expression of potent IFN antagonists while maintaining an acceptable safety profile as recombination to generate an 'armed' replication competent virus is highly improbable. We determined that a Mengovirus-based replicon expressing measles virus P or V protein was able to reduce induction of IFNβ by 100 fold and eliminated IFNα production in CT-26WT cells infected with Mengovirus. Our results demonstrate that type I IFN induced antiviral immunity in CT-26WT cells can be suppressed by heterogeneous virally derived IFN antagonists to overcome resistance to VSV and Mengovirus infection *in vitro.*

406. Novel Approach for Treatment of Pediatric High-Grade Gliomas through the Combination of Oncolytic Adenoviruses and Gene Therapy Encoding a BiTE Directed to the EphA2 Tumor Antigen

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Pediatric high-grade gliomas (pHGG) are amongst the most common malignant neoplasms of childhood, whose outcome remain dismal with conventional treatments. Thus, new therapeutic approaches are urgently needed. Immunotherapy based on Oncolytic Adenovirus (OA) is a promising strategy but its efficacy is suboptimal. We aimed at improving the antitumor efficacy by combining the OA and a gene-therapy with the Bispecific T-cell Engager (BiTE) directed towards the erythropoietin-producing human hepatocellular carcinoma A2-receptor (EphA2), conveyed by a replication-incompetent adenoviral vector (EAd). We demonstrated, by immunohistochemistry and qPCR, the expression of EphA2 in 100% (16/16) of pHGG samples analyzed, its intensity correlating significantly with a worse outcome. We then tested the transgene amplification, after co-infection, on two HGG cell lines (U373, U87) by qPCR and Flow Cytometry (FACS) for the selectable marker ΔCD19, confirming a significantly enhanced production in OA+EAd vs EAd alone ($p<0.01$ and $p<0.001$, respectively). Notably, the FACS analysis of the infected tumor cells after 5 days of co-culture with T-cells showed a significantly increased apoptosis with the engagement of T cells (U373: 82,13%$\pm$5,02%; U87: 35,53%$\pm$5%), as compared to the OA alone (U373:70,28$\pm$3,53%; U87: 3,78%$\pm$1%). To obtain the tumor eradication, we then fully activated T cells by including an anti-CD28 antibody (αCD28), obtaining a further apoptosis enhancement (U373:85,78%$\pm$8,14%; U87:54,7%$\pm$18%). We then characterized the T-cell populations by FACS analysis showing a significant increase of activation markers (Fig. 1); the addition of αCD28 was able to induce a significantly higher production of interferon-γ in EAd or OA+EAd conditions. Finally, we established an orthotopic HGG mouse model and administered OA/Ead/OA+EAd and T cells intracranially. Preliminary data show that the treatment is well tolerated and that the EphA2-BiTE improves tumor control compared to the control groups (Figure 2). In conclusion, the combinatorial approach is able to amplify the production of the BiTE and to determine a significant,

tumor-redirected activation of T cells, resulting in an effective tumor control, further amplified in the presence of αCD28, representing a promising innovative treatment.

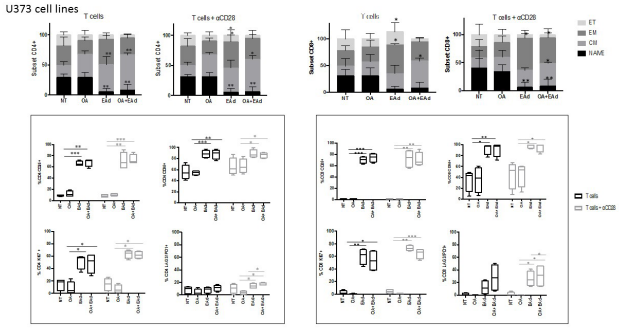

Figure 2. FACS analysis of the T-cell populations

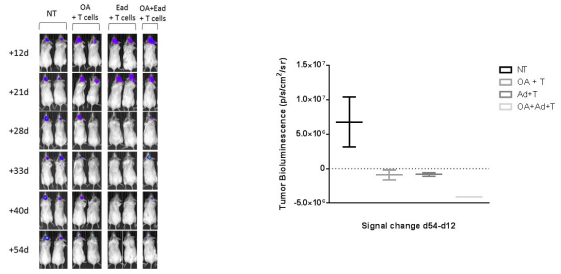

Figure 3. Orthotopic HGG mouse model

407. Combination Treatment of VSV-mIFN-NIS with Ruxolitinib and PDL1 in 5TGM1 Mouse Model

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Vesicular stomatitis virus encoding the IFNβ and NIS transgenes (VSV-IFNβ-NIS) shows potent oncolytic activity in preclinical animal models and the virus is now in Phase I clinical testing after intravenous administration in hematological malignancies. As reported, VSV-IFNβ-NIS exhibits multiple antitumor functions; direct cell killing and stimulation of antitumor immune response and could synergize with immune checkpoint inhibitors to increase the anti-tumor immune response. 5TGM1-C57BL/Ka mouse model is a good multiple myeloma model that is responsive to VSV oncolytic virotherapy: there is VSV dose dependent tumor response and long term antitumor control in cured mice. Using this model, we explored the immune parameters associated with combining VSV-IFN-NIS therapy with immune modulators such as ruxolitinib and immune checkpoint antibodies. Early data showed that addition of ruxolitinib (IFN pathway inhibitor) made tumor cells more compatible to VSV infection in vitro, increasing virus infectivity and titers. Importantly, using IFNβ as a marker, we showed that levels of virally encoded IFNβ were increased in combination of VSV with ruxolitinib and or anti-PDL1, suggesting an increase levels and duration of intratumoral viral replication. Addition of anti-PDL1 antibody to the VSV therapy showed a significant decrease in peripheral blood CD8 T cells, potentially due to increase intratumoral infiltration at these time points. Overall survival and tumor growth rate of mice treated with the combination drugs showed a favorable trend but was not significant. We are currently evaluating additional immune modulating protocols to determine the impact of timing and other drug combination protocols to increase the potency of VSV virotherapy in myeloma.

408. MicroRNA-Detargeted CVA21 Infectious RNA; Delivery of an Oncolytic Virus as Infectious Nucleic Acid

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Although objective responses have been observed in phase I and II clinical trials investigating CVA21 as an oncolytic virotherapy, its monotherapeutic efficacy needs improvement. Oncolytic CVA21 is currently under investigation for combination with checkpoint inhibitor immunotherapies against various cancer types. However, with drug combinations comes inflated costs and the potential for increased toxicity. Although rare in humans, CVA21 can cause myositis (muscle inflammation and paralysis), a toxicity that is more prevalent in immunodeficient hosts. In order to circumvent these pitfalls, we have developed a microRNA-detargeted CVA21 (R-CVA21) with oncolytic activity equivalent to CVA21 virus treatment when delivered as infectious RNA (iRNA) to SCID mice bearing human melanoma xenografts. This formulation of CVA21 is less expensive and easier to manufacture than virus preps; and has significantly improved safety compared to unmodified CVA21. The ribosomal scanning region within the 5' untranslated region (UTR) of the viral genome was replaced with a muscle-specific microRNA response element. Replacement of this region improved the stability of the response element *in vivo*, likely due to the elimination of selective pressures exerted by the limited carrying capacity of picornaviruses. This configuration of the microRNA response element also ameliorated the reduction in iRNA specific infectivity that is endemic to insertion of response elements within the 3' UTR. Furthermore, the specific infectivity of R-CVA21 iRNA could be enhanced by generating *in vitro*-derived transcripts with authentic termini. We are currently scaling our production protocol and evaluating the effects of the immune system on R-CVA21 iRNA versus virus formulations in tumor-bearing animals in the presence and absence of various immunotherapies. Development of this CVA21 iRNA-based virotherapy with enhanced safety and a reduced financial footprint has the potential to significantly improve the efficacy of CVA21 and expand patient populations eligible for treatment.

Adenovirus Vectors and Other DNA Virus Vectors

409. SB100x-Transposase Mediates Integration of a 32.4kb Transposon and High-Level Gamma-Globin Expression after In Vivo HSC Transduction in Mice

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We developed a minimally invasive and readily translatable approach for *in vivo* HSC gene delivery without leukapheresis, myeloablation, and HSC transplantation. The central idea of our 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 HDAd5/35++ gene transfer vector system. The novel features of HDAd5/35++ vector system include: *i)* CD46-affinity enhanced fibers that allow for efficient transduction of primitive HSCs while avoiding infection of non-hematopoietic tissues after i.v. injection, *ii)* a SB100X transposase-based integration system that functions independently of cellular factors and mediates random transgene integration without a preference for genes with one to two integrated vector copies per cell, *iii)* a MGMT(P140K) expression cassette mediating selective survival and expansion of progeny cells without affecting the pool of transduced primitive HSCs by short term treatment with low-dose O^6BG/BCNU. We have recently shown in a mouse model of thalassemia intermedia that the combined *in vivo* transduction/selection approach was safe and resulted in human gamma-globin expression in >60% of peripheral blood erythrocytes, resulting in near complete correction of the thalassemic phenotype. In this study the gamma-globin gene was under the control of a 5kb mini-beta globin LCR containing the cores of hypersensitivity regions HS1 to HS4. We found that the level of gamma-globin expression was only 10-15% of adult mouse alpha globin, which would not be sufficient for a cure of hemoglobinopathies. A large fraction of (randomly) integrated gamma-globin cassettes was silenced and background expression in non-erythroid cells was observed. To address these problems, we employed a 26.1kb version of the beta-globin LCR version to drive gamma-globin expression in the context of an HDAd5/35++ vectors. In vivo HSC transduction/selection in CD46 transgenic mice resulted in stable gamma globin expression in >70% of RBCs, indicating that the SB100x transposase is capable of integrating a 32.4kb transposon. Preliminary HPLC data on RBCs show significantly higher human gamma globin levels (and correspondingly reduced mouse beta globin levels) compared to the 5kb mini LCR. We are currently testing whether the long LCR protects from silencing and whether it mediates more stringent erythroid specificity. With these data in place, we will evaluate the efficacy and safety of this vector in mouse models of thalassemia and sickle cell disease.

410. Single Adenoviral Vectors Armed with HPV Oncogene Specific CRISPR/Cas9 as Efficient Tumor Gene Therapy Tools for the Treatment of HPV Related Cancers

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Human Papillomaviruses (HPV) cause malignant epithelial cancers by expressing viral oncoproteins. Oncogene disruption by CRISPR/Cas9 has been shown to reverse oncogenic pathways and drive cancer cells into apoptosis. To translate these findings towards *in vivo* applications we constructed gene deleted, high capacity adenoviral vectors (HCAdVs) armed with all components of the CRISPR/cas9 machinery specific for the major high risk HPV types HPV16 and HPV18 E6-oncogenes within one single vector. Cervical cancer cell lines Siha, Hela and Caski containing integrated HPV16 or HPV18 genomes and HPV negative A549 lung cancer cells were transduced with HPV type specific CRISPR-HCAdVs. Adenoviral delivery of HPV type specific CRISPR/Cas9 resulted in cell death in HPV positive cervical cancer cell lines whereas HPV negative A549 cells were unaffected. Compared to untreated or HPV negative control cells transduced cervical cancer cells showed and decreased proliferation and viability and increased apoptosis induction and p53 reaccumulation. This suggests that HCAdVs armed with HPV E6 specific CRISPR/Cas9 can serve as potent HPV specific cancer gene therapeutic agents. As HCAdV based on the Adenovirus (AdV) serotype 5 capsid have limitations for in vivo applications we explored a library of 21 different reporter gene expressing AdV serotypes for their ability to specifically transduce HPV positive cervical and head and neck cancer cell lines by assessing reporter gene expression following AdV transduction. The results demonstrate that AdV14 outcompetes the commonly used AdV5 in its ability to transduce these cells. AdV3 showed comparable results as AdV5 whereas other serotypes did not enter efficiently. AdV14 and 3 are promising candidates for the conversion into efficient and specific vectors for treatment of HPV related epithelial tumors. We hope that the versatility of the CRISPR/Cas9 system combined with efficient and tissue specific adenoviral delivery will contribute to establish complementary personalized treatments for each individual HPV related tumor.

411. Serotype-Specific Binding of Prothrombin to Adenovirus Vectors

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Certain serotypes of human adenovirus (Ad) have the ability to bind coagulation factors VII, IX and X (FVII, FIX and FX). It has been shown that hexon trimers form a cavity that can bind with high affinity to a common domain on these coagulation factors: the gamma-carboxyglutamic acid (Gla) domain. FX, in particular, has been intensively studied due to its profound effects on gene therapy with Ad5 vectors. FX enhances Ad5 transduction of cells in vitro, FX enhances Ad5 liver transduction in vivo, and FX shields Ad5 vectors

from neutralization by natural antibodies and complement both in vitro and in vivo. The most abundant Gla-domain-containing protein is FII (prothrombin), which has a plasma concentration that is 10-fold higher than the concentration of FX. Early studies found that FII has no impact on transduction of cells by Ad5 vector, and therefore the potential of FII to interact with Ad viruses has not been further investigated. In the current report, we examine the ability of FII to interact with Ad viruses and with vectors derived from Ad1, Ad2, Ad5, Ad6 and Ad57 (all members of human Ad species C). Although we find that Ad5 is able to bind FII, the affinity for FII is substantially weaker than the affinity of Ad5 for FX. In contrast, we find that FII binds strongly (low-nanomolar affinity) to vectors based on Ad1, Ad2, Ad6 and Ad57. Interestingly, the affinity of these non-Ad5 serotypes for FII is substantially higher than the affinity of these serotypes for FX. Furthermore, we show using hexon-chimeric and hexon-mutant vectors that hexon is the Ad protein that binds FII, and we identify point mutations in hexon that impair binding of both FX and FII, indicating that these coagulation factors bind to the same region of hexon. Thus, although FX is the major coagulation factor that binds to Ad5, other serotypes preferentially bind FII. Because FII is the most abundant Gla-domain-containing coagulation factor in plasma, the potential impact of FII needs to be considered when evaluating gene therapy with non-Ad5 vectors.

412. CRISPR-cas9 Gene Editing for Cystic Fibrosis

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The CRISPR-cas9 system is a versatile tool that have been applied frequently for gene editing; it is also an attractive strategy for gene therapy since it allows permanent correction at the target locus. Despite the apparently advantages of CRISPR-Cas9 technology, one major obstacle remains, which is the potential immunogenic response caused by cas9 protein to the recipient. Cas9 was originally adopted from bacterial system and thus may cause antigenic response leading to the elimination of edited cells by the host. To overcome this, we engineered a Helper-dependent Adenoviral vector (HD-Ad) delivery system packaging both CRISPR-cas9 as well as the donor genes. This HD-Ad system allows transient expression of Cas9, which only creating sufficient time for gene editing, but not permitting immunogenic response elicited by the host since the vector genome is compromised shortly after delivery. In addition, the HD-Ad vectors are capable of delivering gene constructs to the airway basal stem cells *in vivo*. Using this delivery system, we demonstrated successful delivery and precise integration of LacZ gene and CFTR gene to the human AAVSI locus in cultured cells. We also showed elimination of Cas9 protein and vector genome following delivery. Furthermore, we achieved sustained CFTR expression in corrected cells and demonstrated substantial functional correction. Taking things together, our findings showed feasibility of permanent gene integration and correction *in vitro* with limited toxicity caused by Cas9.

413. Biodistribution Analysis of Transgene Expression from a Non-Cytotoxic Herpes Simplex Virus Based Vector

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Replication-defective herpes simplex virus (HSV) vectors have great potential as transfer vehicles for large or multiple transgenes for basic research and gene therapy applications. We recently engineered a new generation of replication-defective HSV vectors for efficient and safe gene transfer to both neural and non-neural cells. Our vector, termed JΔNI5, is functionally deleted for all immediate-early (IE) gene expression to exclude vector-mediated cytotoxicity and its genome is transcriptionally largely quiescent. However, transgene expression cassettes placed between anti-silencing genetic elements in the latency associated transcript (LAT) locus provide robust and long-term transgene expression from the otherwise silent vector genome *in vitro*. Here, we explored the functionality and safety of this non-toxic HSV vector system *in vivo*. JΔNI5 vectors harboring a luciferase expression cassette in the LAT locus (LAT vector) or in the opposite terminal repeat (TR) region (TR vector) were engineered and administered subcutaneously or intraperitoneally into neonatal mice. The LAT vector showed robust transgene expression, whereas almost no expression was observed from the TR vector. When the LAT vector was administered subcutaneously, transgene expression was largely restricted to the injection site, whereas intraperitoneal administration resulted in transgene expression at both the injection site and in the spinal cord, indicating that the distribution of transgene expression depends on the route of administration. Our results suggest that the LAT locus is transcriptionally active in non-neural cells not only *in vitro*, but also *in vivo*, and that this activity is tissue-specific. These findings will be useful in the development of non-toxic HSV-mediated gene therapies. Currently we are further studying the property of non-toxic HSV and improving its transduction efficacy *in vivo*.

414. Enhanced Tropism of Species B1 Adenoviral-Based Vectors for Primary Human Airway Epithelial Cells

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Adenoviruses are efficient vehicles for transducing airway epithelial cells. Human adenoviruses (Ad) are classified into seven species termed A-G. Most species use the coxsackie-adenovirus receptor (CAR) as a primary cellular receptor. Ad group B is notable because it is further divided into groups B1 and B2 and its members use CD46 or desmoglein 2 (DSG2) as cellular receptors. To date, Ad type

2 (HAdV-2) and Ad type 5 (HAdV-5) have been the predominate choices for preclinical and clinical trials using Ad-based viral vectors in the airways. Both HAdV-2 and HAdV-5 belong to species C and use CAR as a receptor. In this study, we screened 14 Ad types representing species C, B1, B2, D, and E. Using well-differentiated primary cultures of human airway epithelial cells (HAE), we examined transduction efficiency at the apical and basolateral surface. Based on GFP or nanoluciferase expression, at least 6 types transduced HAE as well as or better than HAdV-5. HAdV-3, HAdV-21, and HAdV-14 belong to species B, reportedly use DSG2 as a cellular receptor, and had notable transduction properties. We further examined transduction properties of conditionally reprogrammed airway basal cells and primary basal cells from human lung donors. Again, the transduction efficiency of species B members outperformed the other types. These data suggest that adenoviral vectors based on species B may have the enhanced ability to transduce both fully differentiated columnar epithelial cells and progenitor cells in the human airways.

415. AdMG-MYOGp(S)-mMEF2-5/3F, Myogenin Promoter-Controlled Oncolytic Adenovirus, Selectively Kills PAX3-FOXO1$^+$Rhabdomyosarcoma Cells

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Background: Rhabdomyosarcoma (RMS) is a malignant skeletal muscle tumor that occurs mainly in children and adolescents. The specific cytogenetic abnormality of about 80% of patients with alveolar RMS (ARMS) is *PAX3-FOXO1* fusion-gene, and the prognosis of PAX3-FOXO1$^+$ ARMS is much worse than that of the others. This implies that PAX3-FOXO1 irrespective of histology is a critical factor in risk stratification of RMS. Novel therapeutics for the treatment of PAX3-FOXO1$^+$ ARMS are desperately needed. Oncolytic adenoviruses (OAds) selectively replicate in the cancer cells and provide anatomical distraction of solid tumor and immunological stimulation in the tumor locale. One of the way to realize cancer specificity of OAds is controlling the expression of adenovirus E1 gene by promoters which is active only in the target tumor. In general, tumor-specific promoters controlled OAds are safe and effective when the promoter activity shows clear contrast between target tumor and background normal tissue. Myogenic regulatory factors, MYOD1 and myogenin (MYOG), play important roles for the process of skeletal muscle differentiation. Both MYOD1 and PAX3-FOXO1 are upstream regulators of MYOG. Interestingly, when the MEF2 binding site in MYOG promoter (MYOGp) was mutated (mMEF2), MYOD transactivation was abolished, but PAX3-FOXO1 transactivation was not affected. This means MYOGp-controlled OAd with mMEF2 can be activated in only PAX3-FOXO1$^+$ RMS cells, theoretically. **Methods:** Two RMS cell lines, RH30 (PAX3-FOXO1$^+$) and RD (PAX3-FOXO1$^-$), and normal skeletal muscle cell (SkMC) were used the following experiment. Three different of length of MYOGp were cloned, and then pShuttle-MYOGp-GL3B, pGL3B-MYOGp, and AdEasy-MYOGp-GL3B-5/3F were established, respectively. Using these plasmids and viruses, we evaluated which length of MYOGp was the most powerful and specific for PAX3-FOXO1$^+$ cells. Subsequently, to compare their replication and killing abilities in vitro and vivo, we also made AdMG-MYOGp-5/3F viruses, fully competent OAds. **Result:** Luciferase assay revealed that the shortest MYOGp (MYOGp(S)) regardless of MEF2 mutation showed highest activity in Rh30 and 293-PAX3-FOXO1$^+$ 293. Next, in luciferase assay using AdEasy-MYOGp-GL3B-5/3F, MYOGp(S)-controlled OAds showed higher activity in not RD but Rh30, and the difference between mMEF2$^+$ and $^-$ was significant. Based on these results, we made AdMG-MYOGp(S)-5/3F and evaluated their characters. We next compared viral replication and cytolytic effect of MYOGp-controlled OAd in PAX3-FOXO1$^+$ and $^-$ RMS cells. AdMG-MYOGp(S)-mMEF2-5/3F replicated in Rh30 as well as AdMG-MYOGp(S)-5/3F. However, in RD, there were significant difference in virus replication between AdMG-MYOGp(S)-5/3F with and without mMEF2. Additionally, both MYOGp-controlled OAds could kill Rh30 even under low titer condition, while AdMG-MYOGp(S)-mMEF2-5/3F showed one order of magnitude lower cytolysis in RD compared to AdMG-MYOGp(S)-5/3F without mMEF2. Neither of MYOGp-controlled OAds replicated in and killed SkMC at all. These data indicated that AdMG-MYOGp(S)-mMEF2-5/3F more selectively replicated in and killed PAX3-FOXO1$^+$ RMS cell *in vitro*. *In vivo* study, both AdMG-MYOGp(S)-5/3F viruses strongly suppressed tumor growth derived from Rh30 regardless of mMEF2. Moreover, qPCR and immunostaining revealed that AdMG-MYOGp(S)-5/3F spread intratumorally as much as multi-targeted positive control OAd. **Conclusion:** Our findings demonstrated the applicability of MYOGp for the design of oncolytic adenovirus for RMS. With mutation in MEF2 binding site, MYOGp-controlled OAd might be more useful to enhance its specificity and safety.

Nonclinical Studies and Assay Development

416. Preclinical Validation of ^{64}Copper as a Translational Tool for Evaluating the Pharmacodynamics of VTX-801 Gene Therapy in Wilson's Disease

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Wilson's disease (WD) is an autosomal recessive metabolic defect of hepatocyte copper excretion into the bile, caused by absent or reduced ATP7B copper transporter function. The ATP7B transporter has a dual role: it transports copper into the trans-Golgi compartment for incorporation into the plasma protein ceruloplasmin and into the bile for excretion of excess copper stores. Recently, we have demonstrated that the administration of an adeno associated vector (AAV) encoding

a mini version of the human *ATP7B* cDNA (AAV-miniATP7B) provides long-term correction of copper metabolism in WD mice. In anticipation of a future gene therapy clinical trial, we considered the value of using biliary copper excretion as a pharmacodynamic biomarker. For that purpose, we have evaluated the excretion of radiocopper (^{64}Cu) into the faeces as an alternative biomarker in AAV-miniATP7B-treated WD mice. Male and female WD mice were intravenously injected with AAV-miniATP7B at 6 weeks of age. Three months later, radiocopper was injected intravenously in treated mice as well as in control mice of the same age (WT and Atp7B +/-). Abdominal PET analyses were performed 24 and 48h later. 24h faeces, 24h urine and serial blood samples were collected over a period of 72h post radiocopper injection, at which time animals were sacrificed and organs collected (liver, kidney, lung, brain and spleen); the radioactive signal was then measured for each biological sample in a gamma counter. Faecal radiocopper excretion was significantly higher in control mice in comparison to WD mice, in which a signal was barely detectable; while in AAV-miniATP7B treated WD animals, radiocopper faecal elimination was restored. The overall kinetics of radiocopper in blood was also very different in controls and WD mice, and it treated WD mice showed a kinetic similar to WT mice. Finally, the radioactive signal in the liver was much higher in untreated WD mice compared to controls, and was reduced in treated WD mice. In conclusion, faecal excretion of radiocopper represents a very promising biomarker to evaluate the therapeutic efficacy of *ATP7B* gene supplementation in WD patients.

417. Early Versus Late Gestation Approaches in Fetal Gene Therapy

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Progression of in utero gene therapy (IUGT) for various monogenetic disease has improved over the past decades. However, clinical success has been limited by low efficiency of gene transfer and poor expression of transgene level. We have designed a non-human primate (NHP) model of IUGT by injecting scAAV LP1-hFIX (AAV5 or AAV8 pseudotypes) into the fetus at 0.4G (1-10 x 10^{10} vg/fetus, n=6) or 0.9G (4 x 10^{12} vg/fetus, n=5) gestation. Delivered offspring were serially monitored for transgene level, immune expression level and liver transaminase level. Interval biopsies of liver and peripheral tissues were performed to monitor temporal vector distribution through qPCR. Livers were visually examined during laparotomies and histologically examined to ascertain any evidence of hepatotoxicity. Our results revealed that early recipients demonstrated lower median levels than late recipients (15.62 vs. 49.02%, p=0.01) while early AAV5 recipients showed a much lower expression than late AAV5 recipients (2.06 vs. 11.51%, p<0.0001). Overall transgene expression in early AAV8 was similar to late AAV5 subjects. No difference was apparent in the growth velocities of early and late IUGT recipients. Early recipients of both AAV5 and AAV8 showed a mild anti-AAV humoral response with initial reactions approaching but not crossing the positive threshold for the first 150 days post-IUGT. In comparison late recipients showed a more robust positive humoral response for the first 700 days post-IUGT particularly with AAV5-IUGT. At the same postnatal time-points early recipients had 2-3 log-folds lower vector copy per number (VCN) than their late IUGT counterparts in all tissues apart from cerebellum, pancreas, adrenal gland, gonads and peripheral organs: fat, skeletal muscle, skin, umbilical cord. In order to achieve desired outcomes of in IUGT, factors like gestation at intervention, and efficacy of vector should be optimized.

418. Advantages of DNA Barcoding versus Integration Site Analysis for In Vivo Clone Tracking after Transplantation

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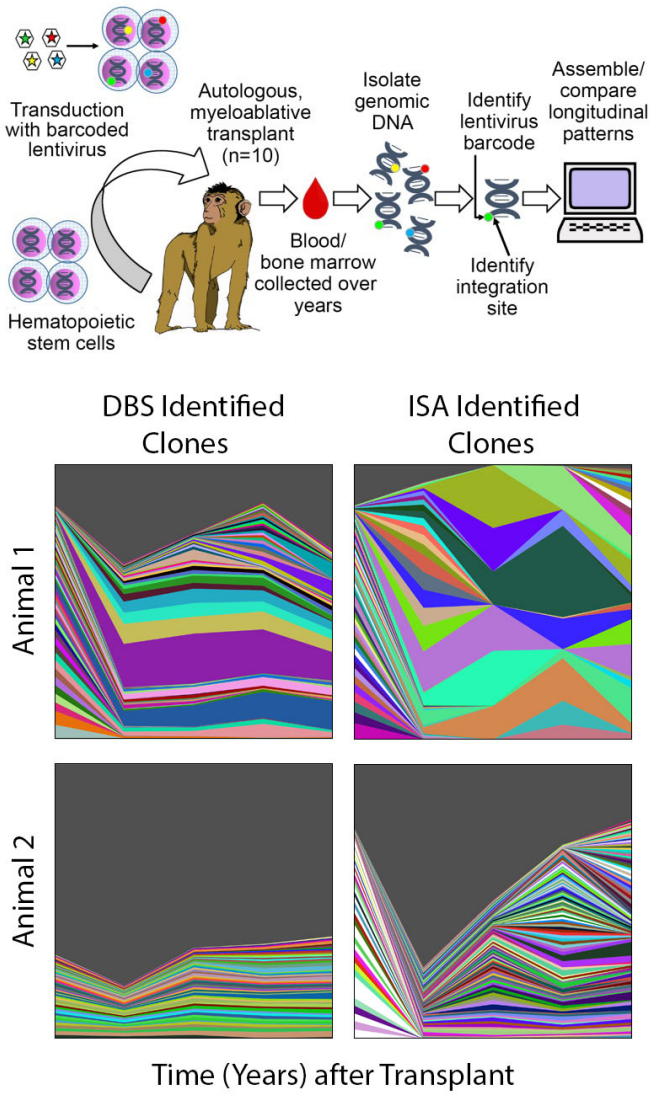
In vivo tracking of retrovirus-tagged blood stem and progenitor cells is used to monitor safety and study hematopoiesis after gene therapy. Sequencing the locus of retrovirus insertion, termed integration site analysis (ISA), is the gold standard. However, for preclinical studies of hematopoiesis, DNA barcode sequencing (DBS) has also been applied. As the field moves towards gene editing strategies, ISA will no longer be a viable option for clone monitoring in vivo whereas DBS presents a possible alternative if barcodes can be safely introduced at edited loci. A key question is how these two techniques compare in their ability to detect and quantify clonal contributions. Here we assessed both methods simultaneously in a nonhuman primate model of autologous, myeloablative transplantation. Blood and bone marrow samples were collected from two transplanted animals over the course of 2 years. Samples were processed for both ISA and DBS clone identification. In animal 1, ISA detected 1,468 unique clones while DBS detected 41,935 clones. In animal 2. ISA detected 4,649 unique clones while DBS detected 19,849 unique clones. We used clone capture data and computer simulation to estimate capture rates for each method and demonstrate that DBS is at least five-fold more efficient than ISA. While ISA and DBS reliably capture the most abundant clones in each animal, the increased sensitivity of DBS allows more consistent tracking of lower frequency clones, especially in animals with lower levels of gene marking. These data suggest re-processing samples is necessary to ensure reliable coverage of clone diversity, regardless of which method is used, but that DBS requires far less re-sampling than ISA. Importantly, we were able to trace lineage and abundance with both methods for safety monitoring, without adverse events observed in either animal. Our analysis demonstrates DBS as a useful guide to maximize ISA interpretation in current gene therapy patients, and as a more informative option for monitoring clonality in gene editing approaches where barcode introduction is possible.



419. Computational Correction of Index Switching among Multiplexed Samples in Integration Site Analysis

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In Next generation sequencing technologies, hundreds or thousands of DNA samples can be sequenced simultaneously (multiplexing) and the obtained sequencing reads can be distinguished by the presence of sample-specific nucleotide sequences (indexes) embedded in the primers used for the DNA amplification. Custom bioinformatics pipelines, by reading the indexes present in the sequencing reads assign them to a specific sample (demultiplexing). Multiplexing however is plagued by index switching, a phenomenon occurring when free index primers are randomly fused to DNA sequences belonging to other unrelated samples of the library pool and resulting in the incorrect assignment of sequences to one or multiple wrong samples. In the field of gene therapy, vector integration site (IS) studies heavily depend on sequencing of DNA fragments (containing proviral-cellular genome junctions) from several samples and are affected by index switching. This issue is particularly relevant in clonal tracking studies, where the level of shared IS between different cell lineages or different time points of the same GT patient are required to define the levels of multilineage reconstitution and estimate the number of stem cells and other calculations. Therefore the spreading of IS between datasets caused by index switching could result in inflated sharing IS levels which could lead to misinterpretation of the results.To evaluate the extent of index switching in IS analyses in our laboratory, we analysed 123,431,269 sequencing reads originating from a pool composed by 54 samples amplified in triplicate, each tagged by two indexes fused to the ends of the PCR products containing the LTR and Linker Cassette (LC) sequences resulting in 162 index combinations (combining a total of 48 LTR and 32 LC indexes). From this analysis we found that >95% of sequencing reads belonged to the correct 162 index combinations while a the remaining 5% of reads belonging to 1374 false index combinations resulting from frank events of index swapping. The levels of swapping were similar among the different LTR and LC indexes with an average of 1709 ± 3469 reads (range 9 to 52000) for false index combinations. We then evaluated the levels of sharing of univocally mapped IS between different samples and found that essentially all samples had different levels of contamination. Overall, 91.5% of IS were assigned to a single sample, 7.25% were found shared in two samples and the remaining 1.25% were present in more than 2 samples. Focusing on a sample from a cell line with 6 known IS we calculated the spreading levels and their relative abundance on other samples. From this analysis we found that at least one of the 6 know IS were found in 13 unrelated samples out of 54 (24%). In 3 out of 13 samples the amount of contaminating reads from this cell line reached levels ranging from 13 to 40% of the entire dataset. These high levels of contaminations justified the development of new approaches for indexing switching correction in IS studies. To this aim we developed a set of probabilistic and logic algorithms that allows to remove contaminating sequences by comparing the level of IS sharing between technical replicates, eliminating sequences where multiple unique molecular identifiers are linked to the same shear site, comparing the relative abundances of each IS and other parameters that will be described.We are now testing and validating in depth this approach in a controlled dataset of known IS. A final refinement of the method will complete the correction of the IS data, allowing to release reliable results without the noise introduced by sequencing artefacts.

420. Toxicology Studies for Hematopoietic Stem & Progenitor Cell Gene Therapy for Cystinosis Revealed an Unexpected Cytotoxicity of Polybrene to the Ctns$^{-/-}$ Sca1^{+}Cells

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Cystinosis belongs to a family of lysosomal storage disorders & is characterized by the accumulation of cystine within the lysosomes of all organs leading to impairment of renal function & other organs such as the eye, thyroid, muscle, pancreas, & central nervous system. Mutations

or deletions in the ubiquitous gene *CTNS* cause cystinosis; & this gene encodes cystinosin, a transmembrane lysosomal protein, which is a proton-driven cystine transporter. The investigational gene therapy is planned to be used for autologous transplantation of hematopoietic stem & progenitor cells (HSPCs) transduced *ex vivo* using the lentiviral vector pCCL-CTNS containing the human *CTNS* cDNA. Once engrafted, the transduced cells are expected to divide & differentiate, thus providing a population of cells with normal cystinosin that can cross-correct other cells in the body. Early proof-of-concept studies showed that transplantation of wild-type HSPCs in a lethally irradiated $Ctns^{-/-}$ mice leads to cystine content decrease in every tissue tested, as well as functional & structural improvement in kidney, eye & thyroid in treated mice compared to mock-treated mice. Following a pre-Investigational New Drug (IND) meeting with the Food & Drug Administration (FDA), we conducted in vivo nonclinical studies in the mouse model of cystinosis using the murine analogous $Sca1^+$ HSPCs isolated from the $Ctns^{-/-}$ mice. pCCL-CTNS-transduced $Ctns^{-/-}$ $Sca1^+$ HSPCs were transplanted in 2-months old $Ctns^{-/-}$ mice (Test mice) & compared to mock-transduced $Ctns^{-/-}$ $Sca1^+$ HSPC-transplanted mice (Control mice) for 6 months post-transplant. Preclinical safety & efficacy were determined by assessing clinical signs, comprehensive histological tissue analyses, Vector Copy Number (VCN) in blood, bone marrow & hematopoietic lineage cells from the $Ctns^{-/-}$ mice & vector integration site analysis. The initial in vivo study had unexpected polybrene-related findings, which were resolved when polybrene was removed from the transduction process. Findings included early death & abnormal Complete Blood Count (CBC) values in the Primary Test mice, especially in Primary Test females. Corrective / Preventive Action procedures were performed to identify the cause of the findings & identified that the use of polybrene in the transduction step of the $Ctns^{-/-}$ $Sca1^+$ cells was cytotoxic for the $Ctns^{-/-}$ mHSCs, reducing their viability & resulting in low post-transduction cell survival affecting bone marrow reconstitution in the transplanted Primary Recipient Test mice. In contrast, polybrene had no cytotoxic effect on the wild-type $Sca1^+$ HSPCs, showing that this unexpected impact was specific to the cystinosis cells, which appear to be very sensitive to polybrene added to the transduction medium. In the subsequent study, removal of the polybrene from the transduction step resolved the issue. The IND for autologous transplantation of HSPCs modified by *ex vivo* transduction using the pCCL-CTNS lentiviral vector for cystinosis was approved December 19, 2018, & the clinical trial is now open at UC San Diego.

421. Preclinical CAR-T Cell Target Safety, Biodistribution, and Tumor Infiltration Analysis Using In Situ Hybridization Technology

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Chimeric antigen receptor (CAR) T cell therapy has proven to be highly effective in treating hematologic malignancies, and major efforts are being made to achieve similar efficacy in solid tumors. These efforts face multiple challenges, including off-tumor target expression and checkpoint inhibition of CAR T cell activity in the tumor microenvironment. CAR T cells are much more potent compared to antibody therapeutics, therefore there is a need for more stringent CAR T target safety assessment to avoid adverse events resulting from "on-target/off-tumor" activity. Furthermore, it is critical to track and monitor CAR+ T cells within the context of intact tissue and tumor to understand the mechanisms underlying off-tumor toxicity and efficacy in tumor killing. In this study we employed the RNAscope in situ hybridization (ISH) technology to assess target expression specificity and to track CAR T cell distribution and activation in xenograft and host tissues using the RPMI-8226 xenograft mouse model, which expresses both *BCMA* and *ROR1*. The anti-ROR1 CAR T cells used in this study recognized both the mouse and human proteins whereas the anti-BCMA CAR T cells recognized only the human protein. RNA ISH revealed that *BCMA* was only expressed in the xenograft tumor and in no mouse organs, while *ROR1* was found to be expressed in the xenograft tumor as well as at low levels in mouse lung and liver. Duplex RNA ISH assay with probes targeting the CAR 3' UTR and either *IFNG* or *GZMB* mRNA allowed highly sensitive and specific detection of CAR T cells and their activation state in both tumor and normal tissues from vehicle, anti-ROR1 CAR T cell, or anti-BCMA CAR T cell treated mice. Activated anti-BCMA CAR T cells expressing *GZMB* and *IFNG* were found only in the xenograft tumor, where *BCMA* was expressed. In contrast, activated anti-ROR1 CAR T cells were found almost exclusively in mouse lung and liver with very few anti-ROR1 CAR T cells being found in the xenograft tumor, consistent with the previously observed pulmonary and hepatic toxicity of anti-ROR1 CAR T cells that was not predicted by IHC analysis of ROR1 protein presumably due to lack of antibody sensitivity. Lastly, we employed a multiplex ISH-IHC approach to confirm the presence of activated anti-BCMA CAR T cells in the xenograft tumor through simultaneous detection of the BCMA CAR 3' UTR, *IFNG*, *GZMB*, and CD3, validating the anti-tumor activity of anti-BCMA CAR T cells. These data thus demonstrate how the RNAscope ISH assay can be utilized for CAR T efficacy and safety/toxicity assessment in preclinical models by detecting very low levels of target antigen expression in off-tumor tissues and monitoring CAR T cell pharmacodynamics and activation in tumor models. This technology has equal utility in understanding both CAR T and TCR T cell activity in patient tumors.

422. In Vitro Evaluation of Potential PRAME TCR-Mediated Off-Target Toxicity Using iPSC-Derived Neuronal Cells

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Clinical studies using TCR-transgenic T cells for adoptive T cell therapy revealed the efficacy of this therapeutic approach but also uncovered possible toxic effects against healthy tissues, such as neuronal or cardiac cells, caused by on-target/off-tumor or off-target toxicity. Due to the lack of adequate *in vivo* models for prediction of potential TCR-mediated toxicity against healthy tissues, physiologically relevant *in vitro* models need to be developed. Here we describe the use of inhibitory GABA neurons and astrocytes derived from iPS cells for

evaluation of potential TCR-mediated neurotoxicity *in vitro*. HLA-A2-positive iPSC-derived cells served as target cells in functional co-culture assays with CD8+ T cells transduced with an HLA-A2-restricted, PRAME-specific TCR. Both cell types were analyzed for expression of PRAME mRNA via qPCR analysis and showed no expression of the antigen, thus not representing a potential target for on-target toxicity. Nevertheless, neuronal cells could still be potentially recognized via PRAME TCR-transduced T cells due to off-target toxicity. To show general susceptibility to recognition and killing via T cells, the iPSC-derived cells were exogenously loaded with PRAME peptide or co-cultured with T cells transduced with an allo-HLA-A2-reactive TCR. Recognition of target cells via T cells was measured via cytokine release of T cells and detection of Caspase 3/7-positive, apoptotic target cells in a real-time killing assay. Since sufficient cell surface expression of HLA-A2 is a precondition for recognition via an HLA-A2-restricted TCR, the neuronal cells were analyzed for HLA-A2 expression via flow cytometry. Interestingly, iPSC-derived neurons only showed cell surface expression of HLA-A2 after IFN-γ treatment. Loading with exogenous peptide did not seem to work as efficiently as for iPSC-derived astrocytes but could be optimized via addition of β2-microglobulin, a component of the HLA complex. Importantly, neither iPSC-derived neuronal cell type triggered activation of PRAME TCR-transduced T cells. Since neuronal cells could behave differently *in vivo* due to their 3D structure, 3D neuro-spheroids were generated and utilized in co-culture assays with PRAME TCR-transduced T cells. Confirming the results generated in a 2D system, 3D neuro-spheroids were not killed via the PRAME-specific TCR, whereas exogenous loading of 3D neuro-spheroids with PRAME peptide led to an efficient killing of the neurons. In summary, we showed that functional *in vitro* assays using neuronal cells derived from iPS cells represent an elegant approach to assess the potential risk of TCR-mediated neurotoxicity *in vitro*. The analyzed PRAME-specific TCR showed a favorable safety pattern against both iPSC-derived astrocytes and GABA neurons in 2D and 3D *in vitro* cultures.

Gene Therapy for Metabolic Disorders: Proof of Concept and Beyond

423. Systemic AAV GGene Therapy Rescues Murine Models of Propionic Acidemia Caused Mutations in Pccb from Neonatal Lethality

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Propionic Acidemia (PA) is a rare autosomal recessive metabolic disorder caused by reduced activity of the enzyme propionyl-CoA carboxylase (PCC). The PCC enzyme is composed of two subunits, alpha and beta, encoded by the *PCCA* and *PCCB* genes respectively and is responsible for the catabolism of propiogenic amino acids, as well as odd-chain fatty acids and cholesterol. The incidence of PA is approximately 1 in 150,000 live-births and is equally likely to be caused by mutations in the *PCCA* or *PCCB* gene. 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. Hypotonia, lethargy, growth retardation/failure to thrive, cardiomyopathy and seizures are variably present but overall are common complications in the patients. PA is chronically managed via dietary restriction, and antibiotic therapy is used in some patients to decrease propionate derived from gut bacteria. Medical management can decrease the severity of symptoms, but the high rates of morbidity and mortality still persist in many with PA, driving care teams to offer elective liver transplantation as a surgical treatment option. Because of suboptimal outcomes observed in PA, there is a pressing need to develop new therapies. Here, we describe the generation of the first mouse models of Pccb deficiency, and the development of effective AAV gene therapy. Four mutant *Pccb* alleles (*Pccb* Ex14 c.1492_1495 pAla498fsX499, *Pccb* Ex14 c.1495_1499del p.Ala499fsX504, *Pccb* Ex14 c.1492_1494del p.Ala499del, *Pccb* Ex14 c. 1492_1501del p.Ala499_Arg501delinsValGly) were generated using CRISPR Cas-9 gene editing to target exon 14 of the *Pccb* gene. Mice homozygous for any mutant *Pccb* allele (*Pccb*$^{-/-}$) were born in Mendelian proportions but died 24-48 hours after birth. The *Pccb*$^{-/-}$ mice also displayed elevated plasma levels of methylcitrate, a disease related biomarker. To test gene therapy in the newly generated PA models, we packaged a transgene, which uses a liver-specific human alpha anti-trypsin promoter (hAAT) to express human *PCCB* cDNA, into an AAV9 capsid. Gene delivery was tested in two different homozygous *Pccb* mutant lines. Pups received 1e11 VC of AAV9-hAAT-PCCB delivered by intrahepatic injection at birth. The treated *Pccb*$^{-/-}$ mice exhibited increased survival and decreased plasma methylcitrate levels. Seventy percent of the treated *Pccb*$^{-/-}$ mice lived more than 30 days (n=6) and appeared healthy, active and tolerant of a normal mouse chow, but were significantly smaller than wild-type litter mates. The treated *Pccb* $^{-/-}$ mice also had a lower mean plasma methylcitrate levels at 30 and 60 days of life day $P \leq 0.0002$ (n=5). In summary, we have created new murine models of severe PA caused by Pccb deficiency, developed candidate AAV vectors, and tested them for efficacy in the *Pccb* mutant mice. Our results provide the critical, and first, preclinical steps in the pathway to develop AAV gene therapy for patients with propionic acidemia caused by PCCB mutations.

424. NGS-Based Multiplexed AAV Library Screening across Species and Preclinical Liver Models

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The liver is a multi-functional organ able to synthetize and metabolize a large number of proteins and molecules. It is affected in many inborn metabolic disorders, which makes it an attractive target for gene therapy applications. Adeno-associated virus (AAV) vectors are an attractive approach to liver-directed gene medicine because they are non-pathogenic and many serotypes are hepatotropic following an intravenous injection. Therefore the field has pursued to select for AAVs in those model systems that would predict the human setting most appropriately. Nevertheless in various preclinical models the efficiency of transduction between serotypes can range over 2 orders of magnitude. Here, we have interrogated 4 preclinical hepatocyte models with complex libraries of AAV in order to assess quantitatively whether and to which extent these models diverge or replicate each other in predicting vector performance. Briefly, 2053 AAV variants, including several currently in liver-directed clinical studies and ancestral AAVs, were administered in (1) C57BL/6J mice, (2) FRG mice transplanted with primary human hepatocytes, (3) rhesus macaque, and (4) ex vivo primary hepatocyte multi-patterned co-culture (MPCC) of human and murine cells. Relative gene transfer and activity data on over 99% of AAV variants in all of the model systems was collected and compared for consistency across models and species. Using novel methods called CombiAAV and AAVSeq, 1:1 barcoding was accomplished which allowed exhaustive readout of gene transfer (DNA) and transcriptional activity (RNA) using Illumina NovaSeq sequencing at various timepoints. Data were analyzed by normalization for relative representation in the injected input viral library preparation and visualized to define the contribution of structural motifs to the hepatotropism observed in each of the model systems. Our data illustrates clear consistencies of performance across species and model systems, however with clear outliers which we are further investigating. Our approach indicates the value of multiplexed screens to address questions in translational gene therapy of clinical candidate vector identification and preclinical model validation.

425. The Combination Therapy of ImmTOR™ with AAV Anc80 is Therapeutic, Safe, and Repeatable in Mice with Methylmalonic Acidemia, and Compatible with the Low Seroprevalence of Anc80 Nabs in the Patient Population

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Methylmalonic acidemia (MMA) is a common and severe organic acidemia most frequently caused by mutations in the enzyme methylmalonyl-CoA mutase (MUT). Elective liver transplantation has emerged as an experimental surgical therapy, which can be effective in controlling the disease symptoms. The intrinsic limitations and risks associated with liver transplantation have led us to pursue AAV gene therapy as an alternative treatment approach for MMA. Here, we describe a therapy where ImmTOR (formerly known as SVP-Rapamycin), a biodegradable nanoparticle encapsulating the immunomodulator rapamycin, is delivered in combination with an AAV vector designed to express a human MUT protein and pseudoserotyped with the synthetic capsid, Anc80. We first established the tolerability of rapamycin in MMA patients and of ImmTOR in a murine model ($Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$) that recapitulates the clinical phenotype of severe MMA. A review of the MUT MMA patient cohort (n=79) identified 18 transplant recipients, of which 7 patients, ranging in age between 6-33 years, received rapamycin over a total of 33 patient years without complications. We next dosed adult WT and $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice with or without 300 μg ImmTOR (n=4-5 per group). Survival, liver function, routine chemistries, plasma methylmalonic acid concentrations and weight were measured before ImmTOR administration and on days 12 and 30 post-treatment. All the treated mice survived without adverse events, and the ImmTOR-treated mutants even showed slight reductions in transaminases and plasma methylmalonic acid concentrations compared to untreated controls. These results indicate that both the MMA patients and MMA mice tolerate rapamycin delivered as either chronic oral therapy or as a single injection within ImmTOR nanoparticles, respectively. Next, $Mut^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mut}$ mice received $5x10^{10}$ vg/kg of an Anc80-luciferase reporter delivered with or without 100 μg ImmTOR (n=5 per group). Consistent with previous reports, ImmTOR blocked the formation of Anc80 neutralizing antibodies (NAb) when co-administered with AAV.

Because vector re-administration may be required in the treatment of MMA, we evaluated the ability of ImmTOR to enable therapeutic re-dosing in MMA mice. In the prime-boost model, $5x10^{10}$ vg/kg Anc80-CB-Luc reporter with 300 μg ImmTOR was first delivered. ImmTOR prevented the formation of Anti-Anc80 IgG antibodies. At day 47, mice were treated with an optimized vector, Anc80-hAAT-MUT, at a dose of $2.5x10^{12}$ vg/kg, with or without 300 μg ImmTOR (n=6 per group). Both groups of mice showed a pronounced and equal reduction in metabolites by day 12 ($p<0.0001$) after the second injection. The MMA dropped from 906 ± 83 uM to 154 ± 1 uM in Anc80-MUT combined with ImmTOR mice. These studies demonstrate that ImmTOR is efficacious and well-tolerated in MMA mice, and allows therapeutic redosing of AAV by blocking the formation of anti-capsid IgG antibodies. Finally, we surveyed Anc80 Nab titers in 27 non-transplanted MUT MMA patients between the ages of 2-32 years; only 2/27 were weakly seropositive (7%). The Nab correlated with the level of anti-Anc80 IgG but not anti-Anc80 IgM antibodies. In aggregate, the patient observations and murine experiments provide critical enabling data for a combination ImmTOR: Anc80 AAV gene therapy to treat MMA patients. The low seroprevalence of Anc80 NAbs in the MMA patient population, and potential for redosing with this regimen, has the potential to make this combination therapy versatile and amenable for repeat dosing.

426. AAV Expressing MDR3 (VTX-803) Mediates the Correction of Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) in a Clinically Relevant Mouse Model

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Background and Aims: Progressive familial intrahepatic cholestasis type 3 (PFIC3, or MDR3 deficiency) is a rare monogenetic disease caused by mutations in the *ABCB4* gene encoding the multidrug resistance protein 3 (MDR3), which result in a reduction in biliary phosphatidylcholine (PC) content. This reduced biliary PC is not able to counteract the detergent effects of bile salts, leading to cholangitis, cholestasis, cirrhosis and ultimately liver failure. We attempted to treat *Abcb4*$^{-/-}$ mice with an hepatotropic AAV vector carrying a codon-optimized version of human *ABCB4* in order to achieve transgene expression and reversion of PFIC3 disease markers via restoration of PL in the bile. **Methods:** HuH-7 cells were transfected with one of six AAV plasmids encoding three different MDR3 isoforms (A, B and C), of either a codon-optimized (co) or wildtype (wt) version of each gene and analyzed by immunofluorescence microscopy. Next, *in vivo* expression was confirmed by IHC following hydrodynamic injection (HDI) of *Abcb4*$^{-/-}$ mice with plasmids expressing isoform A (co and wt). Finally, two-week-old *Abcb4*$^{-/-}$ mice were treated intravenously with hepatotropic AAV viral particles expressing the selected transgene sequence coMDR3-A. Blood samples were harvested during the subsequent 12 weeks and levels of liver transaminases, alkaline phosphatase and bile salts were measured. At sacrifice, liver tissue was analyzed for AAV transduction and transgene mRNA by qPCR and RT-qPCR, respectively. Liver sections were stained for histological analysis and MDR3 quantification via IHC. Bile was harvested from gall bladders for PC concentration measurement. **Results:** From *in vitro* expression tests, only MDR3 isoform A was observed to localize to the membranes of hepatic cells. In addition, HDI studies showed that only coMDR3-A was expressed efficiently *in vivo*, localizing to biliary canaliculi. When AAV-coMDR3A (VTX-803) was administered to *Abcb4*$^{-/-}$ mice, a sustained therapeutic effect was observed as serum biomarkers of liver disease decreased significantly compared to saline-treated control animals and animals treated at a lower AAV dose. Interestingly, male mice achieved a sustained therapeutic effect up through 12 weeks. However, the effect in females lasted through 12 weeks in half of the animals, while in the other half, the effect began to wane after 8 weeks. Upon sacrifice, markers of PFIC3 disease such as liver and spleen size, PC concentration in bile and liver histology were all significantly improved. Sustained MDR3 expression was detected in AAV-treated mice via RT-qPCR and IHC, showing specific localization of the transgene product to biliary canaliculi. **Conclusion:** VTX-803 AAV-directed gene therapy successfully prevented the development of PFIC3 manifestations in a clinically relevant mouse model, with a sustained therapeutic effect up through 12 weeks post-treatment

427. Systemic AAV Gene Therapy for cblC Type Combined Methylmalonic Acidemia and Homocysteinemia Rescues Neonatal Lethality and Provides Lasting Phenotypic and Metabolic Correction Comparable to Chronic OHCbl Injections

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Combined methylmalonic acidemia and homocysteinemia, *cblC* type (*cblC*), is the most common inborn error of intracellular cobalamin metabolism, and is caused by mutations in the *MMACHC* gene. MMACHC transports and processes cobalamin into its two active cofactors, 5′-deoxyadenosylcobalamin and methylcobalamin, necessary for the enzymatic reactions of methylmalonyl-CoA mutase and methionine synthase, respectively. Disease manifestations can be severe, and can include growth failure, heart defects, anemia, neurocognitive impairment, and progressive blindness. Treatment with lifelong daily injections of hydroxocobalamin improves survival and some disease related complications but neurological symptoms and visual impairment persist despite therapy. We used TALENs to create pathogenic *Mmachc* mutations, and selected two alleles for further examination: c.163_164delAC p.Pro56Cysfs*4 (del2) and c.162_164delCAC p.Ser54_Thr55delinsArg (del3). A decreased number of homozygous mutant pups were noted at birth ($p<0.001$), but dissection at embryonic day 18.5 showed mutant embryos were present in predicted ratios. The median survival of the mutant mice was 5 days, with complete lethality by 1 month (del2/del2 n=14; del3/del3 n=66; $p<0.0001$). Mutants were distinguishable from their littermates by delayed fur growth in the first week of life, and hypopigmented ears and tails. At 2 weeks, *Mmachc*$^{del3/del3}$ mutant mice weighed 35% less than littermates (n=12; $p<0.0001$) and displayed the characteristic biochemical features of *cblC*, including significantly elevated plasma methylmalonic acid and homocysteine, and decreased methionine compared to wild-type controls (n=5-10, $p<0.05$). Pathological examination of *cblC* mice at 1 month revealed variable CNS and reproductive changes. To explore systemic gene therapy as a new treatment, we generated two AAVs: rAAVrh10-CBA-m*Mmachc* and rAAV9-CBA-h*MMACHC* that were delivered by a single neonatal intrahepatic injection (1×10^{11} GC/pup), and compared to standard weekly prenatal and prenatal+postnatal hydroxocobalamin (OHCbl) injection therapy. Dramatically improved clinical appearance and increased survival was observed in mutant mice treated with AAVrh10 (n=9), AAV9 (n=11), prenatal+postnatal OHCbl (n=16), and prenatal OHCbl+AAV9 (n=11) ($p<0.0001$), with treated mutants living beyond 1 year and although mutants were smaller than controls, long-term weights between the different treatment groups were comparable. [MMA] was reduced at 6-13 months with prenatal OHCbl+AAVrh10 (182 M; n=3) and prenatal OHCbl+AAV9 (137 M; n=3) treatment, but not with prenatal+postnatal OHCbl (>1500 M; n=4) treatment. Following a single neonatal intrahepatic injection of AAV9, AAV genome copies were widely detected and quantitated by ddPCR, and increased MMACHC protein expression was noted in the liver, heart,

and kidney. In summary, *Mmachc*$^{del3/del3}$ mice recapitulate the disease manifestations seen in humans with *cblC* including decreased survival, reduced growth, and metabolic abnormalities. A single neonatal injection of an AAV vector, expressing either the mouse or human *MMACHC* gene, provided superior long-term metabolic control and produced equivalent survival compared to chronic, injectable OHCbl treatment in the mutant *cblC* mice. Therefore, we suggest that AAV gene therapy could provide a promising new therapeutic approach to treat the systemic, and perhaps ocular, manifestations experienced by patients with this devastating metabolic disorder.

428. Therapeutic Levels of FVIII Generated by CRISPR/Cas9-Mediated In Vivo Genome Editing in Hemophilia A Mice

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Modification of the genome of somatic cells using sequence specific nucleases has the potential to provide lifelong correction of genetic diseases. We used the CRISPR/Cas9 system to insert a functional Factor VIII (FVIII) cDNA into intron 1 of the albumin gene of adult mice by non-homologous end joining (NHEJ) such that transcription of the exogenous FVIII gene was driven by the strong liver specific albumin promoter. To minimize off-target cleavage events we delivered Streptococcus pyogenes Cas9 (spCas9) mRNA to transiently express spCas9 nuclease, and a single guide RNA (sgRNA) targeting mouse albumin intron 1, by encapsulation in an optimized lipid nanoparticle (LNP). This LNP formulation enabled cleavage of up to 60% of murine albumin alleles in the liver after a single IV injection. A donor template composed of a codon optimized human FVIII cDNA lacking the signal peptide flanked by a splice acceptor and polyadenylation signal was delivered to mice using an adeno-associated virus (AAV). Because the FVIII cDNA lacks a signal peptide, the secretion of FVIII protein relies on fusion to the albumin signal peptide encoded in exon 1. In hemophilia A (HemA) mice that received 2e12 vg/kg of the AAV-FVIII donor and 2mg RNA/kg of the LNP, FVIII levels measured with the Coatest® activity assay averaged 30% of normal human levels at 2 weeks post dosing. About 25% of the HemA mice eventually developed antibodies against human FVIII associated with a loss of detectable FVIII activity. In immune deficient NOD *scid* gamma (NSG) mice dosed with 2e13 vg/kg of AAV donor and 2mg RNA /kg of the LNP, FVIII levels measured with a human FVIII specific capture-Coatest® assay averaged 70% of normal human levels and were stable over time. Integration of the FVIII cassette at the on-target site in albumin intron 1 in the liver was demonstrated by droplet digital in-out PCR, which revealed targeted integration frequencies in the range of 0.5% to 3% of the murine albumin alleles. As repeat dosing of the LNP-encapsulated spCas9 mRNA/sgRNA may lead to incremental increases in donor template targeted integration, we evaluated expression of a secreted alkaline phosphatase (SEAP) reporter gene lacking a signal peptide flanked by a splice acceptor and polyadenylation signal such that after integration into the same site in albumin intron 1 the expression and secretion of the SEAP protein is driven from the endogenous albumin promoter. No SEAP activity was detected when only the AAV-SEAP virus was injected. Cohorts of 5 HemA mice that received the AAV-SEAP followed by 1, 2 or 3 doses of LNP encapsulated spCas9/sgRNA had mean SEAP levels in their blood (expressed as micro U/ml) of 3306 +/-848, 6900 +/-2120 and 13117 +/-1318, respectively. These results demonstrate a doubling of SEAP expression after each additional LNP dose. AAV and LNP dose response studies are currently in progress to elucidate the relationship between dose of AAV FVIII donor, frequency of on-target double strand breaks, targeted integration and FVIII levels. Further exploration of these concepts is ongoing and ultimately might provide a durable and curative treatment option for hemophilia patients.

429. In Utero Liver-Directed Lentiviral Gene Therapy Cures a Pig Model of Hereditary Tyrosinemia Type I

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In utero gene therapy is an advanced approach that offers significant advantages over postnatal therapy. In liver disease, it can be curative while also addressing disease before any clinical consequences occur. We show here for the first time that *in utero* gene therapy can cure a large animal model of liver disease, in this case hereditary tyrosinemia type I (HT1). We performed ultrasound-guided intrahepatic injections of a lentiviral vector carrying the human fumarylacetoacetate hydrolase (*Fah*) gene under the alpha-1 antitrypsin promoter in 16 *Fah*$^{-/-}$ pig fetuses at E70±5. Doses ranged from 2.4x10E8 to 1.6x10E9 transducing units/fetus. Sows were maintained on the protective drug, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), throughout gestation, and piglets were maintained on NTBC until weaning. Sixteen treated and seventeen control *Fah*$^{-/-}$ piglets were born from four pregnancies. Six treated piglets were euthanized for early data collection, and five died from complications due to surrogate sows. Positive FAH immunohistochemistry was seen at birth. Remaining treated piglets and control littermates are being followed long-term, and all treated piglets have demonstrated NTBC-independent growth. Liver biopsies at 50 days of life show expansion of FAH+ hepatocytes with no evidence of fibrosis, cirrhosis, or HCC. Liver biopsies at 6 months show near complete repopulation of the liver with FAH+ hepatocytes. Plasma tyrosine levels in dosed animals have normalized. Lentiviral integration was seen in only one of 4 sows and in the liver, as well as several other tissues, in treated piglets and shows a benign integration profile. This report is the first ever to use *in utero* gene therapy to cure a large animal model of a human liver disease.

Next Generation Gene Vector Engineering and Analytics

430. Optimizing In Vivo Targeting of Lentiviral Vectors Pseudotyped with Tupaia Paramyxovirus Glycoproteins Bearing Cell-Specific Ligands

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Our goal is to design strategies allowing systemic delivery and targeting of lentiviral vectors *in vivo* in the context of mouse models of human cancer. To do this, we tested the capacity of IL-13-displaying lentiviral vectors bearing engineered Tupaia paramyxovirus (TPMV) H glycoproteins fused to the IL-13 ligand to target IL-13Rα2 expressing tumor cells *in vivo*. To investigate vector targeting *in vivo,* a single dose of lentiviral vectors pseudotyped with the TPMV-H-IL-13 glycoprotein and expressing firefly luciferase (Fluc) was administered i.v. to immunodeficient mice bearing orthotopically implanted NCI-H1299 non-small cell lung cancer cells tagged with Gaussia luciferase and expressing IL-13Rα2. The vectors were administered three to five weeks post cell transplantation. One week after vector administration, tumor growth and vector biodistribution were monitored using bioluminescence imaging and PCR analysis. Lentiviral vectors pseudotyped with a TPMV H glycoprotein lacking IL-13 were used as a control to determine specificity. Our initial assessment of vector targeting based on bioluminescence imaging and PCR revealed that animals injected with IL-13-displaying vectors showed Fluc expression at the tumor site while control vectors lacking IL-13 did not. Also, animals revealing Fluc expression were strongly positive by PCR for vector sequences using genomic DNA extracted from lung samples, while control animals lacking Fluc signals were not. These results demonstrate the potential of TPMV H glycoprotein pseudotyped lentiviral vectors for cell targeting *in vivo*.

431. Profiling the Heterogeneity of Inverted Terminal Repeat Sequences of Encapsidated Genomes in AAV Preparations by AAV-Genome Population Sequencing

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Many adeno-associated virus (AAV)-based gene therapy strategies are now moving into clinical trials. Although this marks an exciting time for treating human disease, quality control standards have yet to be universally defined and met. Current FDA requirements for AAV-based drugs focus on the presence of impurities that may carry through the manufacturing process. These include, contaminants in plasmid DNAs used in vector production schemes, the percentage of empty capsids in preparations, the presence of encapsidated non-vector nucleic acid contaminants, and detection of adventitious agents, which include viruses, bacteria, fungi, and protozoa. Unfortunately, little attention has been placed on the homogeneity and integrity of vector genomes that are packaged into virions. Since their discovery, wild-type AAV genomes have been demonstrated to package less than full-length genomes in the form of defective interfering particles. Curiously, vector genome truncations have also been documented to exist. Only recently has vector heterogeneity been accurately quantified using AAV-Genome Population sequencing (AAV-GPseq), a single molecule real-time (SMRT) sequencing method we specifically developed to gauge the prevalence of truncation events. Notably, AAV-GPseq can also assess the abundance of non-vector DNA contaminants in preparations and other characteristics of the vector genome that may impact the potency of rAAV designs. Among the strengths of AAV-GPseq is its ability to sequence through the ITRs for both single-strand (ssAAV) and self-complementary (scAAV) genomes. This property has allowed for the quantification of flip and flop configurations as well as assess heterogeneity of ITR ends, which may affect second-strand synthesis, the molecular conversion of vector genomes into stable episomal species, and other properties of ITRs yet to be defined. To this end, we have extensively explored the heterogeneity of ITR ends in vector preparations generated by the standard triple-transfection method in HEK239 cells. We found that of all genomes that do have their most 5' and/or 3' sequences terminate at the T-shaped hairpins, the majority (>99%) do in indeed end at or near the terminal resolution sequence (TRS). In fact, with AAV-GPseq, we can also extrapolate the extent of ITR repair occurring during replication and packaging steps of vector production when mutated/incomplete ITRs are present in vector plasmid preparations. Since we found that vector ITRs are predominantly homogeneous, we expect that the overall transduction of vectors and the persistence of transgenes are not heavily influenced by ITR heterogeneity. We will discuss these findings and other properties of ssAAV and scAAV genomes discovered by AAV-GPseq.

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432. A Novel, Non-Integrative DNA Nanovector System for the Modification and Manufacture of CAR-T Cells

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The capability of introducing functional Chimeric Antigen Receptors (CARs) into naïve Human T Cells represents one of the most promising therapeutic strategies for the treatment of a range of cancers. We have invented a novel DNA Vector platform that provides the opportunity to quickly and efficiently generate genetically engineered T-Cells for Human Immunotherapy. This system represents a valuable alternative to the currently used integrating vector systems such as retro- and lentiviruses or sleeping beauty transposons which present a potential risk of genotoxicity associated with their random genomic integration. Our system is based on a Nanovector technology. It contains no viral components and comprises only clinically approved sequences, it is cost-efficient and secure to produce, it does not integrate into the target cell's genome, but it can replicate autonomously and extrachromosomally in the nuclei of dividing primary human cells thus avoiding the inherent risk of integrative mutagenesis. In preclinical experiments, we have successfully demonstrated human T-Cells genetically modified with this DNA vector platform sustain the expression of a reporter gene for over a month at persistently high levels without decline. We showed that human CD3+ cells could be efficiently engineered with transgenic CAR receptors against the human epitope carcinoembryonic antigen (CEA) and we have demonstrated their viability and capability in the targeted killing of human cancer cells in vitro and in vivo. We showed that CAR-T cells generated with our technology more efficiently killed target cells when compared to T-cells engineered with the current state-of-the-art integrative lentivirus significantly increasing the survival of the mice by inhibiting tumour growth. The anti-cancer activity of our CAR-T cells was also evaluated in comparison to the current FDA approved CAR-T therapy. We demonstrate the functionality of the modified cells with an anti-human CD19 CAR in vitro, and in vivo analysis using xenotransplanted human cancer cells in immunodeficient mice. Using this DNA Vector system, we can efficiently generate large quantities of pure, supercoiled DNA with a single fermentation and we demonstrated the scalability of our approach producing a clinically relevant number of CAR-T Cells that can be used for the treatment of patients. We are currently analysing the molecular behaviour of the vector in the modified cells, and we are also evaluating the impact of the vector on their behaviour and viability with single-cell RNA sequencing and single cell proteome analysis. We believe that this novel DNA Vector system provides a unique and innovative approach to this exciting therapeutic strategy for cancer therapy and we firmly believe that this novel methodology will provide a simpler and cheaper method of CAR T-cell manufacturing.

433. Integration Site Analysis of Ad5 and SV40 Large T Antigen in HEK293T Cells

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The widely used HEK293 cell line was originally derived from human embryonic kidney (HEK) cells transfected with fragments of mechanically sheared adenovirus 5 DNA (Ad5). The insertion sequence, which includes E1A and E1B, was mapped to human chromosome 19 (19q13.2). The HEK293T variant, often employed for virus and vector production, was produced from the parent cell line by stable transfection of the plasmid pRSV-1609 (Rio 1985; DuBridge 1985), which carries the SV40 (Simian Virus 40) allele tsA1609. However, the exact insertion site, structure and frequency of the temperature sensitive SV40 large T antigen containing the pRSV-1609 insertion has never been determined. Since, like E1A, SV40T is capable of malignant transformation under specific conditions, a characterization of the HEK293T cell line, including the structure of the Ad5 and SV40-containing pRSV-1609 insertions, is needed to assess the risks associated with potential host cell DNA contaminations in vector preparations derived from HEK293T cells. First of all, the location and structure of the Ad5 insertion was confirmed via touchdown PCR, which was used to amplify the vector-genome junctions. The obtained results were in line to previous publications (Louis 1997) validating the internal approach. Due to the complications associated with the unknown structure of the pRSV-1609 insertion, an approach similar to Primer Walking was adopted to characterize it. Primers were designed to yield amplicons of different lengths according to the most probable sequence of the pRSV-1609 insertion, based on a recent study (Lin 2014). The amplicons obtained were analysed via Sanger sequencing to reconstruct the sequence of the pRSV-1609 insertions. Furthermore, the use of chromosomal primers, based on the most probable insertion sites on chromosome 3 found by Lin et al., allowed for the identification and characterization via Sanger sequencing of the 3' vector-genome junction. The core pRSV-1609 insertion structure was found to be including the AmpR, the bacterial ori, the RSV promoter, the coding region for the SV40 large T antigen (Large T CDS, including the small T antigen), the large T intron and the SV40 nuclear localization signal (SV40 NLS), as well as the SV40 poly(A) signal. Single Nucleotide Polymorphisms (SNPs) and a 15bp deletion have been observed within the RSV promoter region. Additionally, a truncated construct only containing AmpR and ori has been identified. Lastly, the 3' break point and the exact sequence of the vector-genome junctions of the core pRSV_1609 construct and its truncated form have been determined. Since the core construct and its truncated form share the same 5' vector sequences, the exact sequence structure at the 5' of both insertions could not be determined at this point. Additional experiments using linear amplification methods such as S-EPTS will be employed to identify the junctions and complete the characterization of the insertions. The knowledge of the exact sequence and structure of the pRSV-1609 insertions will also be used to conduct further analyses to achieve the determination of the integrity and copy number of SV40T (allele tsA1609) contained in the pRSV-

1609 insertion. Based on preliminary qPCR data, the copy number of SV40T amounted to one third of that for Ad5 E1A in all HEK293T cell lines analysed so far.

434. Detection and Characterization of Micro-RNAs Found as Residual Contaminants in AAV Vector Batches

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With more than 150 clinical trials and two commercially approved gene therapy products, adeno-associated viral vectors (AAV) are one of the most popular vehicle to deliver therapeutic DNA *in vivo*. Concurrent with this success, regulatory agencies ask for a more comprehensive analysis of process- and product-related impurities in recombinant AAV batches. Amongst those, residual cellular DNA is routinely controlled and is recommended by the FDA not to exceed 10 ng per parenteral dose in order to limit the genotoxic risk or the transmission of foreign coding sequences. Whereas illegitimate DNA has been shown to be external and internalized in AAV capsids, surprisingly, the presence of small cell-derived RNAs, such as micro-RNAs, has never been investigated. Therefore, we have selected a set of six miRNAs that are expressed at a low, medium and high level in the HEK293 and Sf9 cell lines and quantified them in purified rAAV lots by RT-qPCR. Our data demonstrated that miRNA are co-purified with AAV vectors in a proportion that is correlated with their abundance in the producer cells. In particular, miR-19b, a micro-RNA belonging to the oncomiR-1 cluster, is one of the most represented miRNAs found in HEK293-derived vectors. In addition, we have shown that the amount of miRNA is independent of the serotype tested and of the type of AAV particles, i.e. empty *versus* full, and can be reduced using a purification process including an immuno-affinity chromatography followed by a tangential flow filtration step compared to vectors purified by cesium chloride gradient ultracentrifugation. These data, together with filtration assays, suggest that these miRNAs are mainly external to capsids. Our study provides evidence that miRNAs are found as residual contaminants in rAAV batches. Further investigations are required to determine if these small RNAs could be inadvertently co-delivered with AAV vectors in targeted tissues and modify cellular pathways, and interfere with transduction efficiency *via* RNA sensing.

435. Improved Performance of Lenti-Viral Vectors by the Incorporation of Novel, Human Chromatin Insulators Displaying Both Enhancer-Blocking and Barrier Activity

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Introduction: Despite the unequivocal success of hematopoietic stem cell gene therapy for various genetic diseases, gene therapy still faces major challenges. Self-inactivating lentiviral vectors (SIN-LVs), although safer and less susceptible to position effects than γ-retroviral vectors, still carry the risk of genotoxicity (especially when incorporate strong enhancers like the globin-vectors) and position effect variegation/silencing of gene expression. Chromatin insulators (CIs) have been proposed as a means to minimize vector-mediated genotoxicity (enhancer-blocking CIs, EB-CIs) or limit transgene silencing/variegation (barrier CIs, BA-CIs). Nevertheless, up to now, the prototypic and dual functioning vertebrate insulator cHS4, has been associated with several limitations including suboptimal titers due to its large size (1.2kb) and only partial insulation. Recently, human, small-sized (119-284bp) CIs have been discovered and identified as robust EB-CIs reducing the genotoxicity of a γ-retroviral vector *(Liu M, Nat.Biotech 2015)* without detrimentally affecting vector titers. We here, aimed to test whether the characterized as EB-CIs (A1, A2, B4, C1, E2, F1) function also as BA-CIs. Our ultimate goal is the identification of an ideal, dual functioning CI to potentially mitigate both silencing and genotoxicity. **Methods:** We tested six of the 27 CIs identified as strong EB-CIs, incorporated in reporter SIN-LVs (GFP, A1-GFP, A2-GFP, B4-GFP, C1-GFP, E2-GFP, F1-GFP). Transduced normal human CD34+ cells were seeded in methylcellulose or cultured in erythroid differentiation medium or/and transplanted into sublethally ablated NSG primary and secondary (after mouse immunomagnetic CD45+cell depletion) recipients. **Results:** The tested CIs did not negatively affect vector titers (p=ns). After transduction, GFP expression was significantly higher in cells transduced with the insulated A2-GFP-, B4-GFP-, C1-GFP-LVs over the uninsulated vector GFP-LV (% GFP : 43, 44, 46, vs 29, respectively, p<0,005). Post erythroid differentiation, B4-GFP-LV showed higher GFP expression over the parental vector (p<0,05). Myeloid & erythroid colony pools and individual colonies, demonstrated higher GFP expression and mean fluorescence intensity (MFI) when transduced with B4- and C1-GFP-LVs compared to GFP-LV (p<0,001). The increased expression was VCN-independent, given that i) the VCN was similar between the insulated vectors and the uninsulated parental vector (p=ns) and ii) after normalization of MFI per VCN in single split colonies, the superiority in expression remained statistically significant (p<0,001)

for B4- and C1-GFP-LVs. *In vivo*, GFP blood and marrow expression was significantly higher at all times tested, in B4- and C1-GFP-LV- over the GFP-LV-transplanted recipients (p<0,04). **Conclusion:** Our data indicate that the novel CIs have no negative effect on vector titers. B4, followed by C1, apart from being powerful EB-CIs, they also possess strong barrier activity, demonstrating superior, VCN-independent transgene expression. The incorporation of these double functioning elements in therapeutic vectors may provide safer and better *in vivo* performing LVs, an issue particularly important in the context of GT for hemoglobinopathies.

436. CombiAAV Enables Rapid Generation of Combinatorially Diverse Barcoded AAV Capsid Libraries

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Barcoded libraries of Adeno-associated Virus (AAV) capsids are emerging as a powerful new tool in the arsenals of AAV biologists and engineers. Researchers can simultaneously subject libraries of many different AAV capsids to selection and can rapidly generate comparative measures of capsid function through deep sequencing of barcodes present as DNA or RNA. These approaches show incredible promise but can be technically limited with respect to the scope or nature of the sequence diversity that can be included within these libraries. Chief among these technical challenges are A) generating targeted sequence diversity with high fidelity and B) associating each mutant in a library with a known, unique identifying sequence of DNA and/or RNA.Researchers have developed many creative approaches to address these challenges. However, frequently these approaches involve making compromises, such as limiting combinatorial diversity to a relatively short, often linear segment of the AAV genome. Here we introduce our approach to address these challenges through a new process which rapidly generates libraries of tens of thousands of barcoded AAV capsids without limiting where within the capsid's coding sequence diversity can be incorporated.Briefly, we combined commercially available DNA synthesis with scarless molecular assembly approaches to build combinatorially diverse libraries in a series of one-pot reactions. As a proof of concept, we built libraries each encompassing between 8 and 32,768 unique AAV capsids, each with a unique DNA barcode outside of its coding sequence. Upon producing virus from these DNA libraries, we then validated that these unique barcodes can be reproducibly isolated and quantified at the level of viral DNA as well as mRNA. This approach will allow researchers to study the functional impact of mutating multiple residues in combination with each other without constraint on where in the capsid sequence those residues might be located. We believe this will be critical to studying viral phenotypes where conformational motifs play a role, such as receptor preference or anti-capsid antibody evasion.

RNA Virus Vectors

437. Retroviral Replicating Vector-Mediated Prodrug Activator Gene Therapy with Engineered Human Mesenchymal Stem Cells in an Experimental Glioma Model

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Retroviral replicating vectors (RRVs) achieve efficient tumor transduction through selective viral replication in cancer cells. Toca 511, an improved RRV expressing an optimized version of the yeast cytosine deaminase (CD) prodrug activator enzyme, has shown highly promising evidence of therapeutic benefit in preclinical and clinical studies for gene therapy of glioma. In the present study, we engineered human mesenchymal stem cells (MSC) as tumor-homing cellular carriers that produce and release RRV, and evaluated the effect of this mode of virus delivery on the time course of intratumoral RRV dissemination. Multiple human MSC isolates from different commercial sources and tissues of origin (bone marrow, adipose, cord blood) or clinical specimens (fetal liver) were engineered to produce RRV expressing either GFP or CD transgene (MSC-RRV). Cytotoxicity assays confirmed efficient prodrug activator function in U87 glioma cells transduced with vectors produced from MSC-RRV. To evaluate intratumoral migration activity and tumor-homing migration activity *in vivo*, individual MSC isolates were labeled with luciferase or DiI, and injected either directly into U-87 human glioma intracerebral xenografts, or into the contralateral brain hemisphere. MSC-RRV isolates showing the highest levels of intratumoral migration activity were selected, and the efficiency of intratumoral dissemination and tumoricidal activity achieved by these isolates was compared against injection of RRV virus preparations in subcutaneous glioma models. Compared to virus injection, MSC-RRV achieved 1.6x-higher levels of tumor transduction (p<0.05), and 2x-reduced tumor growth (p=0.018) at earlier time points, although differences became insignificant at later time points. Notably, no difference in systemic biodistribution and no evidence of hematopoietic genotoxicity was observed with either delivery method, indicating an acceptable safety profile for MSC-RRV. In short-term survival studies using lower doses of MSC-RRV cells vs. RRV virus injected 14 days post-establishment of intracranial U-87 gliomas, a small but statistically significant prolongation of median survival was seen with intracranial tumors treated with MSC-RRV as compared to RRV (26 vs. 20 days, p<0.05) after only a single cycle of 5-FC prodrug. Thus, MSC can be employed as mobile tumor-homing RRV-producer cells, which release RRV as they migrate to tumor foci *in vivo* and actively penetrate into individual tumor masses, resulting in more rapid tumor transduction and earlier therapeutic

efficacy, which may be advantageous particularly for multi-focal and metastatic disease. This study was funded by the California Institute for Regenerative Medicine (TR2-01791).<!--EndFragment-->

438. Generation of High-Titer Self Inactivated (SIN) γ-Retroviral Vectors in Stable Producer Cells

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The viral vector derived from the Moloney murine leukemia (MLV) γ-retrovirus was the first one developed for gene delivery, and it is commonly used in gene therapy applications. Successful examples of therapies using this vector are Strimvelis, Yescarta and Zalmoxis that have been recently approved for commercialization. However, its strong viral enhancers have contributed to malignant transformations in some hematopoietic stem cell (HSC) gene therapy clinical trials. A SIN version with its enhancers deleted is safer, but its production is cumbersome as high-titer vectors can only be achieved transiently. The aim of this study was to develop a system that could easily generate high-titer SIN vectors from stable producer cells. First, a SIN GFP optimized vector was constructed with the following modifications to increase viral titers: a CMV promoter/enhancer sequence in place of U3 in the 3-'LTR, and the addition of WPRE and 2 poly(A) sequences to decrease the read-through of the MLV poly(A). With this vector, titers of 3 x 10^6IU/ml were achieved in transient transfection and during stable productions in 293Vec-RD114 cells sorted for GFP fluorescence. Next, a *blasticidin resistant* (*BSD^r*) gene under the control of the weak herpes TK promoter was introduced in the retroviral plasmid to facilitate the generation of high-titer stable retrovirus producer cells. Packaging cells were transfected with the SIN plasmid containing the BSD^{r}cassette, or co-transfected with the SIN plasmid and a Hygro^{r}plasmid for comparison. Blasticidin selected cells were 91% GFP positive while only 51% of the cells were fluorescent after hygromycin selection. Furthermore, sorted GFP positive cells were 2.1-fold more fluorescent and had 3.4 more plasmid copies if cells had been selected in blasticidin versus hygromycin. These differences led to the production of vectors at 3.5 x 10^6 IU/ml from the Hygro^{r}cells, and 8.3 x 10^6IU/ml from the BSD^{r}cells. Forty-three clones were screened, and all were positive with titers above 10^6IU/ml. The best clone produced vectors at 1.3 x 10^7IU/ml, in the range of a regular GFP γ-retroviral vector. Vectors with two therapeutic transgenes were also constructed and tested. The first one contains the 1.4 kb *phosphatidylinositol glycan-class A* (*PIG-A*) cDNA and generated titers of 2.5 x 10^6IU/ml in transient transfections, and produced vectors at 7 x 10^6IU/ml in the best 293Vec-RD114 clone. Vectors with the long COL7A1 cDNA (8.9 kb) had titers in transient transfections of 2 x 10^5IU/ml with or without WPRE, and the best stable 293Vec-Ampho clone released vectors at 9 x 10^5IU/ml.Thus, a simple and efficient process was established for the generation of stable 293Vec-derived retrovirus producer cells that release high-titer SIN vectors. We conclude that the stable production of SIN γ-retroviral vectors with the 293Vec platform will be a more efficient, a more reliable and a cheaper option for late stage trials and commercialization than the production in transient transfection.

439. Transient Lentiviral Vector Production Using a Packed-Bed Bioreactor System

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Introduction: Lentiviral (LV) vectors are efficient gene delivery tools that are being exploited for use in a range of gene and cell therapies. Our particular interest is the development of airway-directed gene therapy for the treatment of cystic fibrosis lung disease. As candidate LV-based gene and cell therapies now move into clinical trial phases, a major roadblock to successful commercialisation is the lack of scalable LV production methods. The aim of this study was to develop a packed-bed bioreactor LV production approach that has potential for future scalable manufacturing in a commercial setting. **Methods:** A packed-bed of Fibra-Cel disks was seeded with HEK 293T cells, and three days later transient transfection was performed. A second generation, 5-plasmid LV system and polyethylenimine (PEI) were used to generate a VSV-G pseudotyped, HIV-1 based bicistronic vector containing *luciferase* and *GFP* reporter genes. Total media replacement was performed 8 hours post-transfection with a serum-free harvest media. At both 48 and 72 hours post-transfection, a complete harvest of the vector supernatant was performed, followed by replenishment with fresh media. The supernatant was processed and concentrated using anion-exchange chromatography and ultracentrifugation. Titres were determined using flow cytometric detection of vector-transduced, GFP positive cells. The *in vivo* transduction efficiency of the packed-bed produced LV vector was validated by performing airway gene transfer in 8-week old Sprague Dawley rats. Rats were anesthetised with an injectable mixture of medetomidine/ketamine and were then intubated using an endotracheal tube. The airways were conditioned with 200 µL of 0.1% lysophosphatidylcholine (LPC) to transiently open the airway tight-junctions, allowing the LV to access the basolateral-membrane receptors. One hour following LPC pre-treatment, 250 µL (5 x 10^7 transducing units) of LV vector was delivered intra-tracheally. One week following gene transfer, *luciferase* gene expression was quantified using bioluminescent imaging (Lumina IVIS). **Results:** Unconcentrated LV titres achieved using the packed-bed bioreactor production method were in the range of 10^5 - 10^6 TU/mL, with the highest yields achieved at 72 hours post-transfection. Following purification and concentration, vector yields of up to 10^9 TU/mL were attained. Airway gene transfer performed in rats demonstrated high levels of *luciferase* gene expression as indicated by radiance measures of up to 10^6 photons/sec/cm^2/sr in the lung regions. **Conclusion:** These findings show for the first time that this specific packed-bed bioreactor is suitable for transient LV production. Furthermore, the LV vector generated from this production method yields high *in vivo* gene expression following airway gene transfer in rats. With further optimisation, this packed-bed production approach could offer a potential solution to the ever-growing demands for scalable LV manufacturing. Future investigations include assessing perfusion mode, trialling different transfection conditions and seeding densities, and exploring the use of serum-free growth media.

440. Stealth RNA Vector: Innovative Technology for Gene and Cell Therapy

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Development of ideal gene delivery/expression system is a key for success in gene therapy and in transcription factor-mediated cell reprogramming. Recent success in various gene therapy clinical trials largely rely on lentivirus vectors and AAV vectors, both can induce stable gene expression. On the other hand, these vectors still have various drawbacks as a versatile tool for gene and cell therapy. We have been focused on development of RNA-based technology overcoming the limitation of current vector systems. In general, RNA virus vectors (except retro- and lentivirus vectors) have been regarded only as a tool for powerful but transient gene expression, due to their strong cytotoxic phenotype. However, we succeeded to construct defective and persistent Sendai Virus Vector (SeVdp vector) (Nishimura, et al. (2011), *J. Biol. Chem*., 286, 4760-4771.) capable stable gene expression from cytoplasmic RNA genome, based on unique mutant Sendai virus strain clone 151. Lessons obtained from development of SeVdp vectors lead us to invent Stealth RNA Vector (SRV), a new generation RNA-based gene delivery/expression system suitable for clinical application in gene and cell therapy. SRV was reconstructed from structure-optimized synthetic RNA for escaping the defense system of host cell against invading pathogen. Through this optimization, SRV can be installed with up to 10 cDNA (with total length up to 13,500 nt) on a single RNA genome, and can express them stably and simultaneously, without interfering the function of host genome DNA. This phenotype is quite advantageous for expressing proteins with huge size (*e.g*., coagulation factor VIII and dystrophin), proteins with multiple subunits (*e.g*., IgG, IgM and bispecific IgG), proteins requiring co-factors (*e.g*., lysosomal sulfatases and phenylalanine hydroxyrase), and for reprogramming somatic cells with plural transcription factors. In addition, SRV can be erased from the cells completely by interfering vector-derived RNA-dependent RNA polymerase with siRNA and with some chemical compounds. This phenotype makes SRV ideal platform for cell reprogramming and for gene editing. It is also important for guaranteeing safety in gene therapy, that is not applicable to DNA-based platform. In this presentation, I introduce the recent progress of SRV technology and its application in gene and cell therapy.

441. Manufacture of High Titer Lentiviral Vectors with a GMP Compatible Flow Electroporation Technology

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Our methods for lentivirus vector (LV) manufacture under GMP have relied on either Ca_2P0_4 or PEI based transient transfection into 293T cells. We routinely obtain vector yield of $0.5X10^8$ to $5X10^8$ TU/ml after 300 fold concentration of two harvests of vector containing supernatants collected at 48 hrs. & 72 hrs. post-transfection. Here we report a GMP compatible flow electroporation based transfection method (MaxCyte) which compared to our methods, can increase un-concentrated vector titers up to 10 fold from a single harvest. In small scale studies, we compared vector production with i) our procedure for Ca_2P0_4 based transfection method whereby adherent 293T cells were plated at a density of $5X10^4$ cells/cm^2 & cultured for 4 days & ii) cells that were split the day before transfection (manufacturer's recommendation). To transfect, cells were suspended at $1X10^8$/ml in 0.4 ml of electroporation buffer & mixed with a total of 200 μg of DNA that is comprised of the GFP encoding plasmid, the packaging plasmids, pΔ8.9so & pRSVrev, & pMNDVSV-G envelope plasmid at a molar ratio of 1:1:1:0.1 respectively. Electroporations were performed in the instrument "MaxCyte STX" using proprietary conditions -7 & -9. Transfected cells were cultured for 48 hrs. & supernatants were harvested. Infectious titers as determined by qPCR based quantitation of integrated provirus were: $1.36X10^8$ & $1.19X10^8$ TU/ml for the conditions -7 and -9 with our method & $1.95X10^8$ & $5.6X10^8$ TU/ml for the same set of conditions with the MaxCyte method respectively. This process was scaled up to ~9 fold whereby $3.5X10^8$ cells were electroporated with the same conditions as above. The transfected cells were cultured in a 1-stack culture chamber (Corning) with 84.5 ml media for 2 days upon which the vector enriched supernatants were collected. Infectious titers for the conditions -7 & -9 were $2.12x10^9$ & $3.09X10^9$ TU/ml respectively. Because of inherent variability in cell seeding density, individual cell transfection efficiency, & known potential challenges for Ca_2P0_4 transfection (2XHBS, pH, etc.), an electroporation based transfection procedures which can be standardized & more reproducibly controlled, when integrated into GMP processes that facilitate adaptation of adherent cells to grow in suspension-like state presents an attractive strategy to produce large scale GMP products with consistently high titers. We evaluated the feasibility of culturing adherent cells on Synthemax II (Corning) which are dissolvable microcarriers (DMC) that i) allow anchorage dependent cells to grow in a suspension-like state, & ii) are readily digested & dissolved by an animal-free solution to release the cells for electroporation based transfection procedures. We evaluated a small scale culture whereby $2.5X10^7$ cells were seeded on 0.5 gm of DMC which represents a cell culture surface area of 2500 cm^2 with a culture volume of 250 ml & a 2 fold scale up with $5X10^7$ cells on 1 gm of DMC that provides 5000 cm^2 of surface area for growth & with a culture volume of 500 ml. In both cultures, the cells were allowed to attach & grow on the microcarriers for 6 days at which time the microcarriers were easily dissolved & the total cell numbers for the small scale culture & the latter were enumerated as $1.25X10^9$ & $2x10^9$ respectively. The cells from the latter culture were mixed with 200 μg DNA & subjected to small scale electroporation with the conditions -7 & -9 (as above). Infectious titer values for the conditions -7 & -9 were $1.41X10^8$ & $7.11X10^7$ TU/ml respectively. In summary we present a GMP amenable pathway to generate high titer LV from a single un-concentrated harvest. Currently we are evaluating a GMP stage scale up of the MaxCyte method.

442. Kinetics of Lentiviral Integration and Transgene Expression, in Cell Lines and Primary Human T-Cells, Transduced with VSV-G Pseudotyped 3rd Generation Lentivirus

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Lentiviral and retroviral vectors are extensively used for introducing transgenes into T-cells for preclinical research and clinical applications, including recently approved products such as Tisagenlecleucel for relapsed/refractory B-cell lymphoma. VSV-G pseudotyped 3rd generation lentiviral vectors offer high titers, high transduction efficiency and safety, and have become the vectors of choice for T-cell engineering. Viral T-cell engineering usually involves T-cell activation by CD3/CD28 antibodies, lentivirus transduction, and cell expansion for 9 to14 days. While longer cultures increase cell numbers, they also change the T-cell phenotype to a more differentiated state. Recent data suggests the duration of *ex vivo* culture impacts the persistence and efficacy of CAR-T cells, and cells cultured for shorter duration display a less differentiated phenotype and are highly efficacious in preclinical models. We were interested in evaluating the transduction, integration and expression kinetics of lentivirally introduced transgenes in T-cells to understand the contribution of lentivirus dynamics during *ex vivo* culture conditions to the efficacy and durability of *in vivo* anti-tumor responses. To understand transgene expression kinetics, we transduced CD3/CD28 activated primary human T-cells with lentivirus carrying CD19 chimeric antigen receptor. We analyzed cells for viability, viral genomic integration using quantitative PCR, transcript levels using quantitative RT-PCR, and cell surface expression of the CD19CAR, on and between days three and fourteen after transduction. We also reactivated the cells with CD3/CD28 beads on day fourteen after transduction and analyzed copy number, mRNA levels and surface expression. In all cases, cell viability was >90%. Our data showed efficient integration up to twenty copies per cell of the transgene into the genome during early timepoints and declined to two copies per cell that then was stable throughout the remainder of the culture. The mRNA levels declined over the duration of the culture and increased after re-activation on day fourteen. Surface expression of the CD19CAR declined between days three and fourteen, but increased after re-activation. To address whether these observations were generalizable, we carried out similar analysis of transgene dynamics in T-cells transduced with cytosolic green fluorescence protein, membrane bound Interleukin 15 and the secreted cytokine Interleukin 12. Similar to CAR, we saw efficient genomic DNA integration stabilizing after an initial decline of copy number, decreasing RNA and surface expression levels over time, and an increase in RNA and surface expression after re-stimulation. These observations suggest transgene dynamics could be an important determinant of efficacy of lentivirally engineered cells. Future experiments will aim to understand the reasons for changes in transgene expression over time, as well as upon re-activation.

443. Highly Efficient Clearance of Residual DNA During Viral Vector Biogenesis Using Co-Expressed Secreted Nucleases

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As virus-based vectors/vaccines continue to be developed into promising therapies to treat a variety of genetic disorders, cancers and pathogenic diseases, their manufacture will be required to be up-scaled in order to meet the demand and reduce costs of these next-generation medicines. Additionally, batch-consistency and the reduction of residuals such as DNA are highly desirable, especially for indications were direct *in vivo* delivery of high dose viral vector will be required. Current methods of viral vector production necessarily lead to the presence of contaminating DNA in the final product; this DNA has a number of sources including from plasmid DNA (encoding vector components) remaining after transfection, or host cell DNA resulting from cell death due to shearing or active cell lysis when liberating virions (e.g. for AAV vectors). The current industry standard approach to reduce residual DNA contamination is the use of GMP grade recombinant nucleases (e.g. Benzonase®, SAN and Denarase®) by treating vector harvest material prior to downstream purification such that smaller, degraded DNA fragments are lost by partitioning away from vector virions. However, this approach is expensive when considering large-scale manufacturing, and because the addition of nuclease represents a 'one time' treatment, its effectiveness can be limited. Furthermore, nucleases such as Benzonase® do not perform optimally at physiological salt levels (or above) within media. For lentiviral vector production, this typically means that a second nuclease step is required during downstream processing, requiring the vector substance to be exposed to elevated temperatures and potential loss in vector activity. We have developed a highly efficient alternative approach by use of secreted nucleases in co-production with viral vector manufacture. This can be achieved by either of two modes: [1] co-expression of nuclease and vector components within the same cells or [2] co-culture of vector production cells with nuclease-expressing Helper cells. We will present data in use of nucleases from *serratia marcescens*, *vibrio salmonicida*, and *vibrio cholera*, demonstrating how nucleases with different biophysical properties suit either or both of the two modes described. We will describe optimal approaches and modifications to nuclease proteins that enable extensive clearance of both plasmid and host cell DNA that is achieved when efficacious levels of nuclease is expressed continually during vector virion assembly. The use of secreted nucleases will not only reduce costs associated with viral vector manufacturing and the associated supply chain but may also allow improvements to downstream processing steps such as ion-exchange chromatography that have hitherto had to contend with partially degraded residual DNA; achieving increasing yield and quality of vector.

444. All-In-One Delivery Using LentiFlash Technology, a MS2-Chimeric RNA Delivery Tool Designed for Clinical Applications

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Gene editing and vaccination show great promises for a wide range of therapeutic areas such as genetic disorders, cancer and antiviral strategies. Nevertheless, their use faces a number of challenges especially for clinical development including the delivery technology and expression duration management. As a game-changing RNA carrier, LentiFlash technology overcomes DNA delivery issues since RNAs are delivered directly and transiently expressed into the cytoplasm. As a bacteriophage-lentivirus chimera, it efficiently transfers RNA *in vivo* and *ex vivo* without any risk of integration. Recent advent of RNA-guided gene-editing technology has triggered a major expansion of the RNA-therapeutics field. Safe intracellular delivery, RNA stability and immune responses are major challenges that need to be overcome when using such strategies in a therapeutic context. Transient RNA-mediated expression level and duration must be managed depending on applications. Gene-editing requires a low and short-term expression to avoid off-target effects while vaccination implies a high and mid-term expression to ensure an efficient and durable immune protection. Here, we show that LentiFlash particles deliver non-viral RNA in the entire cell population such as T cells, HSC and muscle cells. We also demonstrate that LentiFlash-mediated transduction preserves the viability and original cell phenotype. In parallel, we compare the expression level and duration depending on the cell type, cell culture and the LentiFlash RNAs species. The results show that LentiFlash particles need to be differently designed for gene-editing (KO, KI, exon skipping) or antigen expression. The combination of genome-editing and immunotherapy approaches that aims to design one-time therapies will be also presented. All these properties offer additional safety considerations for clinical development and human use.

445. Optimization of Benzonase Treatment in Lentiviral Vector Production by Quantifying dsDNA Degradation with PicoGreen

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Lentiviral vectors (LV) are increasingly used as a tool in gene therapy. In recent years, CAR-T cell therapy and other clinical applications require large amounts of highly purified LV. One of the challenges of LV purification is the removal of excessive plasmids transfected and nucleic acids released from producer cells. Nucleic acids in LV preparation can be toxic to cells being transduced and they can interfere LV quantification by qPCR. As a result, Benzonase is commonly used to degrade these nucleic acids. Benzonase is typically added at the beginning of the downstream processes, so that the enzyme and degraded nucleic acids can be removed through succeeding purification steps (Merten OW et al., 2016). While effective, Benzonase is expensive and substantial amounts (usually 50 U/mL) are required to treat large volumes of harvested conditioned medium (CM). Additionally, Benzonase treatment prolongs the processing time. Since Benzonase is stable at 37°C, we hypothesized adding a small amount of Benzonase to the cell culture medium after medium replacement (~ 18 hrs post transfection) could both reduce the amount of Benzonase required to degrade contaminating nucleic acids and reduce the overall LV processing time. To optimize the Benzonase treatment conditions, we developed a sensitive and specific method to monitor dsDNA degradation in CM. PicoGreen is a fluorochrome that becomes fluorescent when bound to dsDNA and the binding site size is about 4 bp (Dragan A.I. et al. 2010). Since Benzonase can digest dsDNA to 2-5 bp, we hypothesized that Picogreen-positive dsDNAs could decrease after Benzonse treatment and PicoGreen could be utilized to monitor Benzonase mediated dsDNA degradation. Indeed, in proof of concept experiments our results showed that dsDNA concentration detected by PicoGreen decreased in a dose-dependent manner with increasing amounts of Benzonase. After validating the DNA detection assay, we conducted experiments to determine if adding Benzonase to the medium of virus production cells at 18 hours after transfection could degrade DNA present in the media as effectively as Benzonase treatment added at the time of virus harvest. Our results showed that a concentration of Benzonase as low as 2.5U/ml added 18 hours post transfection was as effective as 25U/ml of Benzonase added at the time of LV harvest (1 hr at 37°C). Importantly, the cell viability and LV production yield were not affected by the addition of Benzonase to the cell culture medium. The implementation of the method could potentially reduce the costs and the processing times needed for lentivirus production, and therefore, decrease the inactivation of labile LV. Additionally, we established a new method for monitoring Benzonase mediated DNA degradation.

446. Perfusion Cell Culture for an Order-of-Magnitude Increase in Lentivirus Production

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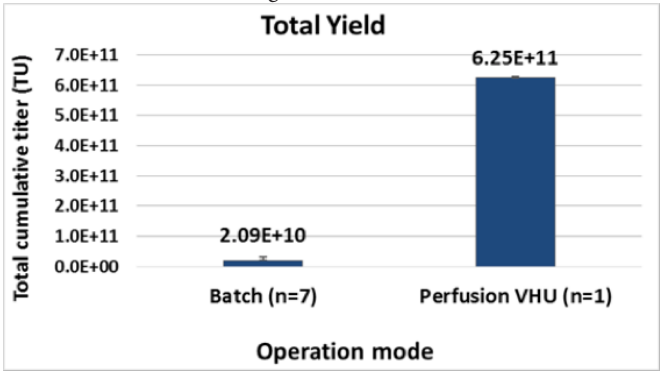
Background: Lentiviral vectors (LV) represent a key tool for gene and cell therapy applications. The production of these vectors in sufficient quantities for clinical applications remains a hurdle, prompting the field toward developing suspension processes that are conducive to large-scale production. The HEK293 stable producer cell line employed grows in suspension, thus offering direct scalability, and produces a green fluorescent protein (GFP)-expressing lentiviral vector in the 10^6 transduction units (TU)/mL range without optimization. This study describes a LV production strategy using a stable inducible producer cell line grown in suspension using a novel perfusion process. **Methods:** A 3.5L bioreactor vessel (working volume 2.7 L), equipped with probes to measure and control different parameters (temperature of 37 °C; pH 7.05-7.1; dissolved oxygen [DO] 40%; agitation 80 rpm) was operated in either batch or perfusion mode. In perfusion mode, cells were retained in the bioreactor using a VHU™ Perfusion Filter System (Artemis Biosystems). Cultures were grown up to 1-1.5 · 106 cells/mL in batch mode. Perfusion was started at 0.5 volume of medium per reactor volume per day (VVD), and increased to up to 1 VVD after induction. The culture was induced after reaching the targeted cell density (5 · 106 cells/mL). After induction, perfusion mode (1 VVD)

was started with fresh medium containing inducers for 3-4 days. In all perfusion runs, harvests were collected, and the LV-containing supernatant was kept on ice or at 4 °C until clarification (once daily) and subsequently stored at -80 °C until quantification using the GTA assay. **Results and Conclusions:** The study demonstrated that LV production in perfusion mode using the VHU filter is outperforming our routine perfusion approach. Using this strategy, the cumulative functional LV titers were increased by up to 30-fold compared to batch mode, reaching a cumulative total yield of $6.25 \cdot 10^{11}$ TU/L of bioreactor culture (Figure 1). This approach is easily amenable to large scale production and commercial manufacturing. Figure 1. Total yield of Batch and Perfusion using the VHU filter.



447. Smartphone Based Titration of Lentiviral Vectors

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Lentiviral vectors are flexible delivery tools that possess attractive features for gene delivery, including permanent integration into the host genome, the ability to infect both dividing and non-dividing cells, a broad tropism for transducing a wide range of cell types, as well as easy manipulation and production. Extensive use in basic research and translational studies including gene editing and chimeric antigen receptor (CAR) expression, have increased the need for accurately determining the infectious titer of a lentiviral vector preparation. Successful transduction of a target cell relies on knowing the infectious titer of the lentivirus preparation, as it permits calculation of the multiplicity of infection and influences the final proviral copy number within the transduced cells. Ironically, many researchers choose not to quantify vector titer due to the labor-intensive and time-consuming assays that take from four hours to two weeks to complete. In this work we present an iOS and Android-compatible smartphone application that analyzes a p24-specific lateral flow assay and delivers infectious unit values (IFU/ml) in approximately 10 minutes. The simplicity of the assay facilitates easy monitoring and optimization of lentiviral production processes to ensure consistency and confidence in downstream applications. The two-step assay consists of adding a small amount of lentiviral supernatant to the lateral flow device followed by imaging and analysis of the results using a smartphone. Densitometric analysis of the observed bands is performed by the intuitive Lenti-X GoStix software that compares the results to an automatically downloaded, lot-specific standard curve. The result is a GoStix Value (GV) that, like an ELISA assay, can be used to normalize virus stocks prior to transduction. In addition, a reference virus with a known infectious titer can be applied to generate an IFU/ml titer for additional unknown samples. Analyzing several vectors made with different packaging parameters, including a lentiviral sgRNA library, we obtained accurate titer values with R^2 values of greater than 0.99 and with coefficients of variation of less than 15% when compared to FACS-based GFP titers. In summary, this highly convenient titration technology can quantify lentiviral vector preparations in approximately 10 minutes, reduce expenses related to labor and materials, and accelerate lentiviral production processes and transduction experiments.

448. Variability Analysis of qPCR, ddPCR and Potency Assays for AAV Vectors: Implications for Future Development

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Adeno Associated Virus (AAV) vectors are currently being developed for multiple gene therapy applications. In order to support process development and quality control activities several critical quality attributes (CQAs) must be monitored. Two of these CQAs are vector genome titer and biological potency. The viral genome titer is also the starting point for many follow-on analytical and biological assays measuring other quality attributes of the AAV product. Thus, any variability in viral genome titers gets carried over into all downstream assays, with the potential to create a systematic bias. The current industry standard for measuring viral AAV genome titers is real-time PCR with quantitation through use of a calibration or standard curve. However, the nature of using a separate standard curve introduces systematic variability. Inter-assay variability observed for qPCR can range from below 10% to greater than 30%. Additionally, site-to-site variability can be greater than 50%, which will greatly affect quantitation accuracy. Recently the field has started to gravitate towards other quantification methods, which provide absolute copy numbers, thereby removing the systemic bias of qPCR calibration curves. Droplet digital PCR (ddPCR) has been touted as the next generation in vector genome quantitation. In order to assess how this systematic variability carries over in downstream assays, an in-house comparison was performed between real-time quantitative PCR and digital droplet PCR for viral genome titer determination. Subsequently, the effect of titers obtained by these methods on a downstream biological potency assay was assessed and data will be presented from four different GMP batches performed by multiple operators. Despite showing low inter-assay variability, the inter-assay %CV for qPCR titer assay were much higher than that for the ddPCR titer assay, requiring more replicate measurements to achieve comparable confidence in the absolute titers. Lastly, we also assessed how these titers affect the outcome of a downstream biological potency assay.

449. High Levels of Native eGFP Detected Following F/HN Lentiviral Vector Transduction of a Variety of Lung Models

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Lentiviral vector pseudotyped with F and HN coat proteins from Sendai virus has been shown to successfully target a variety of cell types in the murine lung. Transduced cells expressing eGFP were identified using immunohistochemistry, an indirect staining method, showing approximately 15% of airway epithelial cells as eGFP-positive following delivery of 8E8 transducing units (TU) of rSIV.F/HN.hCEF.eGFP (Alton *et al.* 2017, Thorax 72:137). In this study, we use native eGFP fluorescence to identify eGFP-expressing cells directly, following transduction of murine lungs *in-vivo* and human, ferret and pig lung tissue *ex-vivo*. **Method:** Mice were dosed intranasally with either 1E8 or 3E8 TU of rSIV.F/HN.hCEF.eGFP (n=4 per dose). In addition, precision cut lung slices (PCLS) of 500μm were prepared from human, ferret and pig lungs and treated *ex-vivo* with 3E8 TU of rSIV.F/HN expressing eGFP or secreted reporter Gaussia luciferase (GLuc) under the control of the hCEF promoter. The PCLS were imaged during culturing for eGFP fluorescence and supernatant was assayed for secreted GLuc activity over a period in excess of 30 days. Lung tissue was fixed, cryoprotected and cryosectioned on day 7 or 28 following treatment. By imaging the cryosections using the EVOS FL Auto 2 imaging system (Thermo Fisher), the entire lung lobe (or PCLS) could be captured by 'stitching' together multiple fields of view. EGFP expression from the mouse lungs was quantified using Image J software to calculate the percentage area of epithelium or parenchymal tissue above a threshold fluorescence (autofluorescence) level for regions of interest. **Results:** Approximately 40% of the mouse airway epithelium in both treated groups of mice (n=4 per dose) exceeded the fluorescence threshold, compared with epithelia from naïve mice where the median percentage area above threshold was zero (ANOVA $P<0.0001$). For PCLS cultured *ex-vivo*, high levels of secreted GLuc activity were detected in supernatant from treated PCLS (n=4-8 per species) compared with naïve slices for all species (t-test of area under the curve $p<0.05$). High levels of eGFP fluorescence were visualised in *ex-vivo* treated human, ferret and pig lung slices in culture. Studies are underway to characterise the transduced cells in each animal species, by combining native eGFP detection with cell type-specific immune staining of cryosections. These studies will evaluate the relative transduction rates and cell types in each animal species to aid selection of the most appropriate system to model human lung transduction.

450. Abstract Withdrawn

451. Establishment of a High Yield rAAV/HBoV Vector Production System Independent of Bocavirus Non-Structural Proteins

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The genome of recombinant adeno-associated virus 2 (rAAV2) remains a promising candidate for gene therapy for cystic fibrosis (CF) lung disease, but due to limitations in the packaging capacity and the tropism of this virus with respect to the airways, strategies have evolved for packaging an rAAV2 genome (up to 5.8kb) into the capsid of human bocavirus 1 (HBoV1) to produce a chimeric rAAV2/HBoV1 vector. While the efficiency of packaging of the rAAV2 genome is similar for the AAV2 capsid and those of other AAV serotypes, the yield of rAAV2/HBoV1 from the prototypic production system was 5% of that for the rAAV2 vector. Using an HBoV1 replication incompetent genome for capsid trans complementation, the prototypic system also expresses all the HBoV1 nonstructural (NS) proteins NS1, NS2, NS3, NS4 and NP1. We hypothesized that the non-essential NS protein expression in the transfection negatively impacts the efficacy of vector production, thus, we investigated the involvement of NS proteins in HBoV1 *cap* expression. Due to the existence of internal proximal polyadenylation (pA)p sites in the *cap* gene, eukaryotic expression plasmid encoding HBoV1 *cap* cDNA failed to express HBoV1 capsid proteins. We discovered that NP1 is the only NS component required for *cap*expression, preventing the premature termination of transcription of the *cap* mRNA from the native genome. Furthermore, we found that silent mutations to null these (pA)p sites can eliminate requirement for NP1, and preventing the expression of all NS proteins from the rAAV2/HBoV1 production system significantly increases the yield. Whereas the expression of capsid proteins VP1, VP2 and VP3 from a codon-optimized *cap* mRNA was highly efficient with >30-fold increase in yield achieved, optimal virion assembly, and thus potency, required enhanced VP1 expression, entailing a separate VP1 expression cassette. Taking these together, a production system in the context of NS protein-free (NS-free) was developed, with one trans helper plasmid encoding VP1 and the AAV2 Rep proteins, and another encoding VP2-3 and components from adenovirus. This NS-free system, with 3-plasmid cotransfection of HEK293 cells, yielded >16-fold more virions than the prototypic system, without reducing transduction potency. This increase in virion production is expected to greatly facilitate both research on the biology of rAAV2/HBoV1 and preclinical studies testing the effectiveness of this vector for gene therapy of CF lung disease in large animal models.

AAV Vectors II

452. Identifying the Receptor Used by a Novel AAV Capsid on Apical Airway Epithelia

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Background: Virus mediated transfer of functional cystic fibrosis transmembrane conductance regulator to diseased airway cells could treat cystic fibrosis lung disease. Most naturally occurring viruses utilize basolateral receptors, are minimally infectious, and are minimally contagious (have a low R_0). We previously passaged a library of chimeric AAV2 and AAV5 capsids on human airway epithelia (HAE) using a directed evolution strategy to select for apical infection efficiency. We selected AAV2.5T which, unlike AAV2 or AAV5, when applied to the apical side of HAE binds to a specific partner and infects efficiently. The Adeno Associated Virus Receptor (AAVR) required by AAV2 and AAV5 is not present in the apical membrane of HAE. To determine whether AAV2.5T can use an alternative receptor, we performed AAVR antibody blockade and CRISPR knockout in cell lines. We found that incomplete blockade of AAV2.5T infection predicted sustained permissiveness after AAVR knockout. Binding kinetics provided further support for the presence of an unknown receptor in these cells. **Hypothesis:** We hypothesize that the unknown AAV2.5T receptor can be identified through a cell line screen and subsequent bioinformatic analyses. **Methods/Results:** We interrogated dozens of cell lines for their ability to be infected by AAV2.5T in the presence of AAVR blocking antibodies. Using bioinformatics, we then generated a list of cell surface proteins highly expressed in permissive cells and not in refractory cells. Among these proteins were known measles receptors. We found that non-confluent Vero cells could be infected with AAV2, AAV5 and AAV2.5T, and those infections could be blocked by AAVR antibodies. However, when Vero cells became confluent and polarized they were refractory to all three viruses. We chose to interrogate measles receptors further, as measles is highly contagious with an R_0>14. The expression of some measles receptor proteins, but not others, produced polarized Vero^{MeVR} cells permissive to AAV2.5T but refractory to AAV2 and AAV5. Moreover, AAVR antibodies did not block AAV2.5T infection of polarized Vero^{MeVR}, while peptide and antibodies against the extracellular region of the exogenous protein blocked infection. **Conclusion:** The present study suggests directed evolution for apical efficiency on HAE selected an AAV that exploits the same receptor as the highly contagious measles virus.

453. Isolating Natural Adeno-Associated Viruses from Primate Tissues with a High-Fidelity Polymerase

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There are currently only two approved adeno-associated virus (AAV) mediated gene therapy drugs on the market. Several clinical trials are underway for naturally isolated or engineered AAVs. To expand the AAV "toolkit" for gene delivery, we screened for new AAVs in primate tissues by polymerase chain reaction (PCR) using Q5 polymerase, an exceptionally high-fidelity, commercially available, PCR polymerase. Q5 polymerase has a fidelity that is ~280 times higher than *Taq* polymerase. We screened 271 human tissues and ~50 nonhuman primate (NHP) samples and obtained 33 new AAV natural isolates, including hu.68 (AAVhu68). Most human isolates were clade B members or AAV2-AAV3 hybrids. Most NHP isolates belonged to clade D, E, or rh.3233-like. The AAV PCR positive rate in human tissues was ~8%: 22% in heart (5/23), 20% in intestine (4/20), 19% in liver (10/54), 5% in kidney (1/20), 3% in lung (1/33), and 3% in spleen (1/34). Compared to their closest natural AAVs in GenBank, the isolates' VP1 DNA variations (aligned regions) per amino acid (aa) variation were 12.3 bp/aa (median 6.9 bp/aa). This suggests that the AAV capsid may be evolutionarily stable at the protein level. An extreme example was an isolate named rh.75, which is 170 bp different from rh.8, its closest hit in GenBank, with only two amino acid differences at the protein level. We vectorized and tested several isolates, most of which performed well and showed good yields. We also evaluated the PCR protocol used to isolate AAVHSC1-17 based on published information. Using a pure AAV9 plasmid as the template, we amplified the plasmid with HotStar HiFidelity Polymerase Kit (Qiagen; fidelity was over 10x greater than *Taq* polymerase). We also used nested PCR (40 cycles + 40 cycles) and cloned the PCR product. We sequenced 19 plasmids, eight of which had the same VP1 DNA as the template. The remaining 11 plasmids were 1-3 bp different from the template, leading to 0 - 2 amino acid changes (nine plasmids carried at least one amino acid change and were called AAV9 PCR isolates). The average DNA difference/amino acid change of the AAV9 PCR isolates was 1.3 bp/aa (median: 1.0 bp/aa). Compared to AAV9, the average variation rate of the AAVHSC1-17 was 1.5 bp/aa (median: 1.5 bp/aa). We vectorized and tested the AAV9 PCR isolates: three had a normal yield and two had comparable Huh7 cell transduction. Our results indicate that natural AAV capsid protein sequences can remain relatively stable even as their DNA sequences experience considerable change. Therefore, caution is required when low-fidelity PCR polymerases are used for natural AAV isolation.

454. Dynamic Delivery of Therapeutic Peptides Driven by Circadian Response Elements Using Adeno-Associated Viral Vectors

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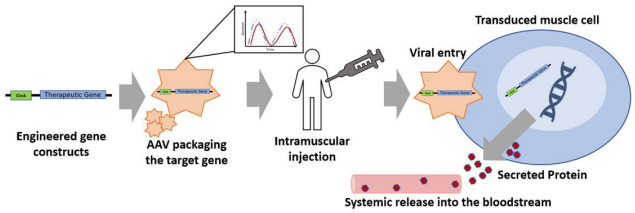
There are currently over 300 FDA-approved therapeutic peptides which cover a wide breadth of indications from diabetes to rare orphan diseases. For many patients treated through traditional pharmaceutical methods, dosing and patient adherence is a constant challenge due to the timing of drug delivery required for efficacy. We are developing a platform drug delivery system for therapeutic peptides using gene therapy to drive secretion with a circadian promoter. Circadian transcriptional response elements receive feedback throughout the day and night from the suprachiasmatic nucleus (SCN) to drive translation of downstream genes.



Using adeno -associated virus (AAV) as a vehicle for delivery, constructs that contain a circadian promoter upstream of the therapeutic peptide will drive secretion dynamically and deliver the therapeutic daily. AAV shows continuing promise in the world of gene therapy and as shown in the figure above, the viral particles can be injected intramuscularly to locally transduce muscle cells and achieve systemic secretion. With the diverse properties of AAV, such as serotypes for muscle, intramuscularly delivery is being targeted for ease of patient administration as well as maintaining long-term transient expression. The rhythmicity of these constructs has been verified *in vitro* in murine muscle cells and human cell lines, as well as observed expression *in vivo*, months after intramuscular injection in mice. Data will be presented to show the activity of the circadian promoter to drive secretion of a reporter protein, as well as initial proof-of-concept studies to secrete therapeutic peptides. Furthermore, dosing in gene therapy is an added complexity which remains abstract compared to traditional pharmaceutical treatments. This presentation will report initial models for dosing of this gene therapy platform and hypothetical dose-response curves compared to empirically-derived data to help extract valuable pharmacokinetic parameters. Dynamic dosing in response to circadian rhythms could alter drug delivery and breakthrough the barriers of classical pharmaceutical therapies.

455. Evaluation of Helper Efficacy in Triple-Transfection Method for rAAV Production

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Recombinant AAV vectors are traditionally produced by triple plasmid transfection method in which the AAV helper function and the AAV cis elements reside in different plasmids. Recombinant AAV vectors (rAAV) retain only the inverted terminal repeats (ITRs). The trans plasmids carry the essential sequences coding for AAV rep and cap genes. It is perceived that wild type AAV virus would grow more efficiently than rAAV vectors due to the "defective" nature of rAAV genome, in which trans genes were not amplified in sync to vector genome replication. In order to evaluate the helper function efficiency in rAAV production, we systemically attempted to compare the rescue, replication, and packaging efficiency of wtAAV and rAAV genomes. First, we compared the competition between a wild type AAV plasmid (containing AAV ITRs, rep, & cap) and a rAAV plasmid when grown together. Interestingly when rAAV genome is small (1.1kb and 1.9kb), it has a competitive edge in growth over wtAAV by using rep and cap expressed from wtAAV. When rAAV has similar sized genomes (3.4kb, 4.3kb, and 4.7kb) to wtAAV; wtAAV grows better than rAAV. When we added the optimized AAV helper (pH22) back, we observed that the growth advantages of wtAAV over rAAV was greatly reduced. Southern blot analysis shows that when rAAV genome is small, it has obvious replication advantage over wtAAV. However, with increasing rAAV genome size that advantage diminishes. By adding cap proteins such as VP1 or VP2, the growth advantages of wtAAV over similarly sized rAAV have no change. In contrast, adding Rep proteins such as Rep 52 and Rep78 reduced the growth advantages of wtAAV dramatically. However, the total of yields of wtAAV and rAAV were also dramatically inhibited in the presence of Rep 78. These results suggest that the helper efficiency of current optimized helper for rAAV production is close to that of wtAAV virus. Further efficiency improvement of rAAV production will likely require a new mechanism to be discovered. Optimization of other components in the transfection system may have more potential than further augmentation of the helper plasmid. The present study also provides a simple and efficient system for screening optimized AAV helper function.

456. Data-Driven AAV Capsid Design

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Adeno-associated virus (AAV) is a promising vector for delivering gene therapies. There has been tremendous effort in the field dedicated to discovering or creating new AAV vectors for improved delivery efficiency and/or specificity to desired cells and tissues. These projects typically create libraries of AAV capsids using DNA shuffling, point mutations, hexa-peptide scanning, or insertion of random peptides. Capsid libraries are then injected into model organisms and desired variants identified by extracting and sequencing genomes from the target tissues. These experiments have generated vast pools of data on capsid tropism, which have primarily been used to identify a handful of top-performing variants for further development as therapeutics. We aim to build on this prior work by using modern statistical techniques to create predictive models of capsid fitness based on protein sequence. Such predictive models could be used to develop libraries centered on optimal regions of the viral fitness landscape, reducing the development and screening time for novel AAV capsids. Specifically, using the AAV Barcode-Seq approach developed by Adachi *et al.* (Adachi *et al. Nature Comm*. 2014), we are codifying the transduction levels of different AAV capsids using next generation sequencing. We are mapping out how different mutations affect the transduction profiles of the various AAV vectors. However, due to the highly non-linear transduction results, we use modern machine learning techniques to analyze the data. We are currently testing several data-driven design optimization schemes for accelerating the screening and development of AAV capsids with desired properties. By applying statistical learning approaches to create generative models, we can guide the future development of optimal AAV vectors for specific applications.

457. Methods Matter: Standard Production Platforms for Recombinant AAV Produce Chemically and Functionally Distinct Vectors

Neil Rumachik[1], Stacy Malaker[1], Nicole Poweleit[2], Lucy Maynard[3], Chris Adams[1], Ryan Leib[1], Giana Cirolia[3], Susan Stamnes[4], Kathleen Holt[4], Patrick Sinn[4], Joseph DeRisi[2,3], Andrew May[3], Nicole Paulk[2,3]

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Background: Different manufacturing approaches have been used in the production of recombinant adeno-associated viruses (rAAVs) for gene therapy. We sought to thoroughly characterize potential differences in rAAV vector lots when produced by the two leading manufacturing platforms, live baculovirus infection of insect cells and transiently-transfected human cell systems. **Methods:** We utilized multiple analytical approaches, including deep proteomic profiling with high-resolution mass spectrometry, isoelectric focusing and subsequent SDS PAGE in combination with enzymatic modification removal, transmission electron cryo-microscopy, thermal capsid denaturation and aggregation assays, next-generation sequencing of packaged genomes, human cytokine profiling in response to vector transduction, comparative functional transduction assessments in murine liver and skeletal muscle tissues *in vivo*, and a variety of primary and immortalized cell types from human and mice *in vitro*. **Results:** Our data support the following key findings: rAAV capsids can be post-translationally modified (PTM); vector lot modifications included glycosylation, acetylation, phosphorylation, methylation and deamidation; these capsid PTMs differ when produced in the baculovirus-*Sf9* and human manufacturing platforms; host cell protein (HCP) impurities were different in vector lots when produced in human versus baculo-*Sf9* platforms; HCP impurities can also have their own PTMs, including N-linked glycans; capsid PTM and HCP impurities were seen across all rAAV serotypes, manufacturers, and purification types; there was no difference in the packaged rAAV genome sequence in either production platform; baculo-*Sf9* vector lots have insect and baculoviral HCP impurities and can have poorer packaging percentages than human-produced vector; both full and empty rAAV capsids have only minor structural differences when produced in either production platform; when given at the same vector genome dose, human-produced rAAVs are more potent than baculovirus-*Sf9* vectors *in vitro* and *in vivo* (P 0.05-0.0001); and lastly, regardless of the manufacturing platform, functional rAAV transduction is sexually dimorphic in the liver when administered IV, but not in skeletal muscle when administered IM. **Conclusion:** Our results demonstrate that baculovirus-insect and human manufacturing platforms produce vector lots exhibiting chemical and functional differences. These findings were reproducible across numerous rAAV vendors, including commercial producers, academic core facilities, and individual lab preparations. These differences have implications for capsid folding, viral replication, receptor binding, intracellular trafficking, expression kinetics, stability, half-life regulation, and immunogenicity. Additional biochemical and mechanistic investigations are needed to understand the impact of these differences on rAAV function and safety.

458. Elucidating the Mechanism of Blood-Brain Barrier Transcytosis by the Engineered AAV-PHP.B Capsids: A Critical Step Towards Developing AAV Capsids for Human Gene Therapy

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Numerous neurological diseases lack satisfactory treatments and are potential candidates for gene therapy. To develop gene therapies that broadly target the central nervous system (CNS), gene delivery vectors must be optimized to cross the blood-brain barrier (BBB). We previously described several novel adeno-associated virus (AAV) vectors, most notably AAV-PHP.B (Deverman et al. Nat. Biotechnol. 2016) and AAV-PHP.eB (Chan et al. Nature Neurosci. 2017) that cross the adult mouse BBB and efficiently transduce neurons, macroglia, and endothelial cells. AAV-PHP.B and AAV-PHP.eB are currently in use by hundreds of laboratories and have been applied across a wide range of neuroscience experiments in mice, including brain-wide correction of genetic deficits and neurological disease modeling. In our most recent work, we show that AAV-PHP.eB has the expected enhanced CNS tropism in a large panel of commercially available mouse strains, but the CNS tropism phenotype is absent in a subset of nonpermissive strains, including BALB/cJ mice. We have used this phenotypic information to identify mouse genetic variants that correlate with CNS transduction permissivity and to ultimately elucidate the molecular mechanism by which AAV-PHP.B and AAV-PHP.eB cross the adult mouse BBB. Notably, this mechanism is independent of known AAV receptor interactions. Moving forward, the knowledge of how the AAV-PHP.B capsids engage cellular receptors to cross the mouse BBB is guiding our ongoing efforts to engineer vectors that can cross the BBB in nonhuman primates and humans.

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459. Quantifying Correct Packaging in Multiplexed AAV Libraries

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Adeno-associated virus (AAV) capsid libraries are an important tool for novel vector discovery and optimization for various therapeutic uses. To maximize the number of capsids screened and to streamline

discovery efforts, vector libraries are frequently produced in multiplex, using single transfections of pooled plasmid libraries. While this process is undoubtedly efficient, it is confounded by the possibilities of cross-packaging in the vector library, wherein capsids package a mismatched genome and thereby give erroneous readouts when screened. Additionally, subunits derived from different capsids may assemble to form mosaic capsids, complicating the results further. These phenomena are known consequences of pooled library production, and while several methods have been proposed to resolve this issue, few studies have quantitatively assessed cross-packaging in libraries. To better understand the relationship between library production methods and cross-packaging, we developed qPCR-based assays to measure the prevalence of correct packaging in simplified library production contexts. We then use these assays to establish optimal transfection conditions which minimize cross-packaging while preserving vector yield. Finally, we demonstrate that more complex libraries produced with these optimized conditions are minimally cross-packaged through a functional in vivo assay. Of note, while optimal transfection conditions theoretically deliver thousands of library plasmids to each cell, the abundance of cross-packaging is surprisingly low, possibly suggesting a mechanism of capsid-genome preference. Together, these experiments comprise one of the first efforts to quantify incorrectly packaged virions in multiplexed libraries and indicate optimal conditions for pooled production of high quality AAV vector libraries.

460. Development of a Peptide ELISA for the Screening of Pre-Existing Anti-AAV Antibodies

Hubert Daniel[1], Mavis Agbandje-Mckenna[2], Nishanth Gabriel[3], Sanjay Kumar[3], Kirsten Coleman[2], Arun Srivastava[2], Rajesh Kannangai[1], Asha M. Abraham[1], Alok Srivastava[4]

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Introduction: Presence of anti-AAV antibodies is one of the major limitations in the use of AAV as a gene therapy vector. Current techniques to detect anti-AAV antibodies are capsid ELISA and transduction inhibition assay (TIA). These techniques, though successful, require large scale AAV production and expertise to perform as well as interpret the results. We report a carefully designed peptide ELISA as an alternative to capsid ELISA to detect anti-AAV antibodies against AAV serotypes 3, 5 and 8 and compare with capsid ELISA and TIA. **Methods**: The B-cell epitopes for screening binding antibody against AAV serotypes 3, 5 and 8 were predicted using Bepipred. Total anti-AAV antibodies was screened using AAV serotype 3, 5 and 8 empty capsids. Anti AAV antibodies were assessed in 100 serum samples collected from 50 healthy and 50 hemophilia A/B individuals after IRB approval and informed consent. Screening for binding antibody was done using serotype specific peptides(1, 3 and 1) for AAV 3, 5 and 8, respectively. A subset of 18 healthy individual samples was also screened for neutralizing antibody against AAV3 using TIA by flow cytometry. AAV3 antibody concentration is reported in mU/mL (Figure 1). **Results**: A cutoff of 10^4mU/mL (OD approx1.0) which was found to be equal to a titer of 5 by TIA was used to calculate the prevalence of significant anti-AAV antibodies for capsid and peptide ELISA. The prevalence of total anti-AAV antibody against serotypes 3, 5 and 8 using capsid antigen ELISA was 64%, 56% and 66%, respectively. Prevalence of binding antibody determined using peptide antigen ELISA for AAV serotype 3 and 8 was 53.8%, 52.5%. Binding antibody prevalence for AAV5 using three peptides separately were 54%, 32% and 34%. The anti-AAV3 NAb assessed using TIA by flow cytometry with a titer of greater than 5 was 50% in the healthy population. There is a significant correlation between total anti-AAV3 antibody ELISA mU/mL and TIA titer with a correlation coefficient r of 0.807 (P=0.0001) (Figure 2). **Discussion**: Our study shows a good correlation between carefully selected specific AAV peptide and capsid ELISAs. This can be used for screening of individuals for anti-AAV antibodies as an alternative to capsid ELISA followed by TIA for recruiting individuals for gene therapy. The difference in positivity between different AAV5 peptides could be due to the difference in antigenicity of peptides emphasizing the need for careful design and selection of peptide sequence for this assay. However, given the easier and consistent production of these peptides compared to AAV capsids, this could help standardization of these assays as well as improving their sensitivity and specificity. Further studies are in progress to evaluate these peptide-based assays for anti AAV antibodies.

Figure 1 Standard curve generated using the quantitated sample

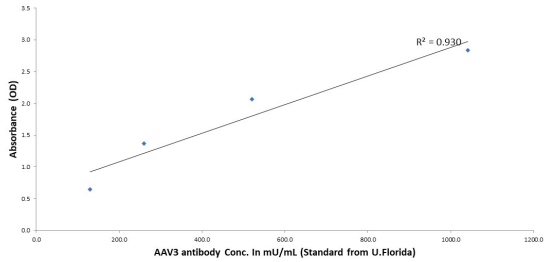
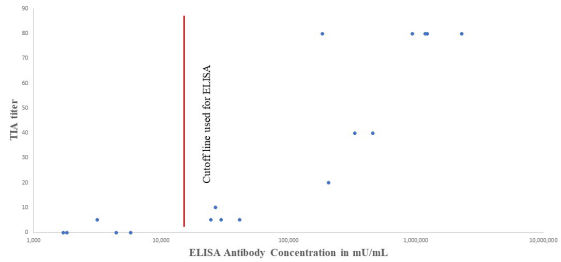


Figure 2 Scatter plot of total anti-AAV3 ELISA titer and neutralizing antibody titer as measured by TIA using flow cytometry (n = 18)



461. AAV Mediated Alpha-1 Antitrypsin Augmentation Prevents Spontaneous Emphysema in SerpinaA1 Null Mouse Model

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Previous work in our lab used the CRISPR/Cas9 system to successfully disrupt all five copies of the serpinA1 gene, leading to mice with undetectable levels of circulating A1AT and, recapitulating the clinical characteristics of human emphysema. Emphysema is one of the primary life-limiting obstructive lung diseases and the leading genetic cause is α-1 antitrypsin (A1AT) deficiency (A1ATD). In this study for the first time we aimed to determine whether optimized rAAV mediated hA1AT serum protein augmentation will alleviate the pulmonary phenotype and is able to prevent the development or progression of emphysema in these mice. Four cohorts of A1AT-KO mice received single treatment via intravenous delivery of either dual-function AAV9.CB-AAT vector at 8 weeks (1st and 2nd cohorts), at 35 weeks (3d cohort) or AAV8.CB-AAT at 20 weeks (4th cohort) of age. Evaluation of respiratory function and histopathology were assessed at 35 weeks (1st cohort), at 50 weeks (2nd and 3d cohorts), or at 60 weeks (4th cohort) of age. Pulmonary mechanics were measured in age-matched, gender-matched treated and control knockout mice. Lung and liver tissue were collected for comparative evaluation of the alveolar diameters and anti-hA1AT immunolabelling, respectively. Mice were bled for serum samples. The maximal pressure-volume loops (PV) of the treated knockout animals showed significantly decreased compliance compare to their untreated controls as evidenced by shift of PV curves downwards. Accordingly, the mean free diameter of the acinar air space complex was significantly increased in untreated compare to treated animals. The measurements indicate that AAV mediated hA1AT protein augmentation can prevent the progression of emphysema. A single injection with the AAV vectors led to life-long expression of detectable levels of normal human A1AT in the serum of A1AT-null mice as determined by ELISA. Finally, we assessed the biochemical activity of circulating hA1AT by analyzing the ability of the mouse sera to neutralize elastase activity. While serum from WT and AAV treated mice exhibited a high elastase inhibitory capacity in vitro, sera from the untreated mice showed a reduced capacity to do so. This indicates that presence of AAV derived serum hAAT is able to restore the circulating serum's antielastase function. In conclusion, these experiments are the first to show that AAV mediated A1AT protein augmentation is able to 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.

462. Internal Ribosome Entry Sites Dramatically Reduce Transgene Expression in Hematopoietic Cells in a Position-Dependent Manner

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Bicistronic transgene expression is desirable in gene therapy and biomedical applications. Internal ribosome entry site (IRES) elements, which were initially discovered in picornaviruses, have been widely used to co-express heterologous transgene products from a message RNA driven by a single promoter. These elements adopt diverse three-dimensional structures and recruit the translation machinery, such as translation initiation factors and RNA binding proteins. It is well-known that the efficiency of IRES-dependent second transgene expression is lower in comparison with that of the first transgene, in most cases between 5 and 50%. Hematopoietic cell gene delivery, such as hematopoietic stem/progenitor cells (HSPCs), and T and B lymphocytes, is a promising treatment for both inherited and acquired diseases. We recently documented a combined strategy for potential genome editing in primary human HSPCs cells, in which transduction efficiency exceeding ~90% could be achieved by capsid-optimized recombinant AAV6 vectors (*Sci. Rep.*, 6: 35495, 2016). In the present studies, we first compared the efficiency of gene delivery using AAV6 vectors with other commonly used non-viral gene transfer systems, such as polyethylenimine, lipofectamine, and electroporation. Among these systems, AAV6 vector was found to be the most efficient into the hematopoietic cells K562. Surprisingly, however, in our attempt to deliver IRES-containing genomes, we observed a complete loss of transgene expression. As a contrast, the IRES-containing genomes led to ~30% and ~6% efficiency in other established human cell lines, HEK293 and Huh7, respectively, compare to their counterparts without IRES. Interestingly, the inhibitory effect of IRES in hematopoietic cells was bi-directional and is contrast to previous studies in which the presence of IRES in a bicistronic vector did not interfere with expression of an upstream gene. Additional mechanistic studies revealed that there was no obvious difference between the internalized vector genomes, either with or without the IRES element. Whereas the levels of IRES-containing message RNAs were ~2-4-fold lower than their counterparts without the IRES, there was ~100-1,000-fold decrease in the level of the transgene product. In addition, a position-dependent effect was observed, in which the IRES element located only between a promoter and the transgenes showed the inhibitory effect. Further studies are currently underway to evaluate the involvement of components of the cellular translation machinery in this phenomenon. Our results suggest the use of an alternative strategy, such as the 2A system, for achieving bicistronic transgene expression in hematopoietic cells.

463. Site-Directed Mutagenesis of Intelligently Designed AAV Vectors Further Improves Their Transduction Efficiency

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Most new recombinant adeno-associated virus (AAV) vectors have been generated from natural isolates or intelligent design. Especially, those vectors derived from capsid libraries present enormous advantages and potential for human gene therapy. For instance, a vector identified from a shuttled AAV capsid library, designated LK-03, was recently used in a Phase I/II clinical trial in patients with hemophilia A (NCT03003533). Highly encouraging results were obtained in that h.FVIII levels ranging from ~16-49% were achieved at a relatively low dose of $5x10^{11}$ vgs/kg. However, at the highest dose of $2x10^{12}$ vgs/kg, two patients experienced severe adverse events (https://www.biopharmadive.com/news/spark-gene-therapy-hemophilia-data-stock/529526/). Thus, It is clear that additional strategies are needed to achieve higher levels of transgene expression at lower vector doses. In this pursuit, we systematically evaluated various steps in transduction by two commonly used, intelligently designed AAV vectors, AAV-DJ and LK-03, using specific pharmacological inhibitors. Overall, the two vectors shared similar transport pathways as AAV serotype 2. For example, soluble heparin significantly inhibited vector binding and internalization. Both vectors were sensitive to EIPA, a fluid-phase uptake inhibitor. Inhibitors of endocytosis, endosomal acidification and Golgi transport significantly reduced the transduction efficiency of LK-03 and AAV-DJ vectors, albeit AAV-DJ vectors appeared to be more resistant to these inhibitors. Proteasome inhibitor, MG132, on the other hand, significantly increased the efficiency of transgene expression from both vectors, suggesting phosphorylation and/or other modifications of these capsids by cellular enzymes. We have previously reported that site-directed mutagenesis of specific, surface amino acid residues on naturally occurring AAV capsids leads to improved transduction by these vectors. Such a strategy has yielded positive results with AAV2 vectors (NCT02161380). Thus, we also performed site-directed mutagenesis of surface-exposed tyrosine (Y), serine (S), aspartic acid (D) and tryptophan (W) residues on AAV-DJ and LK-03 capsids. Our results demonstrated that the S269T-AAV-DJ and Y705+731F-LK-03 mutant vectors significantly enhanced the transduction efficiency, compared with their WT counterparts. To our knowledge, this is the first report that documents that site-directed mutagenesis of specific surface-exposed amino acids can be used to further enhance the transduction efficiency of AAV vectors that are derived from capsid libraries.

464. Differential AAV Serotype Tropism Using Different Constitutive Promoters in the Rodent CNS

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Novel AAV capsids that display unique CNS cellular tropism have significantly contributed to an ever expanding application of AAV-mediated gene therapy to a variety of CNS disorders. The ability to localize AAV transduction to specific cells not only attenuates off target cell transduction, but also reduces the amount of virus required to achieve a therapeutic effect. Because AAV cellular entry relies upon the specific capsid structure, substantial research has focused upon creating novel AAV capsids that exhibit specific properties which are validated using strong constitutive promoters driving reporter gene expression. Due to the packaging constraints of AAV, short but effective constitutive promoters have been constructed in order to provide maximal space for a therapeutic sequence. To this end, we compared the tropism of common AAV serotypes with two different constitutive promoters driving transgene reporter expression; a 1.5 kb CMV enhancer/ chicken beta actin (CBA) hybrid promoter and a 0.8 kb shortened version with an MVM intron (CBh) added. In AAV2, both promoters supported substantial gene expression in the rat striatum that was overwhelmingly localized to neurons. However, surprisingly when these promoters were packaged into AAV9, a differential cellular tropism emerged. As expected, AAV9-CBA vectors exhibited reporter gene expression predominantly in neurons. However, in marked contrast AAV9-CBh vectors exhibited reporter gene expression in non-neuronal cells. Clearly, for AAV9 a small change in a constitutive promoter dramatically shifted cellular reporter gene expression patterns while AAV2 did not exhibit such a dramatic change in cellular expression between the two constitutive promoters. These results suggest that for novel chimeric AAV vectors, the cellular tropism may be determined by yet unknown interactions between the capsid, the promoter, and specific cellular mechanisms.

465. Elucidating the Mechanism of Species Specificity of Recombinant AAV Capsid Vector-Mediated Transduction

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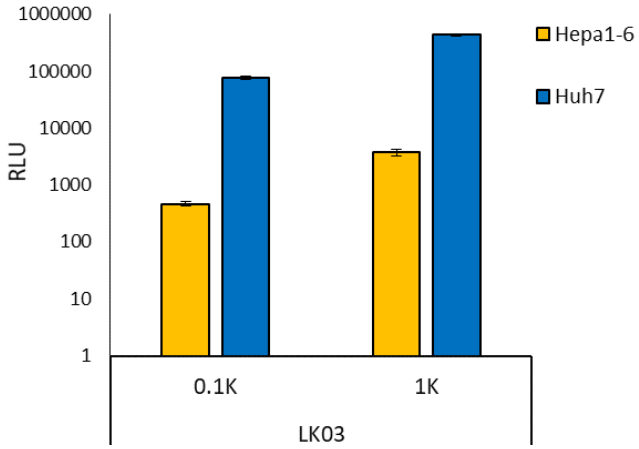
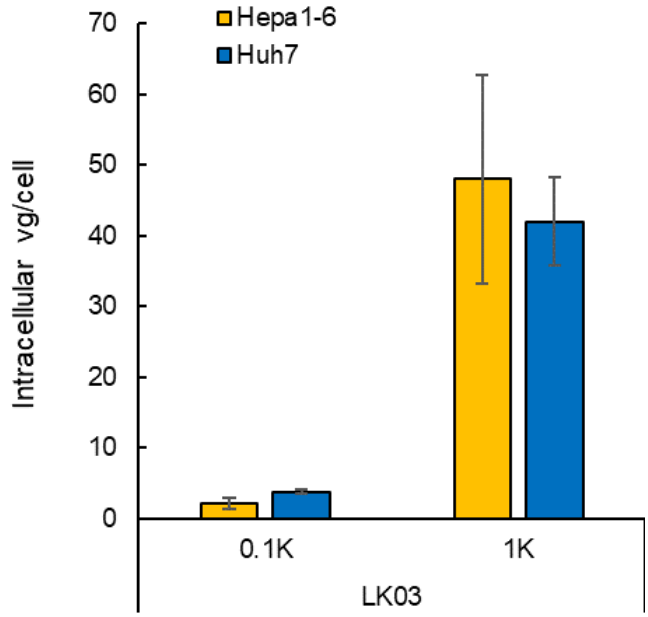
Recombinant adeno-associated viral (rAAV) vectors are of great interest as a potent vehicle for gene therapy and have already shown some promising clinical outcomes in patients with certain monogenic diseases. However, there can be quite a bit of discordance between rAAV transduction properties amongst different species making it difficult to select the optimal capsid for human clinical trials based on preclinical animal studies. In addition, transduction as defined by rAAV-mediated transgene expression does not necessarily correlate with cellular uptake of the vector. Thus, we elected to study the mechanisms responsible for the observed species discordance in rAAV transduction. Previously, we selected several rAAV capsids from screening DNA-shuffled capsid libraries and identified some

potent chimeric rAAV capsids (e.g. AAV-LK03 (Nature 2014), NP59 (Molecular Therapy 2017) that are robust at transducing human liver and human cells but show very poor transduction of mouse liver or mouse cells in general. In this study, we investigated each step of rAAV transduction from cellular uptake to transgene expression in cultured hepatocyte cell lines, Huh7 (human) and Hepa1-6 (mouse) infected with AAV-LK03 vector expressing the luciferase reporter. Another serotype, AAV-DJ was used as a control since it can efficiently transduce in both human and mouse cells (Journal of Virology 2008). While there was more than 10-fold difference in transgene expression of AAV-LK03, both cell binding and internalization resulted in similar uptake of the vector into both the mouse and human hepatoma cells lines (see Figure). We next measured the nuclear vector copy number, and found no significant difference between the two species. These data together suggest that cellular binding, uptake, transport and nuclear uptake cannot explain the species difference in transduction. Our data implicate that uncoating steps of AAV capsid proteins or other capsid processing mechanisms may be involved in the different transduction efficiency of AAV-LK03 in human and murine hepatocytes. Further studies are ongoing and will be required to resolve the molecular events in the nucleus responsible for transduction differences. Unraveling these differences may allow for better predictions of how to select optimal vectors for a given human application.





466. Structure-Guided Engineering of Surface-Exposed Loops on the AAV Capsid

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The use of adeno-associated viruses (AAV) as gene delivery vectors is a promising avenue for the treatment of many severe, unmet medical needs. Mining the natural diversity of AAV sequences present in primate tissues has allowed identification of over a hundred variants distributed in clades and the establishment of the NAV platform of gene transfer vectors in which therapeutic sequences are packaged into various capsids including AAV8, AAV9, and AAVrh10. These vectors have been intensely used in preclinical gene therapy studies and over twenty NAV-based gene therapy products are currently in clinical development. Early studies using the AAV2 scaffold have shown that capsid variants with enhanced utility for gene transfer potential may be engineered. The desirable improvements include potency, escape from immunity barriers, cell type and tissue specificity, and manufacturability. For instance, the variable region eight (VR-VIII) capsid loop has been used extensively for insertion of small peptides since it was first determined to be amenable. More recently, the PHP.B variant of AAV9, which includes a 7-mer insertion within VR-VIII has been shown to possess significantly improved transduction properties in the mouse brain. We propose that an in-depth analysis of surface-exposed loops on the AAV capsid is warranted to determine candidates for capsid engineering via insertional mutagenesis. Here we present work using available high-resolution structures to identify regions of high flexibility and low structural similarity between serotypes and define regions amenable to insertion that do not affect AAV packaging efficiency to provide opportunities for peptide randomization or insertion of purification tags and homing peptides. Three criteria we used for selecting surface loops that might be amenable to short peptide insertions were: 1) minimal sidechain interactions with adjacent loops,

2) variable sequence and structure between serotypes, and 3) the potential for interrupting commonly targeted neutralizing antibody epitopes. Surface exposed loops that have not been investigated thus far in the context of clinically relevant serotypes were selected for further investigation. We constructed a panel of peptide insertion mutants and screened for viable capsid assembly, peptide surface exposure, and potency. The best constructs were then used as templates for insertion of identified homing peptides to test if these peptide insertion points could possibly be used to retarget NAV vectors to tissues of interest.

467. Wild-Type and Recombinant AAV: Novel Insights into Mitochondrial Integration and Trafficking

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We previously showed that recombinant (r)AAV-1 can integrate within the mitochondrial genome (mtDNA) in human and mouse muscle upon intramuscular administration (Kaeppel C et al. Nat Med 2013). Mammalian cells typically contain ~1,000-2,000 mitochondria but, due to divergent energy requirements, this average encompasses cell types with low mitochondrial contents like fibroblasts (~300 mitochondria), as well as mitochondria-rich cells like cardiomyocytes (~5,000 mitochondria). As rAAV mitochondrial integration sites (mtIS) were reported in muscle, we investigated whether rAAV mitochondrial integration could be a passive event only arising in cell types presenting high mitochondrial contents. We established a simplified model by infecting cells bearing high (primary skeletal muscle cells (SkMC), immortalized and iPSC-derived cardiomyocytes) and moderate (HEK293T) mitochondrial contents with wild-type (wt) or rAAV-2 to subsequently identify integration events by LAM-PCR. Immortalized and iPSC-derived cardiomyocytes returned similar results with a total of 55 and 21 wt- and rAAV-2 mtIS for the immortalized cells and 55 and 26 mtIS upon wt- and rAAV-2 infection of iPSC-derived cardiomyocytes. Most likely because of the lower cell input employed, SkMC yielded less mtIS, 9 and 8 for the wt- and rAAV, respectively. However, quantitative PCR performed on iPSC-derived cardiomyocytes and SkMC showed that AAV mitochondrial integration frequency was comparable in both cell types with $3{,}00 \times 10^{-7}$ and $3{,}08 \times 10^{-6}$ mtIS/AAV genome for wt- and rAAV-2, respectively, in iPSC-derived cardiomyocytes and $2{,}67 \times 10^{-7}$ and $2{,}26 \times 10^{-6}$ mtIS/AAV genome within SkMC. In both cases rAAV-2 mitochondrial integration was one-log higher when compared to the parental virus, which may reflect the absence of the Rep-mediated targeted integration. The comparison of these data with the currently in progress analysis of LAM-PCR amplicons from AAV infected HEK293T cells will reveal if AAV mitochondrial integration depends on the target cell mitochondrial content. To shed some light on the trafficking pathway enabling wt- and rAAV mitochondrial integration, we investigated if mitochondria-associated membranes (MAM), representing the connection sites between the endoplasmatic reticulum (ER) and the mitochondria, could play a role. We determined whether increased numbers of ER-mitochondria contact sites correlated with higher rAAV mitochondrial integration frequencies by using stably transfected cell lines overexpressing proteins known to affect the number of MAM (cyclophilin D, glucose-regulated protein 75 or mitofusin-2). These cells were then infected with a rAAV-2 for the subsequent isolation of total- and mtDNA. LAM-PCR amplicons were obtained in all fractions and the ongoing sequencing data analysis will show if shifts in the number of MAM can alter rAAV mitochondrial integration rate. This study focusing on rAAV mitochondrial integration and trafficking will be valuable to finer evaluate the risk of gene therapy approaches targeting mitochondria-rich tissues, as well as to assess rAAV´s potential for the treatment of mitochondrial disorders.

468. AAV44.9 and Clade E/F Intermediate Virus has Unique Glycan Binding and Transduction Activity

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Recent isolation of novel AAV serotypes has lead to significant advances in our understanding of parvovirus biology and vector development for gene therapy by identifying vectors with unique cell tropism and increased efficiency of gene transfer to target cells. AAV44.9 is a natural isolate originally found as a contaminate of laboratory stock of SV15 adenovirus. Its sequence homology places it between clades E and F similar to Rh.8. Little is known about the biology of these intermediate viruses. Recent studies have suggested these isolates have utility as vectors for gene therapy. We have studied the binding activity and biodistribution of one isolate 44.9 and finds suggest unique binding activity and transduction profile. Antibody neutralization studies suggest a lower frequency of neutralizing antibodies compared with AAV2. Glycan array studies of AAV44.9 identified binding to terminal glucose containing molecules. This interaction is unique among AAVs and through biochemical assays was found to be necessary for transduction. To better understand its activity in vivo and general biodistribution mice were transduced by a variety of routes of transduction. Subretinal injection shows extensive transduction of photoreceptors. Intracerebroventricular injection showed transduction of the cortex, olfactory bulb, cerebellum, choroid plexus and brain stem similar to AAV9. Like AAV9, AAV44.9 also shows transduction of hepatocytes and is able to rescue a lethal model of Methylmalonic acid deficiency in new born mice.

469. Applying Coarse-Grained Modeling and Phylogeny-Based Approaches to Predict Formation and Function of Genetically Modified Adeno-Associated Virus Capsids

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Adeno-associated virus (AAV) is increasingly leveraged as a gene therapy vehicle due to its inherent nuclear delivery and cell entry properties coupled with relatively mild immunogenicity. To improve vector tissue- and cell-specificity, researchers are implementing a variety of mutational strategies for generating novel AAV-based vector libraries. Such mutational strategies frequently create large populations of non-forming or non-infective variants that reduce the effective depth of these libraries, costing time and resources through the screening process. We evaluated the effectiveness of two different computational techniques for predicting the fitness of proteins to the task of screening AAV mutants *in silico*. These approaches may facilitate the development of AAV capsid libraries with higher proportions of functional variants, accelerating screening and identification of promising therapeutic candidates. We applied the frustratometer, a computational tool that uses thermodynamic modeling of protein structures to identify key regions facilitating binding and structural transformation, to the AAV2 capsid. Through this approach we identified candidate residues favoring assembled and disassembled states of the capsid. As an alternative computational strategy, we used direct-coupling analysis (DCA), a statistical framework based on the principle of residue coevolution within proteins, to make an additional set of predictions about the functionality of these capsid mutations. We then conducted capsid mutagenesis to quantify the impact of altering these residues on virus formation and transduction. Our frustratometer-based metric shows some correlation with virus assembly (R^2=0.36, p=0.02, N=14). Interestingly, a metric based on the collection of DCA parameters is correlated with virus transduction (R^2=0.86, p=0.008, N=6) in the small population of residues we studied, suggesting that global models of amino acid connectivity could be useful in predicting AAV function *in silico.* Our results indicate that coevolutionary models may be able to elucidate the complex relationships that drive the evolution of multifunctional virus domains while accelerating the process of viral vector design.

470. Potential of AAV-GGF2 Delivery in Gene Therapy of Heart Failure

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Glial growth factor 2 (GGF2) is an isoform of human neuregulin-1β which is critical for normal heart development. Recombinant GGF2 peptide is currently under development as a possible treatment for heart failure. To avoid a frequent dose of recombinant peptide, here we explore the possibility of GGF2 gene delivery for heart failure treatment using an Adeno-associated virus (AAV) vector, which has been documented as a safe, effective delivery system in both animal studies and human clinical trials. To achieve this goal, GGF2 under the control of liver-specific hAAT promoter was built into rAAV vector and pseudotyped with AAV8 capsid. The resulting vector was administered to the heart failure mouse model created by left anterior descending (LAD) coronary artery ligation. Mice were divided into five groups, each was administrated with either PBS as control or various doses of AAV8-GGF2 (2×10^13 virus particles, 2×10^12 virus particles,2×10^11 virus particles, 2×10^10 virus particles) by tail vein injection. In addition, 14 normal mice were similarly divided into five groups and administrated with PBS or various doses of AAV8-GGF2. Unexpectedly, the 10-week survival rates of the mice with 2×10^13 virus particles, 2×10^12 virus particles,2×10^11 virus particles in the MI group as well as the mice with 2×10^13 virus in the normal group were all 0%, and the 8-week survival rates of the mice with 2×10^12 virus particles,2×10^11 virus particles in the normal group were 66.6%, and the 8-week survival rates of the mice with PBS and 2×10^10 virus particles in both MI group and normal group were 100%. Gross anatomy showed enlarged livers in dead mice. Traditional H&E staining of liver slices showed that all liver tissues from dead mice had various degrees of lymphocyte infiltration and sharply demarcated centrilobular necrosis with loss of hepatocyte cytologic details. Taken together, these results indicate that AAV8-mediated GGF2 expression causes hepatotoxicity and proper control of GGF2 expression level is essential for the clinical application of AAV-GGF2 delivery in heart failure.

471. AAV Purification Method Influences Long-Term Efficacy in a Feline Model of Neurologic Disease

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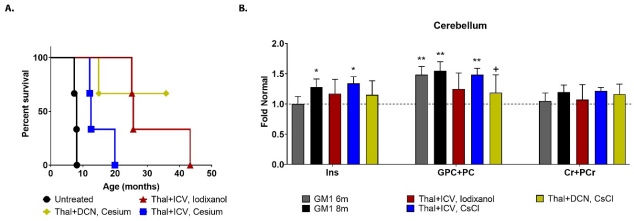
Adeno-associated viral (AAV) vectors are produced by numerous transfection and purification methods, which have been shown to influence overall efficacy in some instances. With the recent approval of AAV for human clinical use and several other AAV vectors in clinical trials, manufacturing processes to optimize feasibility and yield are an area of intense focus. Because anecdotal evidence in our lab suggested differential efficacy of AAV vectors based on purification methods, we

directly compared density gradient centrifugation of AAV particles using cesium chloride (CsCl) versus Iodixanol. After transfection of HEK293 cells to produce a single vector lysate, one-half of the lysate was purified by CsCl and the other half with Iodixanol. The AAVrh8 vector expressed a cDNA for feline lysosomal β-galactosidase, the enzyme deficient in GM1 gangliosidosis, which proved highly efficacious in previous studies to treat GM1 cats by intracranial injection. In the current study, GM1 animals received 7.5e+11 vg of either CsCl or Iodixanol purified vector via thalamic and intracerebroventricular (Thal+ICV) injection. As a control for injection route, another cohort of GM1 cats was treated with CsCl purified vector by injection of the thalamus and deep cerebellar nuclei (Thal+DCN). Animals were followed to a common neurological humane endpoint (inability to stand), and all treatment cohorts had a significant increase in lifespan compared to untreated GM1 cats (Figure 1A). Where untreated GM1 animals reached humane endpoint at 8.0 ± 0.6 months of age, the mean lifespan of Thal+ICV groups was 14.8 ± 3.6 months for CsCl and 31.4 ± 8.4 months for Iodixanol. There are two ongoing animals in the Thal+DCN, CsCl group, but the mean lifespan to date is 28.9 ± 9.9 months. The development of neurological symptoms was attenuated in all treatment groups at a level that correlated well with overall survival. In postmortem samples, lysosomal β-galactosidase activity in the brain was normalized after Thal+ICV treatment, showing 0.4 - 1.7 fold normal activity for the Iodixanol cohort and 0.0 -1.0 fold normal activity for the CsCl cohort. In the single postmortem sample for the Thal+DCN, CsCl cohort, activity was 0.4 - 2.2 fold normal, which is comparable to the previously published results. CSF biomarkers of disease progression, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), were normalized partially or fully in comparison to untreated animals, with the most correction found in the Iodixanol cohort. Magnetic Resonance Spectroscopy (MRS) analysis of brain metabolites included the gliosis marker myoinositol (INS), demyelination indicators glycerophosphocholine and phosphocholine (GPC+PCh), and metabolism markers creatine and phosphocreatine (Cr+PCr) (Figure 1B). For each metabolite in the Thal+ICV cohorts, correction was most pronounced after treatment with Iodixanol purified vector. Our preliminary data show clearly enhanced efficacy using Iodixanol purification of AAV vectors when delivered at a moderate dose through Thal+ICV injection.



472. A Novel Analytical Platform for the Accurate and Rapid Simultaneous Determination of AAV Vector Empty-Full Ratio and Particle Titer

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Adeno Asociated Virus $itAAV$it is well characterized and widely used as a gene delivery vehicle. In recent years, great improvements have been made in AAV vector design, production and purification. However, a satisfactory analytical methods for AAV vector titer, especially empty-full ratio determination, are still lacking. Existing analytical solutions that offer indirect empty-full determination are extremely slow, tedious, capital intensive, require large samples of high purity, and high levels of user training and expertise. Here we describe a groundbreaking duplex assay platform that is designed to enable the simultaneous quantitation of vector DNA by TaqMan assay and viral particle count by proximity ligation assay. This leads to the simple, fast, and direct determination of AAV full and empty ratio with a time-to-result of about 4 hours. The utilization of a novel universal AAV binding ligand also makes this a true universal assay capable of analyzing all AAV serotypes. This novel approach represents a paradigm shift in AAV analytics, and enables users to accelerate their work by obtaining vital information about vector samples with unprecedented speed, ease, and accuracy.

473. CAP-GT Platform: A Scalable AAV Production System Using Helper Virus-Free Adeno-Associated Viral Vector Packaging/ Producer Cell Lines Based in Human Suspension Cells

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Over the past decade, unprecedented insights into the molecular mechanisms of inherited and acquired human diseases pathogenesis shifted gene therapy to the center of clinical medicine. Results from clinical trials evidencing low immunogenicity, broad targeting spectrum and long-lasting transgene expression efficiency appointed recombinant adeno-associated virus (rAAV) as the most promising gene transfer vehicle. Consequently, the expanding clinical demand for rAAV production underscored a critical need for scalable manufacturing processes capable of generating large yields of pure rAAV particles. Using an innovative approach based on its proprietary serum-free suspension cell line, CEVEC has developed CAP-GT cells harbouring all the elements for rAAV assembly stably integrated into their genome. At first, we have established a helper virus-free packaging suspension cell line with stable integration of *Rep* expression under a Tet-inducible promoter. Second, *Rep* expressing stable clonal cell lines that produced higher titers of rAAV were stably transfected with the adenoviral helper functions E2A, E4orf6 and VA RNA. Third, stable integration of the capsid function and transgene flanked by the ITRs resulted in a producer cell line system. Successful selection of

cells stably expressing all the required genetic elements was ensured by drug resistance. With further protocol optimization and process development, we were able to achieve conditions for high rAAV yields and quality. We believe that our stable rAAV production platform tackles the challenges posed by the high demand for rAAV production at industry scale by offering a reproducible, scalable and cost-efficient delivery of high-titre and high-quality viral vectors.

474. High Content Imaging-Aided Assessment of AAV Transduction Efficiency on Human Pluripotent Stem Cell-Derived Motor Neuron Cells

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One of the most important factors affecting the success a gene delivery system is its transduction efficiency. Here, we used human induced pluripotent stem cell-derived neuron progenitor cells (NPCs), motor neuron progenitors (MNPs), and motor neuron (MNs) to screen adeno-associated virus (AAV) capsids to maximize transduction efficiency. NPCs and MNPs can be easily dissociated into single cells, making it possible to score AAV transduction efficiency with flow cytometry analysis; however, for terminally differentiated MNs the dissociation process impairs cell integrity and viability. Scoring transduction efficiency by examining green fluorescent protein expression using a traditional microscope is time consuming and tends to produce subjective bias. Therefore, we evaluated the use of a high content analysis (HCA) system to quickly and objectively score for transduction efficiency. Due to its automated microscopy and multi-parameter image process program, HCA is able to generate quantitative image data from transduced mature MNs. To validate the HCA method, we transduced NPCs and MNPs with different capsids of AAVs (AAV1, AAV2, AAV3B, AAV6.2, AAV9, and PHP.B), and then compared HCA with flow cytometry and manual image analysis. The results were consistent across all three methods, with AAV6.2 having the highest transduction efficiency and expression level in NPCs and MNPs. Next, we analyzed the transduction efficiency of AAVs on mature MNs with HCA and manual image analysis. With both methods, AAV6.2, AAV2, and AAV3B had the high transduction efficiencies and expression levels in terminally differentiated MNs. In summary, HCA provides a highly efficient way to evaluate transduction efficiency of AAV capsids using an *in vitro* cell culture system, amenable to automated high-throughput screening.

475. Investigation of a Serine/Threonine Motif in Multiple Adeno-Associated Virus Serotypes

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The use of adeno-associated virus (AAV) to treat certain diseases has been approved by the Food and Drug Administration, and numerous clinical trials are ongoing for various applications. Despite this, many unanswered questions regarding the mechanisms behind basic viral functions remain. Recently, we identified a serine/threonine (ST) motif, three amino acids in length, that is highly conserved across most AAV serotypes near the N-terminus of VP1 and VP2 subunits. Alanine scanning mutagenesis of this region in AAV serotype 2 (AAV2) shows that this motif, particularly the first serine (S155), is critical for virus transduction. Furthermore, mutation of the first serine to alanine results in reduction in transduction that correlates with a decrease in viral genome transcription but is not associated with changes in virus formation, internalization, and cytoplasmic and nuclear compartmentalization. Here, we study the importance of the ST motif across multiple AAV serotypes. Analysis of the ST region in AAV serotypes reveals three different motif variants: SSS (serotypes 1, 2, 3, 6, 11), SST (serotypes 4, 8, 7, 10), and SSA (serotype 9). We performed alanine scanning across the ST motif in multiple AAV serotypes and studied how these mutations affect virus formation and transduction efficiency. Our preliminary results show that alanine replacement of the first serine does not significantly alter the titer of serotypes 4, 7, 8, and 9. However, alanine replacement of the entire ST motif significantly reduces AAV9 titers. In AAV4, single and triple alanine mutations reduce transduction in Cos7 cells to 40% and 48% of wild-type levels, respectively. In AAV9, single and triple alanine mutations reduce transduction in CHO-Lec2 cells to 44% and 3.2% of wild-type levels, respectively. Taken together, this data suggests that the ST motif is important for transduction across multiple serotypes and may, in the case of AAV9, also be important for virus formation. Serine and threonine residues are known to be targets of phosphorylation. To study if phosphorylation may play a role in the AAV ST motif, we substituted the first or all three amino acids with aspartic acid residues, which are structurally similar to phospho-serine. Our preliminary results show that when the entire ST motif is replaced with aspartic acids, the formation of some serotypes such as AAV2 and AAV4 are severely compromised. Other serotypes, such as AAV8 and AAV9, form at acceptable titers. The introduction of aspartic acids rescued the *in vitro* transduction of AAV9 to wild-type levels. Overall, this data suggests that phosphorylation or lack of phosphorylation of the AAV capsid at the ST motif may be important to both virus formation and virus function. We are currently completing characterization of these phosphomimetic mutants. Thus far, our results indicate that the ST motif is important in AAV function across multiple serotypes, and that transduction can be restored using phosphomimetic residues in certain serotypes. Further in-depth study of this motif will contribute to our understanding of how AAV forms and functions, and aid in its development as a controllable delivery vector for gene therapy.

476. Dual AAV Vectors for Delivery of Large Genes

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Hearing loss affects 1 in 500 newborns in the United States. In developed countries, roughly 80% of congenital hearing loss is due to genetic causes. Importantly, the majority of these cases are non-syndromic and recessive, making these cases ideal targets for a gene replacement strategy. Hearing loss is an especially attractive indication for gene therapy as local delivery of virus to the inner ear circumvents the need for high titer or large volumes of virus as well as the possibility of negative systemic effects. However, the inner ear has a disproportionately high number of large genes that exceed the 4. 7 kb carrying capacity of standard adeno-associated viruses (AAV). One approach to large gene delivery is to use a dual AAV vector approach in which a gene is divided in half and each half is packaged separately in two viruses. When cells are infected with both of the viruses, the halves will recombine creating an intact gene capable of generating a full-length, functional protein. Three dual vector strategies have been used to deliver large genes in discovery efforts and one is currently in use in clinical trials: 1. Overlapping dual vectors make use of homology arms to recombine each half of the gene, 2. Trans-splicing dual vectors use splice donor and acceptor sequences to splice together the transcript, and 3. Dual hybrid dual vectors which use a combination of both homologous recombination and splice donor/acceptor sequences. We have developed *in vitro* and *ex vivo* assays for comparing the efficiency of recombination for several dual vector strategies in cochlear tissues. These assays are critical for iteratively identifying the optimal configuration of the dual AAV for each gene with a reasonable throughput. Here we demonstrate recombination of two halves of a reporter gene *in vitro* using PCR and Sanger sequencing, we show full length functional protein using immunofluorescence *in vitro*, we demonstrate dual vector recombination efficiency in hair cells of the cochlea using cochlear explants *ex vivo*, and we show recombination of dual vectors *in vivo*. Using these assays we can compare recombination efficiencies of various recombination strategies and permutations of dual vector constructs.

477. Development of Novel and Highly Efficient mRNA Trans-Splicing Dual Vectors

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Recombinant adeno-associated virus (AAV) vector-mediated gene delivery is the gold standard for treatment of inherited diseases. The major limitation of AAVs is their limited cargo capacity which precludes their use for treatment of diseases caused by mutations in large genes. The most popular approaches to overcome this limitation are dual AAV technologies (DATs), for which the coding sequence is split and packaged into two separate AAV vectors. The full length genome is then reconstituted within the target cell after co-transduction by the two split genome vectors. For DATs, one of the following strategies is used: i) homologous recombination via overlapping regions ii) intermolecular concatamerization and splicing via artificial splicing elements, iii) a hybrid approach combining both techniques or iv) protein *trans*-splicing with split-intein. Preliminary reports on the split-intein technology suggest high reconstitution efficiencies with this approach. However, reconstitution generates equimolar amounts of a soluble intein peptide as a byproduct, which raises potential safety concerns. The first three strategies aim at reconstitution of the coding sequence at the viral DNA level. However, the reported reconstitution efficiencies for these strategies are too low (up to 10 % for *in vivo* approaches) to support sufficient gene expression for most therapeutic applications. Thus, there is an unmet need to develop novel DATs with higher reconstitution efficiencies yielding higher levels of gene expression. Here, we present a novel dual vector strategy for reconstitution of genes via *trans*-splicing at the mRNA level. For this, both parts of the coding sequence are transcribed, base-pairing of the two mRNAs is facilitated via complementary binding domains and *trans*-splicing is induced creating a full-length mature mRNA. To test this strategy, a splice reporter assay was developed by splitting the coding sequence of the cyan fluorescent protein variant cerulean into two non-fluorescent halves. Both parts were equipped with a complementary binding domain, all splicing elements necessary for *trans*-splicing, a promoter, and a polyadenylation site. In this setting, the cerulean fluorescence can be used as an indicator for successful mRNA reconstitution. Cerulean reconstitution efficiencies were analyzed and quantified via confocal microscopy and western blotting from co-transfected HEK293 cells. In parallel screening experiments, the efficiencies of different sets of binding domains and splice sites were evaluated. By combining the best set of splice sites with an optimized binding domain, reconstitution efficiencies up to 60 % were achieved. In conclusion, we demonstrate remarkably high reconstitution efficiencies using a novel mRNA *trans*-splicing dual vector approach. This method can be used for new DAT-based therapies of hereditary disorders caused by mutations in large genes which cannot be treated with conventional AAV-based gene therapy approaches.

478. Assessment of an Adherent HEK293 Cell Transfection Process for Scalable AAV Production in the iCELLis® 500 Fixed-Bed Bioreactors

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Adeno-associated virus (AAV) vectors are potent gene therapy vectors, used to deliver therapeutic transgenes to target tissues. Gene therapy clinical trials often require high titer vector preparations to adequately deliver the therapeutic transgene, in great excess of research-level production utilized in many laboratories. AAV is often produced utilizing the transient transfection of adherent HEK293 cells with multiple plasmids encoding the AAV capsid serotype, transgene to be packaged, as well as viral Rep and adenoviral helper proteins in tissue culture flasks. To assess large scale production of AAV in

adherent HEK293 cells, we evaluated AAV production utilizing the iCELLis Nano bioreactor (Pall Corporation). This fixed-bed benchtop bioreactor was used to develop a transient transfection process. Based on conditions developed in the pilot iCELLis Nano bioreactor system, we scaled this production to the large scale iCELLis 500 bioreactor (200 m^2 and 333 m^2 surface area). A HEK293 cell seed train was utilized, using the Xpansion® 200 bioreactor (Pall Corporation) to generate sufficient cell numbers for seeding the bioreactor. Transfection reagents were scaled for efficient transfections. Media components, such as glucose as well as O_2 and CO_2 were evaluated and replenished as needed throughout the run using the perfusion process of the iCELLis 500 bioreactor. Following a production phase, $>10^{16}$ vector genomes were isolated from crude harvest of the bioreactor. This data demonstrates that a scalable closed manufacturing process for AAV can be developed using the iCELLis 500 fixed-bed bioreactor.

479. Analytical Ultracentrifugation as a Qualified, Stability-Indicating Method for Monitoring AAV Capsid Integrity and Degradation

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As AAV gene therapy products mature, there is an increasing need for methods to measure integrity and degradation of the AAV capsids. In this context, sedimentation velocity analytical ultracentrifugation (SVAUC) has emerged as a very powerful method to assess purity and composition of AAV products. The scope of our study was the development of an improved SV-AUC method for the characterization of AAVs with high resolution and higher throughput, by applying the novel Optima™ AUC hardware and improved data processing with UltraScan. A qualification based on the principles of ICH Q2(R1) was designed and executed to demonstrate the capabilities of the SV-AUC method. The species quantified included low molecular weight species (25-50S), empty capsids (50-72.5S), full capsids (72.5-120S) and aggregates (> 120S). Specificity was demonstrated with respect to formulation components and to degraded samples. Repeatability and intermediate precision were demonstrated across three runs with two different operators. Linearity of full capsids and of aggregate species was demonstrated by mixing degraded and non-degraded samples in pre-defined ratios and showing a linear relationship ($R^2 \geq 0.95$) between expected and measured aggregate and main species levels throughout the tested range. The results showed that SV-AUC is capable of separating empty from full capsids, as well as from degradation products, including aggregates and lower molecular weight products. It was also demonstrated that the method is capable of monitoring capsids even in the presence of a large excess of a proteinaceous formulation component. Integration and relative quantitation of the various species can be reliably performed. LOQ for the aggregate species was determined to be 1%. In order to demonstrate the stability-indicating properties of the SV AUC method, AAV samples were subjected to forced degradation conditions and analyzed by SV-AUC and dynamic light scattering (DLS). Incubation at pH 2 led to the formation of aggregates which were observed in SV-AUC. Under the same conditions, low levels of particles with diameter larger 100 nm were observed by DLS. On the other hand, short term incubation at 55°C led to higher levels of particle formation as observed by DLS, but no significant aggregate formation was observed by SV-AUC. In conclusion, SV-AUC is appropriate for monitoring AAV quality in real-time, accelerated, and forced degradation stability studies. The developed method allows simultaneous analysis of up to 14 samples per run at high resolution. SV-AUC is also a powerful method for demonstration of comparability between AAV batches produced by using different manufacturing processes. For these purposes, the method is a useful addition to the AAV toolbox and is complementary to other methods for monitoring AAV degradation via aggregation, such as DLS.

480. Novel Human Serotype-Based Adeno-Associated Viral Capsid Variants with Remarkable Hepatic Transduction Efficacy and Therapeutic Potential

Nadja Meumann[1], Joachim Schwäble[2], Julie Lucifora[3], Qinggong Yuan[4], Albrecht Piiper[5], Christian Schmithals[5], Philip Meuleman[6], Ann-Christin Franke[1], Li-Ang Zhang[7], Ulrike Koitzsch[8], Karin Huber[9], Margarete Odenthal[8], Michael Ott[4], Erhard Seifried[2], Hildegard Büning[1]

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Adeno-associated viral (AAV) vectors have proven their potential as *in vivo* delivery tools, especially in liver-directed clinical trials. However, anti-AAV immune responses as well as cell-related barriers still pose a challenge. Consequently, research focusses on the development of second generation AAV vectors, which facilitate clinical trials with lower vector doses and comprehensive pre-clinical assessment across species. Here, we describe results of our high-throughput *in vivo* selection of an AAV peptide display library for AAV capsid variants that meet these challenges. In detail, we identified two AAV2-derived capsid variants, MLIV1 and MLIV3, with optimized liver transduction capacities after three rounds of selection in the absence of helper virus co-infection. Both rMLIV capsid variants package with efficiencies equal to the parental serotype. Transduction efficiencies were at comparable levels in primary human and murine hepatocytes, and were clearly superior

to rAAV8 and the parental rAAV2. To determine *in vivo* cross-species applicability, we will report results of our on-going trial in humanized uPA-SCID mice. Upon systemic administration in BALB/c mice, both AAV variants showed a strong liver tropism (up to 51 fold higher than spleen; up to 18-fold higher than lung; 3-fold higher than heart) that was accompanied by significantly reduced accumulation in common off-target tissues. As compared to parental AAV2, accumulation of AAV variants was 182-fold lower in spleen, up to 24-fold lower in lung and 7-fold lower in the heart. Furthermore, compared to the parental serotype, AAV variants transduced the liver with up to 26-fold higher efficiency, and rMLIV3 reached transduction levels comparable to rAAV8. Remarkably, application of these novel vectors in a preclinical hemophilia B mouse model revealed therapeutic effects for both variants, with MLIV3 showing FIX levels and activity comparable to rAAV8, a serotype successfully used in human clinical trials. In summary, we identified two novel AAV2-based liver-directed capsid variants, which show promise for overcoming cross-species barriers. Moreover, due to favorable hepatotropism and improved transduction efficiencies, these novel human-serotype-based variants may represent a valuable advance in the field of liver-directed gene therapy.

481. Development of a Quantitative In Vivo Potency Assay for AVXS-101 Using the Delta 7 Mouse Model of Spinal Muscular Atrophy

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AVXS-101, an adeno-associated virus serotype 9 (AAV9) gene therapy for spinal muscular atrophy (SMA) used the delta 7 mouse model of SMA to demonstrate potency and determine dosing for the phase 1 clinical trial in Type 1 SMA at Nationwide Children's Hospital (NCH). AveXis has used the delta 7 mouse model of SMA to demonstrate comparability of the AveXis manufacturing product to the NCH produced material, and a conversion of titer based on the potency of the AAV9 gene therapy from quantitative PCR (qPCR) to digital droplet PCR (ddPCR). The delta 7 mouse has proven very reliable across colonies and investigators, with a median survival of homozygous mutant pups between 15-17 days. Detailed dosing examination using AVXS-101 with titer determined by ddPCR allows the identification of multiple doses that are on the linear portion of the dose response, where median survival of the mouse model is increased until the dose of 1.1e13 vg/kg when the mouse model is effectively rescued. The highly reliable performance of AVXS-101 in the delta 7 model has led to the qualification of a quantitative potency assay based on this model. Thus, a quantitative method of potency, as compared to a reference standard, has been developed for the AVXS-101 drug product.

482. A Novel Method for AAV Vector Production on Corning Dissolvable Microcarriers (DMC)

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The field of clinical gene therapy has advanced rapidly and now accounts for over 2200 clinical trials initiated since 1989, of which ~65% are being conducted in the USA (Edelstein, M. The Journal of Gene Medicine Clinical Trial site. October 2015). Due to their efficacy and safety, adeno-associated viral (AAV) vectors have been shown to be ideally suited for gene transfer approaches used in clinical settings. However, producing large scale quantities of recombinant AAV vectors to support large animal pre-clinical studies and early-phase clinical trials has been an ongoing challenge for academic researchers and for biotechnology companies. The conventional AAV vector production process utilizes adherent human embryonic kidney (HEK) 293 cells and transient transfection methods such as calcium phosphate or polyethyleneimine (PEI). While this adherent platform is relatively efficient in terms of vector particles produced per cell, it has disadvantages such as lack of scalability, risk of contamination, being labor/time intensive, and lack of process monitoring and control elements. All these factors contribute to the high cost of large scale AAV vectors manufacturing for clinical trials. In this study, we propose a new complete process of manufacturing AAV vectors in suspension mode using adherent cells. This process includes three steps: 1) The adherent 293-T cells are expanded in suspension using Corning Dissolvable Microcarriers (DMC); 2) Cells are harvested from DMC and transiently triple transfected using electroporation; 3) After electroporation, 293-T cells are recovered on DMC or other microcarrier-free suspension format. In addition, we have demonstrated a simpler DMC dissolution method for AAV vector harvest. The data indicate that adherent 293-T cells yielded 10-fold higher AAV viral genome particles and infectious titer than suspension 293-F cells under the same optimum electroporation conditions. Adherent 293-T cells expanded using DMC (3D surface) in suspension showed the comparable genome particles and infectious titer as the cells expanded using traditional Tissue Culture treated or CellBIND treated vessels (2D surfaces), and post-electroporation surfaces (2D or 3D) had minimum impact on the total AAV viral genome particles or infectious titer generated. In summary, by combining Corning Dissolvable Microcarrier and electroporation-mediated transient transfection, we demonstrated the feasibility of producing AAV vector in suspension mode, without the need of developing a new suspension cell line and chemically defined serum-free medium. This new process offers the advantages of both increased AAV vector yield and scalability, which are critical for commercial production.

Synthetic/Molecular Conjugates and Physical Methods for Delivery of Gene Therapeutics

483. Electroporation-Mediated Gene Delivery to the Esophagus Yields Persistent, High Level Gene Expression

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Gastroesophageal reflux disease (GERD) is characterized by the erosion of esophageal mucosal lining caused by recurrent stomach and bile acid exposure. This erosion results in the loss of epithelial barrier function through cell injury and disruption of tight and adherens junctions and leads to dilated intercellular space (DIS), a hallmark of GERD. In this study we employ an *in vitro* cell culture system that mimics the esophageal squamous epithelium using an immortalized primary esophageal keratinocyte cell line that can be grown and differentiated into stratified squamous epithelium within 3-6 days. We have found that treatment of these stratified cell cultures with bile salts at low pH (as experienced with GERD) reduced the abundance of functional tight junctions and decreased transepithelial resistance. Furthermore when we overexpressed the beta-1 subunit of the Na^+,K^+-ATPase, which has been shown by our laboratory to increase the expression of tight junction proteins and restore epithelial barrier function in lung tissue, we see a marked increase in junction proteins in stratified squamous epithelial tissue. Our goal is to utilize a gene therapy strategy to express the beta-1 Na^+,K^+-ATPase in the esophagus, restore effective barrier function, and prevent progression of GERD. However, there is a paucity of research describing methods for in vivo gene delivery to the esophagus in any animal model. To this end, we have developed an electroporation-mediated gene transfer to deliver naked plasmid DNA to the distal region of the rat esophagus. Gene transfer is achieved by performing an abdominal mid-line transsection surgery to isolate the distal region of the esophagus, gastroesophageal junction and proximal stomach. The esophagus is externally bathed in plasmid DNA and eight square wave pulses are delivered by an electrode that spans 1.5 cm of the targeted distal esophagus tissue. The transfected esophagus is returned to the abdomen and the incision is closed. Animals showed no short or long term effects of the surgery. High level of gene expression is achieved within 24 hours of electroporation in the esophageal mucosa and expression persists up to at least 12 weeks post delivery. This is the first use of electroporation to transfect the esophagus and one of the first reports for directed in vivo esophageal gene transfer. Our goal now is to test our therapeutic approach with the Na^+,K^+-ATPase beta-1 subunit.

484. Gold Nanoparticle for Nonviral Gene Therapy of Hemophilia B

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The current standard therapy for hemophilia B comprises a life-long frequent and expensive prophylactic administration of FIX. Thus, our aim was to develop a novel nonviral genetic therapy for hemophilia B by transferring a normal copy of FIX into hepatic cells, thereby producing the missing protein. As carriers for the DNA, we used 5nm laser-ablated gold nanoparticles (AuNPs), as these AuNPs are nontoxic and nonimmunogenic. Testing different formulations of linear and branched polyethyleneimine (PEI) in liver cell lines revealed that the nonintegrating DNA was most efficiently bound to the AuNPs via 22 kDa deacetylated linear PEI, that also ensured endosomal release of the vector after cellular uptake. We reached high-level FIX production by systematically testing different viral and human-derived promoters, optimized for FIX expression by in-/excluding introns, activating mutations and/or codon-optimization (co). Initially, promoter optimization was carried out in a lentiviral approach with limiting dilution of a self-inactivating vector, as this leads to polyclonal cell cultures with a single integrated FIX expression cassette. We transduced HT1080 cells with <0.2 infectious viral particles/cell and then selected the transduced cells with puromycin. After three days, we reached factor levels up to 184%. In parallel, we tested plasmid transfection of ten different FIX expression constructs in HT1080 and CHO cells including no, one or two introns, as well as wildtype or codon-optimized FIX with and without the Padua mutation. Here, codon-optimization alone leads to high-level FIX production without the inclusion of any intron. After transfection with equimolar plasmid concentrations, we reached factor activities of 57.4% with the CMV promoter in HT1080 cells, while the human EF1 alpha promoter with a shortened, splice-optimized first intron led to factor levels of 46.3% when FIXco carrying the Padua mutation was used as transgene. When our AuNPs were used to introduce the same plasmids into cells, we reached FIX activities of 197% with the co FIX Padua and even 304% with the same cDNA expressed by our human EF1 alpha promoter. When we transfected primary rat hepatocytes with AuNPs and different DNA vectors encoding FIX Padua, active factor levels up to 50% were measured. Interestingly, this high activity levels were achieved with the CMV promoter, whereas factor activity levels only reached 13.4% with the optimized EF1 alpha promoter. Therefore, we concluded that the human promoter is not optimally suited to promote high-level transgene expression in rat hepatocytes. In order to improve the gene transfer efficiencies and factor level activities in primary rat hepatocytes *in vitro*, we next established a layer-by-layer approach with two layers of PEI. In addition to the fact that higher PEI amounts lead to improved transfection efficiencies, this approach also allows to use a second type of PEI with specific characteristics for selective cell targeting. To this end, we included a second PEI variant that carries galactose residues

(JetPEI-Hepatocyte) to target the asialoglycoprotein receptor, that is highly expressed on primary hepatocytes. In experiments with cell lines, we demonstrated that a second layer is not detrimental and can even increase the gene transfer efficiencies further. In the next step, this approach will be employed to improve the FIX production in primary rat hepatocytes and ultimately allow efficient targeting of the AuNP-PEI-DNA complexes to hepatocytes *in vivo*.

485. Whole-Body I-124 PET Imaging of Adeno-Associated Viral Vector Biodistribution in Non-Human Primates

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The development of a genome-independent, *in vivo* imaging biomarker for vector biodistribution in humans would be useful for the assessment of both on-target and off-target effects in a wide range of gene therapy applications. We previously demonstrated that adeno-associated virus (AAV) capsids could be stably radioiodinated using two independent labeling techniques [Kothari P, et al, *Scientific Reports* (2017)]. Here we apply the method to evaluate the organ biodistribution of AAV gene transfer vectors in naive and immunized non-human primates. Radiolabeling of AAV9 and AAVrh.10 capsids with ^{124}I allowed for visualization of organ biodistribution of vector using positron emission tomography (PET), immediately and several days after administration. Labeling was accomplished using the Iodogen method. We achieved up to 1 mCi of labeled vector for each synthesis, demonstrating the potential for translation to human use. The radiolabeling yield of ^{124}I-AAV9 and ^{124}I-AAVrh.10 product was 11.8% ± 6.1% corresponding to 2.18 ± 1.20 ^{124}I atoms per capsid on average. Each serotype (5E12 gc) was administered via intravenous (IV) or intracisternal (IC) routes to African Green monkeys (*chlorocebus aethiops*), whose adult average head and body length of approximately 45 cm allowed transverse positioning in our Siemens Biograph human PET scanner, which in turn facilitated rapid whole-body imaging. PET (1 hr scans) was performed on the day of vector administration and for 3 successive days, at a spatial resolution of 5.0 X 2.0 X 2.0 mm^3 (**Figure 1**). Control animals injected with [^{124}I]NaI exhibited rapid clearance of activity to the thyroid and gut by either IV or IC delivery routes. Following IV administration to immune naïve animals, distribution of both AAVrh.10 and AAV9 were dominated by liver uptake, while IC administration was dominated by CNS activity, but also with systemic diffusion and significant liver distribution. In addition, preimmunity to the vector capsid appears to significantly affect biodistribution, shifting from predominance in the liver to the spleen in both routes of vector administration. Of note, activity in brain parenchyma was limited in all cases to a small percentage of injected activity (<5%). This technology can be directly translated to humans, and potential applications include the development of quantitative viral vector dosimetry, which should aid in therapy planning, rapid prototyping of novel vector designs involving capsid modifications that effect biodistribution, and dynamic studies of viral infection processes.

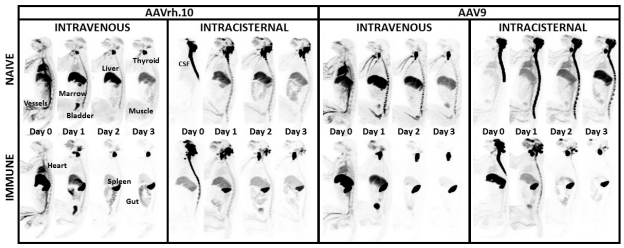

Figure 1. Imaging vector biodistribution in non-human primates after ^{124}IAAV administration. Each animal was scanned on 4 successive days starting on the day of administration. PET scanning on Day 0 started immediately after intravenous injection of ^{124}IAAV and approximately 20 min after intracisternal injection. Vector was first administered when the animal was serotype naive, and again after development of serotype immunity approximately 10 wk later.

486. Hydrodynamic Intrabiliary Infusion of Naked DNA Vectors in Weaned Pig for a Liver-Directed Gene Delivery

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Therapeutic vectors for gene delivery and translation to clinical application remain challenging factors for human gene therapy. We established hydrodynamic intraportal injection of naked DNA vectors in domestic weaned pigs as a large animal model for liver gene transfer (DOI:10.1089/hgtb.2014.140). Here we improved the procedure and developed a surgical method that allows vector administration via the bile duct as a potentially safer alternative. For both hydrodynamic intraportal and intrabiliary injections, laparotomy and transient clamping of V. porta, V. cava caudalis, and Aa. hepaticae were performed. For intrabiliary injection, access via the hepatic duct was established by enterotomy and insertion of a catheter through the papilla. In addition, common bile and cystic ducts were clamped. Injection conditions for both methods were 10 ml/sec and a volume of 100 ml containing between 2-12 mg of vector DNA, and vessels remained clamped for up to one minute after injection. Titrating of vector DNA dose to up to 12 mg resulted in 100% transfection of hepatocytes upon intraportal injection (day 3). In comparison, we found that the intrabiliary injection method was less stressful, showed a stable circulation and faster recovery for the pig, and a transfection rate of 100% by delivering 12 mg of vector DNA (day 3). While further optimization is ongoing, preliminary results indicated a 10 to 100-fold lower copy number and luciferase activity compared to the intraportal injection. In summary, the intrabiliary procedure for hepatocyte

transfection was less invasive over the intraportal access and may allow injections by endoscopic retrograde cholangiopancreatography (ERCP) access.

487. Bipolar Electrotransfer Enhances Gene Delivery

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Gene electrotransfer (GET) is a gene therapy that uses short-duration, electrical pulses to permeabilize living cells aiding in the delivery of exogenous DNA. For majority of GET applications, monopolar pulsing protocols across pairs of electrodes placed at the treatment site *in vivo* have been successfully applied to achieve gene delivery. A notable drawback to exposing tissues to strong electric fields is the stimulation of excitable cells, such as neurons and muscle cells, resulting in mild to severe outcomes of twitching, pain or in the case of gene delivery to the cardiac muscle, ventricular arrhythmias. Successful GET depends on optimization of electrical field strength, pulse number, pulse duration, and pulse frequency for specific cell types achieving high levels of both permeabilization and viability activity. Literature has shown, that high-frequency, short-duration bipolar bursts may minimize muscle twitching during irreversible electroporation (IRE) of tumor tissue. We hypothesize the IRE bipolar pulsing protocols can be adapted for gene delivery. Here, we examine the application of bipolar pulsed electric fields for gene delivery and expression *in vitro*. B16F10 melanoma cells were exposed to plasmid DNA encoding firefly luciferase and subjected to bipolar pulses. Optimal pulse conditions were determined experimentally by varying electric field strength between 600 and 900 V/cm, pulse duration between 10 and 2,500 microseconds, and frequency between 5 and 500 Hz. Relative gene expression was measured using the IVIS Spectrum Imaging System for bioluminescence. Transfection efficiency was evaluated microscopically and metabolic activity was quantified via PrestoBlue Assay. Bipolar GET with more than 500 pulses of 20 μs duration enhanced gene expression with bipolar GET mediated plasmid DNA delivery to cultured cells over no pulsing controls ($p<0.05$). Increases to the applied electric field strength above 600 V/cm exceeded the irreversible electroporation threshold resulting in 20% enhanced cell death ($p<0.001$) without significant change to expression. However, at 100 Hz, expression increased with pulse number ($p<0.0005$) to a threshold where afterwards, significant change to expression was not observed. Conversely, metabolic activity was unaffected by escalated pulse numbers until the threshold was exceeded and viability subsequently decreased. Therefore, optimization of pulsing parameters in the microsecond range for bipolar GET protocols can result in enhanced gene delivery.

488. A Strategy for Therapeutic Targeting in Pathological Cells with Mutant Mitochondrial DNA Using Mitochondria Targeted Minor Grove Binders

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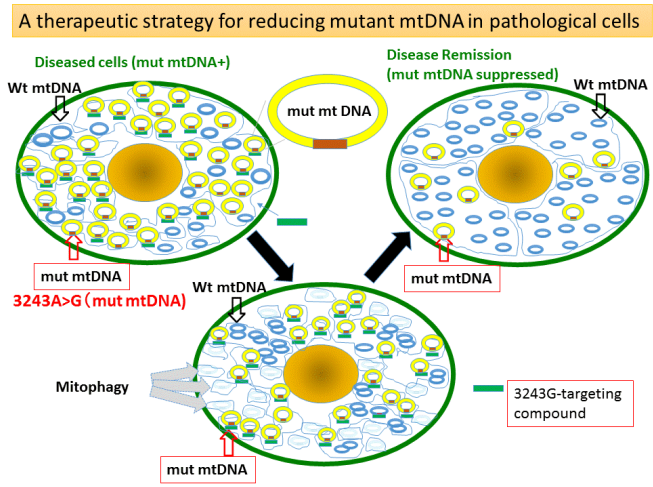
Mitochondria are essential intracellular organelles that regulate energy metabolism, cell death, and signaling pathways that are important drug targets not only for mitochondrial diseases but also for cancer, cardiovascular, and neurological diseases. Hetero/homo-plasmic functional variants of mitochondrial DNA (mtDNA) in patients with mitochondria-related diseases are often involved in pathological state of the diseases. A strategy reducing pathogenic mtDNA mutations must be a clinically relevant approach. We initially synthesized DNA minor grove binder (MGB) compounds targeting the A3243G mutation and have no success in reducing mutant mtDNA. Because mtDNA exists in several hundred to several thousand copies in one cell, it is necessary to increase retention time of MGB in the mitochondria in order to influence mtDNA replication. We therefore developed lipophilic cation-based compounds conjugated with MGB to intend to inhibit mutant mtDNA replication in the pathogenic cells using 3243G Low cybrid and 3243G High cybrid with a different proportion of A3243G mutation harboring ρ0 cells from HeLa cell. They showed mitochondria localization and long-time retention. As a result of long-term exposure, the total copy number of mtDNA tended to increase, while copy number of mutant mtDNA tended to decrease. Intriguingly a conjugate induced inhibition of cell proliferation and apoptosis in cells harboring A3243G mutant mtDNA as the copy number increased. Hence, there is a possibility that the conjugates act on target mutant mtDNA and suppress the proliferation of tumor cells harboring the target mutant mtDNA, which can lead to suppress cancer proliferation, as well as the tendency to change the copy number of the mutant mtDNA. Thus, lipophilic cation- MGB conjugates are promising therapeutic drug candidates for mtDNA mutation-related diseases.



489. Nanoparticle Mediated Non-Viral Delivery of Messenger RNA in Human T Cells Towards Development of CAR T Therapy

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The FDA's recent approval of Kymriah® and Yescarta® CAR T cell therapies for treatment of non-Hodgkin lymphoma and B-cell acute lymphoblastic leukemia, and the breakthrough clinical results, represent just the beginning of the translation of many additional CAR T and other cell-based therapies to the clinic. However, the clinical translation and widespread adoption of cell-based therapies is hampered by the safety, manufacturing and performance limitations of current technologies for modulating gene expression in cells. For example, while viral vectors are in clinical use and provide stable long-term gene expression, there are safety concerns pertaining to immunogenicity and insertional mutagenesis and substantial manufacturing challenges. Non-viral delivery of nucleic acids such as messenger RNA (mRNA) result in transient gene expression and address some of these safety concerns. However, conventional non-viral methods such as electroporation and chemical reagents often require a trade-off between efficiency and cell viability while neglecting issues of sustainable and consistent manufacturing. The objective of this work is to demonstrate the manufacturing and performance advantages of a new nanoparticle (NP) technology for gene delivery to cells. This is presented as a case study for CAR T therapy development by showcasing NP-mediated delivery of messenger RNA (mRNA) to human T cells ex vivo. The NPs were formulated using chemically-defined components and mRNA, and manufactured at microliter volumes using microfluidic mixing on the NanoAssemblr® Spark Platform. The formulated NPs encapsulated a mRNA encoding EGFP as a reporter gene to evaluate transgene expression. The total manufacturing time for the mRNA-NPs was less than 5 minutes. The mRNA-NPs had a diameter of 100 to 150 nm, with a polydispersity index of 0.1 to 0.3 and a mRNA encapsulation efficiency of more than 80%. Donor-derived human T-cells were isolated from fresh whole blood following standard protocols. Dosing T-cells with these mRNA-NPs resulted in high transfection efficiency of EGFP while maintaining exceptional viability and normal proliferation rates post-activation. In addition, EGFP expression levels were found to be very homogeneous across all positively-expressing cells. Further optimization of formulation and treatment parameters as well as T-cell activation status were found to have significant effects on maximizing EGFP expression. Subsequent testing in multiple donors with a range of ages demonstrated high reproducibility regardless of donor sex or age. This case study highlights the potential of this new NP technology for effective gene expression in T-cells ex vivo. The results demonstrate the ease and speed of manufacturing NPs from chemically defined raw materials using a scalable microfluidics mixing process. This is favorable over manufacturing viral vectors using a complex and time-consuming biological process. It should also be possible to greatly simplify cell therapy manufacturing because the NPs are added to cells in a single step. In addition, these results show the impressive performance of the NPs in human T-cells to transiently express a gene of interest while maintaining high viability. This is a notable advantage over electroporation and chemical reagents and could lead to improvements in the quality attributes and efficacy of cell therapies. In conclusion, this new NP technology provides an innovative solution for overcoming the manufacturing and performance limitations of existing non-viral methods for gene delivery. This NP technology should help enable the development and clinical translation of next-generation CAR T therapies and other cell-based therapies.

490. Efficient Gene Editing of Primary Human T Cells using Solupore Ex Vivo 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. Solupore® uses reversible permeabilization to achieve rapid intracellular delivery of cargos with varying compositions, properties and sizes [1]. 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. The process is rapid and cargo transfers directly into the cytoplasm by diffusion in an endocytic-independent manner. We have termed the method 'soluporation'. Non-viral methods, such as soluporation, that enable intracellular delivery of various cargo types for clinical applications have been proposed as attractive candidates as next-generation delivery modalities because of potential benefits for production, safety and regulation [2]. We previously reported that soluporation does not perturb gene expression or cell surface markers in T cells, unlike electroporation [3]. Furthermore, cell proliferation and *in vivo* engraftment is superior in soluporated cells compared with nucleofected cells [3]. Here we report successful scaling of the technology and demonstrate efficient gene editing of primary human T cells using several gene editing platforms. Previously we reported that soluporation could be carried out in a 96 well format. At that scale, approximately 5 x 10^5 primary human T cells can be soluporated with cargos including mRNA with 80% transfection efficiencies. However, pre-clinical and clinical studies will require significantly larger numbers of engineered cells. The method has now been scaled and semi-automated such that up to 8 x 10^7 T cells can be soluporated. GFP mRNA was delivered to either CD3 bead-activated T cells or PBMC initiated T cells. GFP expression was analysed by flow cytometry at 24 hr post-soluporation. Transfection efficiencies >80% were achieved in both cell types. The feasibility of using soluporation to deliver gene editing tools was also evaluated. mRNA encoding for three different proprietary gene editing molecules was delivered to either CD3 bead-activated T cells or PBMC initiated T cells. Edit efficiencies in excess of 50% were achieved. This study demonstrates that the Solupore® technology can be successfully scaled to address clinically-relevant numbers of primary human T cells. Furthermore, efficient levels of gene editing can be achieved across multiple gene editing platforms. Thus the Solupore® technology is gentle yet highly reproducible, automated, and scalable and has the potential to enable a broad range of T cell engineering applications. References 1. O'Dea et al. Vector-free Intracellular Delivery by

Reversible Permeabilization. PLOS ONE. 2017. 12(3):e0174779. 2. Stewart et al. In vitro and ex vivo strategies for intracellular delivery. Nature. 2016. 538(7624):183-192. 3. O'Dea et al. In Vivo Engraftment of T Cells Transfected using Solupore is Superior Compared with Electroporation-based Systems. Molecular Therapy. 2018. 26(5S1):423.

491. An Efficient, Modular and Scalable Mitochondrial Delivery Vector System for RNA and DNA - Toward Mitochondrial Gene Therapy

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Defects of mitochondrial DNA are associated with mitochondrial disease, with cancer and with aging. Mitochondrial gene therapy represents a promising treatment option but is hampered by the lack of efficient mitochondrial delivery vectors. The long non-coding ß2.7 RNA of the human cytomegalovirus was described to localize to the mitochondria of mammalian cells and to bind and stabilize mitochondrial complex I. We identified four conserved structural subdomains within the ß2.7 RNA which govern the mitochondrial targeting activity. The most active subdomain alone resembled the activity of the full-length ß2.7 RNA. Tetrameric domain repeats arranged in tandem showed three-fold higher activity, depicting the modularity and scalability of this mitochondrial delivery vector system. Targeted mitochondrial delivery of antisense RNA triggered up to 97% knockdown of mitochondrial genes MT-ATP6/8 leading to a reduction of mitochondrial ATP levels and cell viability. ß2.7-mRNA chimera transcribed in the nucleus were successfully targeted to the mitochondria triggering recombinant intra-mitochondrial EGFP expression. A mitochondria-targeting RNA domains attached to a single-stranded circular DNA via complementary base pairing, co-delivered the 6,400 nt DNA into the mitochondria. Finally, the shortest mitochondria-targeting ß2.7 RNA-derived subdomain of 100 nt in length, protected human dopaminergic neuroblastoma cells from 6-hydroxidopamine-induced reactive oxygen species in a Parkinson's disease model. Currently, we investigate the potential of extended dodecameric and icositetrameric domain repeats to enter the mitochondria and to co-deliver 'healthy' mitochondrial genomes. This powerful mitochondrial delivery vector system can be explored toward mitochondrial gene therapy of human diseases including cancer, controlling of inflammation and immunity, and for anti-aging.

492. Cytoplasmic Nucleic Acids Sensing in Mouse Myoblasts after Plasmid DNA Transfection: Potential Involvement of Multiple Proteins

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Cytoplasmic nucleic acids sensing represents a first line of organism defense against the pathogens, activating signaling cascades that lead to cytokine secretion and inflammation to build an appropriate immune response. Despite extensive advances in understanding the mechanisms of pattern-recognition receptors (PRRs) action within last two decades, many important details of this process still remain unknown. Our previous studies had proven that electroporation of plasmid DNA (pDNA) into the different tumor cell lines leads to upregulation of PRRs on both mRNA and protein levels in a cell line-specific manner. Upregulation of type I interferon was also detected on both mRNAs and protein levels after pDNA electroporation, pointing to involvement of innate immune pathways in cellular response to the presence of pDNA in the cytoplasm. However, the sequence of events after pDNA electroporation has not yet been clarified. Our group used molecular and proteomics techniques to identify PRRs that can detect pDNA in cytoplasm as soon as 15 minutes after electroporation and to monitor involvement of different PRRs in cytoplasmic pDNA sensing for up to 4 hours. Our data point to ZBP1/DAI protein as an initial sensor of pDNA with the potential later involvement of other PRRs. Better understanding of the molecular mechanisms of nucleic acid sensing by PRRs may allow us to manipulate the process of innate immune activation, developing new and improving existing therapies for diseases such as cancer.

493. A Conjugatable, Adenovirus-Derived Tight Junction Opener Targets Tumors and Enhances Cancer Therapy

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Although the field of cancer therapy has advanced significantly in the last few years due to new therapeutic approaches such as checkpoint inhibitors, several key problems remain for effective treatment of tumors. The physical barriers which tumors use to block entry of therapeutics mediate drug resistance to even the most advanced treatment approaches. A primary mechanism hijacked by cancers to evade killing is a protein which facilitates junction formation, desmoglein-2 (DSG2), which is part of the desomosomal complex, and has repeatedly been observed to be upregulated in multiple cancers. This upregulation results in a physical barrier that prevents the entry of small molecule drugs, biotherapeutics, or immune cells. To address this problem, we have developed a DSG2-targeting therapeutic which

is a junction-opener ("JO"). JO is derived from the adenovirus 3 knob protein which causes the transient opening of tumor tight junctions and is specific for tumors due to the exposed nature of DSG2 in cancers versus healthy tissue. JO acts as a tumor-specific homing molecule because cancerous cells have little polarization making the desmosome-like structures accessible to external targeting molecules. Leveraging this, we built on our previous work targeting tumor tight junctions by additionally developing a conjugable form of the JO (termed "JOC-x"), allowing therapeutic attachment using simple maleimide chemistry. We explored these conjugates by simulating two therapeutic modes, Abraxane® and Doxil®. Conjugation was carried out using a maleimide activated form of the carriers (serum albumin and PEGylated liposomes). The conjugates retain DSG2-binding activity and are stable in solution, and are expected to enhance homing and efficacy of the payload in xenograft tumor models and ultimately in therapeutic settings. When successful, this approach will revolutionize multiple therapy approaches in cancer by eliminating the tolerizing tumor microenvironment while targeting delivery to cancers that are sensitized to treatment by the targeting approach itself.

494. Lipid Nanoparticle Formulations Optimized for Delivery and In Vivo Gene Editing Using Novel Synthetic RNA Guided Nucleases (sRGN)

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Lipid nanoparticles (LNPs) are a robust and effective technology for delivering nucleic acids to the liver, including multi-component systems incorporating both mRNA and gRNA for gene editing. However, typical mRNAs used for Cas9 expression are large and complex RNA payloads. A separate abstract details the engineering of a set of novel synthetic RNA Guided Nucleases (sRGN). When formulated as mRNA into LNPs, we report here the improved performance of sRGN compared to the widely used SpCas9 genome editing endonuclease. We believe that the small size (~3.5 kb) of sRGN mRNA is responsible for the observed packaging advantage into LNPs compared to SpCas9 mRNA (~4.4 kb). When evaluated *in vivo* in rodent, LNPs harboring alternative sRGNs each showed comparable or higher editing efficiency to SpCas9-LNPs—even at equimolar doses. Toward understanding the mechanism for these LNP delivery improvements, cryoTEM analysis of sRGN-LNPs gives evidence for improved LNP morphology and physicochemical characteristics, which suggest a better quality LNP formulation with these smaller payloads. Interestingly, sRGN-LNPs also showed enhanced particle stability compared to SpCas9-LNPs; sRGN-LNPs were less prone to aggregation over time and retained *in vivo* potency significantly longer than SpCas9-LNPs. Over the same storage conditions, a ~70% drop in editing efficiency was observed with SpCas9-LNPs whereas a potency drop as small as ~20% was observed with sRGN-LNPs. Overall, these novel sRGN-LNPs represent a promising alternative to known CRISPR/Cas delivery systems showing initial favorable *in vivo* gene editing performance and enhanced particle stability.

495. New Ramified Cationic Amphiphiles as Novel Efficient Gene Delivery Systems

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Cystic fibrosis (CF) is an autosomal recessive genetic disorder that affects the gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). The alteration of CFTR channel induces a dysfunction of ions transportation conducting to mucus dehydration. Mucosal accumulation leads to bacterial colonization and lung failure which is still remaining the main cause of death. One solution to restore CFTR expression consists to introduce a transgene encoding CFTR directly toward the airway epithelium. Due to is safety, aerosolisation of a such transgene represents the most suitable choice to achieve pulmonary epithelium. Non-viral vectors, like cationic lipids, have the ability to compact and protect nucleic acids to form lipoplexes. Despite a low transfection efficiency compare to viral vectors, they present the advantage to be re-administrable due to their poor immunogenicity. Repeat delivery is necessaire because of the transient transgene episomal expression and the renewal of epithelial airway. One of them, GL67A (Alton et al, Lancet 2015) was recently tested in clinic with a certain success. Yet, the improvement of delivery synthetic vectors is required to deeply change the CF phenotype and to equal the efficiency of viral vectors. Hence, we focused on the incorporation of a ramified poly carbons chain on the lipid part. Then we evaluated their transfection capacity when they were formulated alone or mixed with either 1:0.5, 1:1 or 1:2 cationic lipid / DOPE ratios (mol:mol). Complexation assays were performed using 0.25µg of an optimized CpG free plasmid encoding a luciferase reporter gene (Hyde et al, Nat Biotech, 2008) with different charge ratios (CR) of cationic lipid (CR 0.5 to CR 8). The related nanocomplexes were tested on three different pulmonary cell lines A549, 16HBE14o- and Calu-3, in order to determine their efficiency for gene transfection and their cytotoxicity. The reporter gene activity was evaluated according to the expression of luciferase 24h after transfection. First results after deposition showed luminescence signals two log higher than the standard lipophosphoramidate KLN47, used as a positive control (Picquet et al, Bioconj Chemist, 2005). We also reported that the supplementation of DOPE is beneficial for an efficient formation of the complexes. Indeed, 1:1 cationic lipid / DOPE ratio showed a better transfection efficiency in comparison with cationic lipid alone. Moreover, these vectors present a slight cytotoxicity on Calu-3. This particular cell line has the ability to differentiate and product mucus when cultured in air liquid interphase. One of the greatest challenge for CF gene therapy is to overcome the mucus barrier. In CF context, airway epithelium are recovering by a thick and viscous mucus which is

impeding lipoplexes to reach pulmonary cells. Ongoing investigations are running to determine the ability of this new ramified cationic amphiphiles to penetrate the CF mucus.

496. Dual-Targeting Smart Nanoparticles for SiRNA Delivery and Cancer Therapy

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Hepatocellular carcinoma is the most common type of primary malignancy in the liver. We developed a siRNA delivery system, HLPR, through modular assembly of the different components. This has a condensed siRNA-peptide inner core which is surrounded by a lipid layer and a thin hyaluronic acid (HA) coating from which a specific EGFR-targeted amino acid sequence (peptide) partially protrudes. The HA and the anchored peptide on HLPR were responsible for targeting CD44 and EGFR which are overexpressed on the tumor cell surfaces, respectively. HLPR was relatively stable in the circulation and reached the tumor *in vivo* through passive and active targeting. Then the HLPR nanoparticles entered the tumor cells mainly through the EGFR-mediated pathway followed by the separation of HA from the remaining parts of the nanoparticles. The HA-uncoated complexes escaped the endosome through the membrane fusion function of DOPE and released their cargoes (siRNA and/or peptide/siRNA) in the cytoplasm. The HLPR nanoparticles showed significant inhibition of the engrafted liver tumours without any associated toxicity to the mice. This is a platform that could find applications in other solid tumours.

497. Development of Novel Injector: Pyro-Drive Jet Injector (PJI) Application to Intradermal DNA Vaccination

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Vaccination has progressed with time, there have been lots of different types developed to protect against and cure diseases. DNA based vaccines (DNA vaccine) is one type of vaccination; it was developed as an alternative to the conventional protein based vaccine. Even DNA vaccination could apply to different fields such as cancer or infection disease, DNA vaccine delivery still needs to be improved. To resolve this issue, we investigated the potential of pyro-drive jet injector (PJI; Daicel Corporation PJI). Here we determined the suitable injection conditions to deliver DNA to epidermis or dermis region on both rat and murine models. Under suitable injection conditions, 3D microscopic analysis results showed the PJI injection delivered Cy3 labeled plasmid into the nucleus at higher frequency than by traditional syringe method. Additionally, after PJI injection with luciferase expression plasmid, luciferase expression was 7-37 times higher than traditional syringe injection group. Even though PJI was able to induce protein expression after expression plasmid injection by PJI, we wanted to know whether PJI is able to be used as a DNA vaccine device. To investigate antibody induction performance of PJI, we used ovalbumin (OVA) expression plasmid for antibody induction model. When OVA plasmid was injected by PJI, OVA antibody was detectable in the rat model. PJI injection stimulated antibody production dose dependently following the plasmid injection. In contrast, traditional needle syringe method did not result in robust antibody production or was undetectable. In conclusion, we have not only determined the applicable PJI injection conditions to target the epidermis or dermis regions but have also shown the protein expression and antibody production abilities by PJI. Our results showed that PJI is a reliable method of DNA delivery, resulting in subsequent protein expression and has the potential in reshaping DNA vaccine delivery method.

498. Systemic Delivery of Helical Polypeptide Induces Tumor-Specific Apoptosis

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A cationic helical peptide (CHP) has been intensively used as a gene or drug delivery carrier, and as antibiotics for therapeutic purposes in biomedical fields. CHPs are insufficient as delivery systems because they have a short chain length giving rise to a low helical propensity. In an effort to resolve these inherent limitations, artificial cationic helical polypeptides (ACHP) have been recently devised by further modifications of the side chains in functional group-bearing polypeptides. ACHP possess higher cell-penetrating property than conventional cell-penetrating peptides. However, these ACHP do not possess specificity toward diseases and penetrates into cells indiscriminately like their predecessors. To address these limitations, we have added pH-sensitive anion-donating groups to a helical polypeptide enable the peptide to selectively penetrate into tumors through pH-dependent formational change of peptide following exposure to acidic tumor microenvironment. The mitochondria-destabilizing helical polypeptide undergoing pH-dependent conformational transitions led to efficient targeting of tumors and disruption of mitochondrial membranes, thus leading to tumor-specific induction of apoptosis. This work presents a promising peptide therapeutic system for cancer therapy.

499. Generating Extracellular Vesicles from hMSCS in Scalable Xeno-Free Manufacturing

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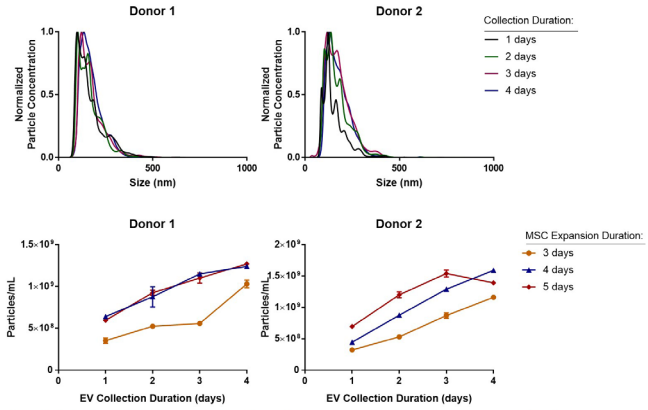
Introduction: Extracellular vesicles (EVs) are released from many different cell types. They can contain both proteins and nucleic acids inside a lipid membrane that serves to protect the cargo. EVs similarity to liposomes has been noted and EVs are increasingly being investigated as both a cell-free therapy and a drug delivery vehicle (Lim 2015). In particular, mesenchymal stem/stromal cells (MSCs) have been used in over 800 clinical trials, and offer a well-studied cell source to generate EVs. A critical barrier in the development of MSC-EVs as a commercial therapy, however, is generating the large amount of EVs that will be required per dose. In this study, two strategies were investigated to maximize EV yield. First, timing parameters for culturing human bone-marrow derived MSCs and collecting MSC-EVs were evaluated to increase EV yield. Second, conditioning the cells was investigated by adding an EV activator during the EV collection period. **Methods, Results, & Conclusion:** MSCs were first cultured in an expansion media (RoosterNourish™) in a fed-batch system. The cultures were then switched to a collection media (RoosterCollect™-EV) with or without an EV activator (EV Boost™). After various timepoints, the conditioned media was harvested. The EVs in the collected conditioned media were analyzed for particle concentration and particle size distributions by nanoparticle tracking analysis, protein expression, RNA expression, and wound healing capability. Particle size distributions were similar among the different conditions with no significant differences observed in media collected from the varying expansion duration and collection duration groups (Figure). Particle concentration (particles per L of media) was used as a measure of EV yield. Particle concentration increased with increasing culture time in expansion media, with confluent cultures generating the most EVs. An EV activator could further increase particle concentration. The EV yield from the low EV activator culture at 24hrs was still about 45% greater than the yield of the culture without the activator after 48hrs. After only 6hrs of collection, the high EV activator concentration increased EV yield to about the same level as without the activator after 48hrs of collection. Optimizing protocols for EV yield will become increasingly important as MSC-EVs move towards the clinic as cell-free therapies or drug delivery vehicles and lot sizes of clinically-relevant doses are required. Pre-conditioning of cells or conditioning cells during EV collection offers one possibility to further increase EV yield and decrease process time.



Top Particle size distributions normalized to particle concentration for each group, are similar for particles collected from conditioned media of up to 4 days of cell culture. **Bottom** Particle concentration increases with increasing collection duration and expansion.

500. Biocompatible and Biodegradable Smart Polymeric Nanogels as Biologics Delivery Vehicles

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Introduction: Amphiphilic degradable hydrogels have been the focus of much research due to their biomimetic and biocompatibility characteristics. Two fields of stimuli-responsive scaffold erosion and controlled therapeutic cargo delivery have benefited tremendously from advances in designing new degradable hydrogel systems. Extensive work in fabrication of biodegradable hydrogels include crosslinked network construction via radical polymerization from different combinations of amphiphilic block copolymers of polyethylene glycol (PEG) as the hydrophilic segment and polylactide (PLA) as the hydrophobic hydrolytic ally degradable segment with (meth) acrylate end functionalities. Both of these polymer chains (PEG and PLA) are biocompatible and FDA-approved in a variety of biomedical applications. Combinatorial effect of PEG and PLA in the copolymer by changing their structural order, and molar ratios have enabled scientists to manipulate several properties of the hydrogel network such as permeability, degradation rate, and mechanical integrity. **Rationale:** Our solution incorporates using our proprietary technology, the polymeric globular nanogels with tunable size between 1.5-20 nm with large surface area to volume ratio, to solve multivalent bio-conjugation of drug/antigen/aptamer/gene-editor/gene either on their surface and/or interior, or precursors for tissue regeneration. Their ultra-small size, enables them to participate in non-active and active transport across the cell membrane. In terms of mechanical integrity, these particles are elastically deformable, which put them in the classification between colloids and hard spheres. As reactive macromers, aqueous dispersions of the nanoparticles can readily be covalently interconnected to yield in situ micro- or macro-scopic polymer networks. The nanogels and the corresponding polymer networks are also degradable with tunable mode and rate of degradation in molecular scale that is translated into tunable mode and rate of erosion in macroscopic scale. The degradation/erosion tunability is embedded in the molecular design aspect, rather than based on the introduction of new monomeric components to the

nanogel structure. The molecular weight of polymeric by-products is less than 30 kDa, which is the cutoff to be excreted through the kidneys. The non-exhaustible design strategy allows us to create a platform that provides cargo-specific vehicles.

501. Development of Pyro-Drive Jet Injector: Transdermal Administration with the Biphasic Pressure Delivery System and it's Plasmid DNA Expression

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Jet injection is a promising method to achieve effective and efficient administration of drugs, and especially expected for DNA vaccinations. However, conventional jet injectors (CJIs) driven by springs and gas have not been used yet for DNA vaccination in the present clinical practice. We considered CJIs as required further improvement, and addressed on developing jet devices employing pyrotechnics that are based on explosive combustion pressure (pyro-drive jet injector; PJI). Furthermore, the optimized PJI for intradermal administration was compared with traditional needle syringe regarding the intradermal plasmid DNA expression in the laboratory animals. The jet pressure of PJI was determined using a load cell. The explosives (ignition powder) resulted in the first peak of pressure and the peak value increased dose-dependently. The combination with the other kinds of explosives (smokeless powder) made the following second peak of long lasting pressure. The pressure profiles were compared with injection processes using transparent urethane gels and porcine skins, pigs, rats and mice. The comparative analyses of those data revealed that the transdermal penetration depth was controlled by modulating the amount of ignition powder generating the 1st peak, and that the injected liquid volume into the tissue increased by adding smokeless powder generating the 2nd peak. The bi-phasic delivery of PJI was highly reproducible and made injection depth controllable in the transdermal tissue layers including intradermal, subdermal and subcutaneous. Those data indicate that the PJI with optimized pressure profile can be widely applicable to ranges of human mimic large laboratory animals as well as small animals. Next, transdermal administration of Cy3-labelled or luciferase-coded plasmid DNA using PJI and traditional needle syringe in pigs and the following plasmid distribution and expression analysis was conducted. In the PJI administration, laser-confocal microscopic observation revealed that Cy3-plasmid distributed preferentially in the intradermal tissue. Interestingly, the merged image of Cy3 and nuclear DAPI staining indicated that the major population of cells was Cy3-posivitve/DAPI-positive in the center of administrated region, and that the intra-cellular co-localized staining was preferentially observed in the intra-nucleus. In the needle syringe administration, only a few number of Cy3-positive cells were observed and the number of frequency was definitely lower. It was also found that the level of luciferase activity expressed in the tissue was much higher with PJI. Those data indicate that PJI with optimized pressure profile can be applicable to plasmid DNA distribution and expression study, suggesting that PJI might be more effective compared with needle syringe in DNA vaccination. Thus, these findings demonstrate that our PJI could be a novel promising alternative of administration devices for laboratory and clinical use. The capability for gene and cellular therapy would also be supported by companion presentations in this meeting.

502. BIUXX

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RNA- and DNA-based drugs can specifically turn any gene on or off. However, whether the drug is made out of siRNA, mRNA, Zinc Finger Nucleases, CRISPR, or others types of DNA / RNA, all genetic drugs are limited by one universal problem: drug delivery. When injected, DNA and RNA drugs tend to accumulate in the liver. Non-liver delivery remains a significant problem. Engineers and chemists have designed thousands of chemically distinct nanoparticles to deliver these drugs to target tissues. After synthesizing these chemically diverse nanoparticle 'libraries', the nanoparticles are typically screened *in vitro.* However, *in vitro* conditions typically lack an immune system, kidney, spleen, pulsatile blood flow, and other factors that affect the nanoparticle *in vivo.* For example, we recently compared how 400 different nanoparticles delivered genetic drugs *in vitro* and *in vivo;* we found no correlation. We therefore reasoned that a method to rapidly screen thousands of nanoparticles directly *in vivo* would allow us to rapidly and efficiently discover nanoparticles with novel tropisms. To this end, we developed a series of increasingly advanced DNA barcoded nanoparticle systems. Here we report three such systems that can (1) measure hundreds *in vivo* nanoparticle biodistribution with 100,000,000x more sensitivity than fluorescence, (2) quantify how hundreds of nanoparticles functionally deliver mRNA to dozens of cell types in a single mouse, or (3) quantify how hundreds of nanoparticles functionally deliver siRNA to dozens of cell types in a single mouse. Finally, we describe a bioinformatics pipeline to iteratively use these large datasets in order to 'evolve' LNPs with new tropisms. Using these new assays and new analytical pipeline, we have identified LNPs that deliver different many types of therapeutic RNAs to new cell types *in vivo.*

503. Angio-PhaGel: Phage Based Angiogenic Extracellular Matrix Niche Engineering

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Although stem cell niche plays a vital role in stem cell differentiation towards different lineages, an artificial stem cell niche achieved so far is not successful to fulfill the complex microenvironment of the stem cell. Here, we demonstrated engineered hybrid phage matrices that possess cell adhesive and angiogenic peptides with a suitable scaffold by formulating polyacrylamide hydrogel incorporating phage in different stiffness to guide adult stem cells (ASC) and could achieve higher stiffness favoring osteogenesis and lower stiffness favoring adipogenesis. In this study, we present a specific phage based angiogenic matrices by modulating physical and biochemical cues in differentiation of ASC, providing convenient artificial stem cell niche.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

504. Surrogate Biomarkers to Assess Efficacy of Liver-Directed Therapies in Methylmalonic and Propionic Acidemias

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Background: Methylmalonic acidemia (MMA) and propionic acidemia (PA) are two inborn errors of metabolism affecting the distal steps of propionate oxidation and, consequently, share clinical and biochemical manifestations. Liver and/or kidney transplantation (LT/KT/LKT) are employed to stabilize severely affected MMA and PA patients, while intense efforts to develop genomic therapies for both disorders are underway. Disease-specific biochemical parameters, such as plasma methylmalonic acid and 2-methylcitrate are highly variable and are affected by dietary intake and renal function. With the aim of developing additional biomarkers that correlate with hepatic enzyme activity and clinical status, we have previously established that *in-vivo* 1-^{13}C-propionate oxidation and plasma FGF21 concentrations improve significantly in response to AAV8 hAAT MUT gene therapy in a transgenic murine model of MMA (*Mut*$^{-/-}$;Tg$^{INS\text{-}MCK\text{-}Mut}$). **Methods**: Through two dedicated natural history protocols, we evaluated 75 patients with MMA and 31 with PA at the NIH Clinical Center. In the MMA cohort, 20 patients had previously undergone liver (n=4), kidney (n=4) or combined organ transplantation (n=12), while in the PA cohort 3 patients received a liver transplant. To assess methylmalonyl-CoA mutase (MUT) or propionyl-CoA carboxylase (PCC) activity, sodium 1-^{13}C-propionate was administered as a single oral or gastric bolus. Baseline VCO_2 production (ml/min) was measured by indirect calorimetry. Isotopomer enrichment ($^{13}CO_2/^{12}CO_2$ ratio) and cumulative percentage of isotopomer dose metabolized was measured using isotope ratio mass spectrometry in breath samples collected serially for two hours. A previous set of healthy volunteers (n=19) who were studied in an identical fashion served as controls. Plasma FGF21 levels were measured using ELISA. **Results**: We observed restoration of propionate oxidation to control levels after liver transplantation in both MMA and PA patient cohorts. The cumulative percent dose of 1-13C-propionate oxidized after two hours increased from 26.9 ± 12.7 to 47.2 ± 7.0% in the MMA cohort (mean ± SD, p=0.008) and from 15.9 ± 13.3 to 67.6 ± 2.9% (p<0.0001) in the PA cohort. Improved propionate oxidation was associated with a drastic decrease in FGF21 levels in transplanted patients. Circulating levels of FGF21 fell from 6383.6 ± 8268.5 pg/ml to 771.7 ± 822.1 (mean ± SD, p<0.0001) in the MMA cohort and from 2316.8 ± 2912.9 to 201.1 ±240.9 (p=0.002) in the PA transplant recipients. These new biomarkers paralleled canonical biochemical improvements: in the MMA cohort, serum [MMA] (uM) decreased from 1164.8 ±1498.2 to 352.2 ± 423.5 (p<0.002) and in the PA cohort, total methylcitrate (nM) decreased from 28689 ± 21123 to 13145 ± 3813 (p=NS). **Discussion**: After liver and liver-kidney transplantation in MMA and PA patients, 1-13C propionate oxidation is restored and plasma FGF21 concentrations are reduced. These biomarkers should be studied in concert with canonical metabolites to examine therapeutic effects of experimental liver-targeted genomic therapies for disorders of propionate oxidation.

505. 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. It is caused by biallelic mutations in one of three genes that encode subunits of the branched-chain ketoacid dehydrogenase complex (*BCKDHA*, *BCKDHB*, and *DBT*). Severe ('classical') MSUD is fatal without treatment. Dietary BCAA restriction is the mainstay of treatment but is difficult to implement, 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 risks of surgery 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 segregates with a population-specific carrier frequency of 4.5%. Patients homozygous for *BCKDHA* c.1312T>A have no residual BCAA oxidation and become 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 Australian Shorthorn and Hereford cattle as early 1986 and rediscovered within Central Indiana herds in 2015. Newborn calves homozygous for *BCKDHA* c.248C>T have a phenotype similar to the human disease. In this study, we aim to leverage the bovine model to develop a gene replacement therapy for *BCKDHA* variants of MSUD. We first designed AAV vectors expressing a codon-optimized human *BCKDHA* gene (opti-*BCKDHA*) and validated protein expression in various cell lines. The opti-*BCKDHA* cassette was packaged into AAV9 that, via systemic injection, efficiently targets the liver and skeletal muscle, tissues which exhibit highest BCKDHA expression in wild type animals and normal humans. We delivered rAAV9-opti-*BCKDHA* into wild type neonatal mice by systemic injection and used escalating doses to determine safety and efficacy of gene delivery. Large-scale rAAV manufacturing 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* within a few 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. *Co-first authors; #Co-corresponding authors.

506. Brain Connectome Analysis of Mice with Mucopolysaccharidosis Type I: Prospects for Evaluation of Gene Therapies

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Mucopolysaccharidosis type IH (MPS IH) is a disease caused by mutations in the gene that encodes the lysosomal enzyme alpha-L iduronidase (IDUA). Current treatment for MPS IH involves enzyme replacement therapy (ERT) by intravenous administration of IDUA. This therapeutic approach is capable of ameliorating peripheral aspects of the disease, but ERT of IDUA enzyme is thought to be incapable of penetrating the blood-brain-barrier to correct neurological deficits in the central nervous system (CNS). Studies using different vector systems to deliver *IDUA* gene constructs to either the liver by hydrodynamic delivery of Sleeping Beauty transposons or the CNS using intranasal delivery of AAV demonstrate partial restoration of enzyme function within the brain and, in the case of AAV, amelioration of spatial memory deficits. The neural circuits that mediate spatial memory function include areas of brain such as the hippocampal formation, thalamus, and retrosplenial cortex. We employed resting state functional magnetic resonance imaging (rs-fMRI) to assess brain connectivity in these areas of brain in MPS IH mice at 9 months of age when spatial memory impairments are quite evident. Animals were anesthetized and placed in a high field magnet at 9.4T to determine rs-fMRI of the brain by assessment of the BOLD signal. Seed analysis of the hippocampal formation in wild- type mice revealed extensive connectivity with wide areas of the cortex, thalamus, and brain stem. In contrast, seed analysis of the hippocampal formation in MPS I mice revealed a paucity of dorsal and ventral hippocampal activity, and sparse connectivity with the areas of cortex, thalamus, and brainstem. Seed analysis of the thalamus in wild-type mice revealed extensive activity in the thalamus and connectivity with areas of sensory cortex. In contrast, seed analysis of the thalamus in MPS I mice exhibited markedly reduced activity in the thalamus and functional connections to the cortex. Finally, seed analysis of the retrosplenial cortex revealed extensive activity along the rostro-caudal axis of this region of brain and extensive connectivity with subcortical structures. Seed analysis of the retrosplenial cortex in MPS I mice reveal markedly reduced activity in this region of the cortex and few functional connections with subcortical areas of brain. These results demonstrate the utility of rs-fMRI to assess the functionality of the brain connectome in wild-type mice and the marked deficits in brain activity and connectivity of age and sex-matched MPS IH mice. These findings offer the potential power of applying rs-fMRI to assess quantitatively the efficacy of gene therapies to correct neurological disorders.

507. Site-Specific Genome Editing by CRISPR/Cas9 for Hemophilia A in Human and Non-Human Primate Cells

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Hemophilia A, the commonest severe bleeding disorder, is a X-linked recessive disorder that results from mutations in the gene for blood clotting factor VIII (FVIII). Currently, there is no cure for Hemophilia A, and the best available treatment requires frequent intravenous infusion of recombinant FVIII. To enables permanent FVIII expression, our approach was to use CRISPR/Cas9 genome editing, combined with non-homologous end joining DNA repair, to integrate FVIII transgene in the *Alb* locus. To achieve this, vectors encoding Staphylococcus aureus Cas9 (saCas9), guide RNA (gRNA) and codon-optimized human B domain-deleted human FVIII (BDD-F8) Donor were tested. Proof of concept of this approach was done initially in F8 knockout (F8KO) mouse strain, a mouse Hemophilia A model, demonstrating elevated plasma FVIII levels and activity, lowered clotting times and decreased blood loss following treatment (See abstract # 2019-A-523-ASGCT for details). Here we present the results of *in vitro* studies in human and Non-human primate (NHP) cells, validating the feasibility of this approach in multiple species and supporting future clinical development. For human system, three human cell-lines: HepG2 liver hepatocellular carcinoma cells, HepaRG hepatic stem cell line, and HEK-293T cells were used. For NHP system, we used the Rhesus RF/6A chorioretinal cell line. Initially, selection of the most efficient guide-RNAs for human and NHP systems (*Alb*-hg3 and *Alb*-Mg2) was done in the HEK-293T and RF/6A cells, respectively, by T7E1 endonuclease surveyor assay and Next Generation Sequencing. For both systems, cells' co-transfections of saCas9, gRNA and BDD-F8 donor vectors were followed by SaCas9 over-expression validation by qRT-PCR, immunofluorescence staining or western blot analysis. BDD-F8 insertion at the *Alb* locus and fused *Alb*-BDD-F8 mRNA product was confirmed by sequencing. Genome editing efficiency was determined by droplet-digital PCR (ddPCR) using species-specific assays for detection of insertions and deletions (indels) and BDD-F8 insertion. Detected indels abundance for HEK-293T, HepG2, HepaRG and RF/6A were 8%, 6.5%, 0.7% and 27%, respectively. Detected indels abundances were higher following co-transfection of saCas9 and gRNA only, without BDD-F8 donor. BDD-F8 insertion abundance and BDD-F8 mRNA expression levels were evaluated as well. *In-silico* Off-target analysis for *Alb*-hg3 gRNA predicts 98% on-target specificity with 5 possible off target sites, each showing 3-4 mismatches among the 21 nucleotides of the *Alb*-hg3 gRNA site. Preliminary results from Guide-Seq cell-based off-target analysis supports a sole target for hg3 gRNA on *Alb* locus. Taken together, these results support the feasibility of our genome editing approach as potential hemophilia A treatment.

Further studies in NHP and humanized FRG mice are ongoing to evaluate *in vivo* insertion efficiencies, as well as to characterize BDD-F8 expression and secretion.

508. Abstract Withdrawn

509. AAV-Mediated TAZ Delivery Improves Mitochondrial Functional and Structure in Barth Syndrome Patient-Derived Fibroblasts

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Barth Syndrome (BTHS) is a rare, often fatal, X-linked mitochondrial disorder caused by mutations in the tafazzin (*TAZ*) gene, an important acyltransferase involved in cardiolipin metabolism. Impaired cardiolipin remodeling leads to disorganized mitochondrial cristae structures and severe mitochondrial dysfunction. BTHS patients have cardiomyopathy, skeletal muscle weakness, and increased fatigability. Initial characterization experiments in 2 BTHS patient-derived dermal fibroblast cultures representing 2 distinct mutations in the *TAZ* locus (Exon 2: 170G>T, and Exon 2: 140-152del13) revealed: 1) decreased mitochondrial circularity and roundness ratios based upon evaluations of electron micrograph images, 2) increased mitochondrial fragmentation based on a TOMM20 immunofluorescence-based mitochondrial length scoring system and the total numbers of mitochondria per area of fibroblast (higher numbers of smaller mitochondria are present in BTHS cells), 3) decreased cell viability as determined by succinate dehydrogenase-based MTT assays, 4) reduced ATP production, 5) reduced mitochondrial membrane potential based upon TMRE assays, and 6) decreased oxygen consumption as well as a reduced ability to respond to mitochondrial stress as determined by extracellular flux assays in BTHS affected cells as compared to unaffected healthy controls. While the BTHS fibroblast line harboring a missense mutation was less severely affected, both BTHS lines were significantly dysfunctional in all of our assessments as compared to healthy control lines. We hypothesized that adeno-associated virus (AAV) mediated *TAZ* gene delivery would correct the mitochondrial dysfunction observed in BTHS patient derived fibroblasts regardless of the severity of the underlying mutation in the *TAZ* locus. We therefore assessed AAV-*TAZ* gene replacement through examination of mitochondrial function and structure in the BTHS lines. BTHS-affected and healthy control fibroblasts were seeded and half the wells from each line were treated with AAV-*TAZ* and the other half AAV-*GFP* at a multiplicity of infection of 100,000 vector genomes per cell using a previously described AAV2 triple tyrosine mutant capsid developed by the Srivastava lab. Gene expression assays showed that *TAZ* expression is increased ~50 fold in AAV-*TAZ* treated fibroblasts. AAV-*TAZ* administration significantly reduced mitochondrial fragmentation and improved ATP production, cell viability, mitochondrial membrane potential, and oxygen consumption. Future directions for this project will involve further analysis of specific mitochondrial gene and protein expression levels. Evaluation of the differences in mitochondrial structure and function in these cells will provide additional phenotypic information for BTHS, may help to reveal a novel functional role for tafazzin, and will determine the extent of correction following gene replacement therapy to BTHS cells harboring different mutations in the *TAZ* locus.

510. Long-Term Consequences of CRISPR/Cas9 Gene Editing in a Mouse Model for Hereditary Tyrosinemia Type I

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CRISPR/Cas9 gene editing has a tremendous therapeutic potential, but currently there is virtually no data on long-term consequences of such an intervention. We have recently developed a new therapeutic concept called metabolic pathway reprogramming, which couples the power of CRISPR/Cas9 technology with a strategy from pharmacology, namely, to inhibit a metabolic pathway rather than directly edit a disease-causing gene. We previously demonstrated the efficacy of this approach by using CRISPR/Cas9 to convert lethal type I tyrosinemia into benign type III tyrosinemia in mice. In present study, we evaluate the long-term effects (12 months) of CRISPR/Cas9 gene editing using gene therapy vectors in type I tyrosinemia. While we observed a correction of the metabolic phenotype we could observe an increased rate in tumor formation. We are currently evaluating on multiple levels the tumorigenesis and will share these results. In summary, long-term evaluation of genome engineering is critical for clinical translation and our study highlights potential limitations.

511. A Gene Editing Approach to Eliminate Hepatitis B Virus In Vivo with an ARCUS Meganuclease Evolved to Prevent Off Target Cutting

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Hepatitis B virus (HBV) infects more than 300 million people worldwide and can cause liver disease and liver cancer. We have developed an ARCUS nuclease that targets the hepatitis B viral sequence to help drive the inactivation of integrated and unintegrated virus as a potential treatment. The initial nuclease was shown to reduce extracellular HBsAg in HBV-infected primary human hepatocytes by 97%. To produce a nuclease that is both safe and efficacious for clinical use, we developed a pipeline combining a sensitive, in-vivo off-target detection assay with a directed nuclease optimization system. The off-target assay has been optimized for the unique properties of ARCUS nuclease-created double-stranded breaks and has a lower limit of detection than other currently used assays (GUIDEseq, CIRCLEseq). The nuclease optimization system works in conjunction with the off-

target assay to produce next-generation ARCUS nucleases that can discriminate against identified off-target sites. By alternating between identifying potential off-target sites and eliminating nuclease activity against those sites with optimization, we were able to generate a clinical candidate nuclease that is more active against the intended target site than the original nuclease and has zero detectable off-target sites within the human genome.

512. Developing a Neuronal Stem Cell Gene Therapy for Mucopolysaccharidosis Type III B Using CRISPR/Cas9 Technology

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Lysosomal storage disorders are life-threatening inherited conditions. Mucopolysaccharidosis IIIB (MPS IIIB), also known as Sanfilippo syndrome, is a childhood devastating degenerative lysosomal storage disorder mainly affecting the brain. It is caused by the mutation in NAGLU gene (N-acetyl-glucosaminidase) that encodes for an enzyme that is involved in the breakdown of a large sugar molecule, heparan sulfate proteoglycan. To date, there is no treatment for this disease, but enzyme replacement therapy (ERT) is in clinical development. We previously demonstrated that lentiviral correction of murine *Naglu*$^{-/-}$ neural stem cells derived from *Naglu*$^{-/-}$ induced Pluripotent Stem Cells (iPSC), have the potential to restore NAGLU activity and correct the CNS lysosomal pathology (Clarke et. al 2018). This study focuses on developing a stem cell-mediated gene therapy (SCGT) using a CRISPR Cas-9 system to evert toxicity associated with viral vectors. We designed gRNAs to be used in combination with a nickase Cas-9 to insert a functional copy of the hNAGLU or rhNAGLU-IGFII gene downstream of the mouse endogenous promoter. In a second strategy, we inserted hNAGLU and NAGLU-IGFII downstream of a strong exogenous promoter, chicken beta-actin (CAG) to obtain overexpression of NAGLU. Upon successful genetic modification of murine iPSCs, here we show that we can insert a functional copy of hNAGLU into the first exon of mNaglu resulting in NAGLU expression and rescue of NAGLU enzymatic activity. Our results show that our overexpression strategy results in several folds increase in NAGLU enzymatic activity compared to cells that rely on the endogenous promoter. We have also reprogramed patient-derived human fibroblast to create MPS IIIB iPSCs, and will correct the cells using the same gene correction design and strategy.

513. The ProTide Prodrug CERC-913 Improves mtDNA Content in Primary Hepatocytes from DGUOK Deficient Rats

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Loss-of-function mutations in the deoxyguanosine kinase (DGUOK) gene result in a mitochondrial DNA (mtDNA) depletion syndrome. DGUOK plays an important role in converting deoxyribonucleosides to deoxyribonucleoside monophosphates via the salvage pathway for mtDNA synthesis. *DGUOK* deficiency manifests predominantly in the liver; the most common cause of death is liver failure within the first year of life and no therapeutic options are currently available. In vitro supplementation of deoxyguanosine or deoxyguanosine monophosphate (dGMP) were reported to rescue mtDNA depletion in DGUOK-deficient patient-derived fibroblasts and myoblasts. CERC-913, a novel ProTide prodrug of deoxyguanosine monophosphate, was designed to bypass defective DGUOK while improving permeability and stability relative to nucleoside monophosphates. To evaluate CERC-913 for its ability to rescue mtDNA depletion, we developed a primary hepatocyte culture model using tissue from DGUOK-deficient rats. *DGUOK* knockout rat hepatocyte cultures exhibit severely reduced mtDNA copy number (~10%) relative to wild type by qPCR and mtDNA content remains stable for up to eight days. CERC-913 increased mtDNA content in DGUOK-deficient hepatocytes up to 2.5-fold after four days of treatment in a dose-dependent fashion, which was significantly more effective than dGMP at the same concentration. Gene expression associated with mtDNA improved after four days of CERC-913 treatment, but disease-associated differences in protein expression were unchanged. Reduction in cell viability noted in DGOUK-deficient cells between four and eight days improved with CERC-913 treatment. The Seahorse Mito Stress Test showed significant difference between untreated wild type and DGUOK-deficient cells on oxygen consumption rate (a key parameter of mitochondrial function), however normalization was not detected after CERC-913 treatment. Overall, these early results suggest that primary hepatocyte culture is a useful model for the study of mtDNA depletion syndromes and that CERC-913 treatment is capable of improving several disease-associated phenotypes in this model.

514. Enzyme Replacing Therapy in MPS-1 Mice by Enzyme Produced In Vivo by Shielded Engineered Allogeneic Cells

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The lysosomal storage diseases (LSD's) are heterogeneous group of disorders caused by mutations in lysosomal enzyme processing

proteins. This results in the buildup of unprocessed precursors leading to a complex array of clinical findings which can and usually involves several organ systems including viscera, bone, muscle, joints, heart, and brain. There have been several strategies to ameliorate such diseases including enzyme replacement therapy (ERT), chaperone therapy, bone marrow transplant, gene therapy, and small molecule therapy. We hypothesize that spheres made with Afibromer™ biomaterials will shield genetically engineered human cells from immune rejection while also preventing the fibrotic foreign body response around the administered spheres, thus enabling a sustained therapeutic effect reflected in reduced substrate accumulation and a sustained therapeutic effect. Mucopolysaccharidosis type 1 is caused by mutations in the gene encoding iduronidase (*IDUA*). Lack of IDUA activity leads to loss of glycosaminoglycan (GAG) catabolism and accumulation GAGs in all tissues. A mouse knock out (KO) model for MPS-I exist and it displays the same biochemical and clinical features to the human disease severe phenotype (Hurler Syndrome). We implanted non-virally modified human cells optimized for IDUA secretion into the intraperitoneal space of MPS-1 KO mice. Mice were followed up to 21 days post-administration. At intervals, serum was tested for a trisaccharide biomarker and total GAG levels. Total GAG analysis will be performed in liver, spleen and kidney post-termination. A significant reduction in the trisaccharide biomarker and total GAGs was observed already after 10 days of treatment. Additional analysis to 21 days post-administration are ongoing and will be presented. These preliminary data confirm that IDUA secretion by shielded engineered human cells are an alternative to frequent enzyme delivery or gene therapy approaches, with several potential important advantages. Shielded Living Therapeutics is also a viable platform for the treatment of multiple LSDs.

515. Efficient Gene Correction of AGXT Mutations Causing Primary Hyperoxaluria Type 1 in Patient-Derived Fibroblasts

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Primary hyperoxaluria type 1 (PH1) is a rare disorder of glyoxylate metabolism characterized by the accumulation of oxalate due to a deficiency of the peroxisomal hepatic enzyme L-alanine: glyoxylate aminotransferase (AGT). Clinical presentation varies, ranging from occasional symptomatic nephrolithiasis to nephrocalcinosis and end-stage renal disease with systemic involvement. PH1 is produced by mutations in the *AGXT* gene. Gene corrected autologous hepatocyte-like cells generated by means of cellular reprogramming is proposed as an alternative therapeutic cellular source in patients with PH1. The development of optimized tools for the specific gene correction of the mutated gene, *AGXT*, is essential to address this strategy. A first approach aimed the specific correction of a founder mutation described in Canary Islands that accounts for most of PH1 patients in this area. Different guideRNAs (gRNA) to drive Cas9 cleavage to the proximity of the Canary Islands specific mutation in exon 7 (c.853T>C) and different single-stranded oligodeoxynucleotides (ssODN) were designed to correct the mutation. Proper recombination with ssODN as repair templates resulted challenging, with a high proportion of partial insertions of these DNA templates. Specific correction of the mutation was achieved in PH1 derived fibroblasts with around 4% efficacy. Position and length of the ssODN was crucial for minimizing undesirable partial insertions of the DNA template. As a second alternative for the correction of PH1 by gene editing we aimed the introduction of the whole corrected cDNA of the *AGXT* gene by knock-in near the starting codon of the endogenous gene. This strategy would correct almost all of the different mutations present in PH1 patients. Using as a repair template an optimized version of *AGXT* carrying a positive-negative selectable marker, and appropriate CRISPR/Cas9 assisted tools, more than 80% of the drug resistant clones exhibit the correct insertion at the desired site, being the design of templates again essential for an efficient knock-in generation. Corrected clones, either by specific correction or knock-in strategies, will be reprogrammed following the reprogramming protocol previously optimized in healthy donor derived fibroblasts, offering a new cellular source for PH1 treatment.

516. Evaluation of New Approaches to Wilson Disease GeneTherapy in the Jackson Toxic Milk Mouse Model

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Wilson disease, a recessive disorder caused by mutations in the ATP7B copper transporter, leads to accumulation of copper in tissues resulting in hepatic, neurologic, and psychiatric symptoms. Current treatments are copper-modulating drugs which cause side effects and do not restore normal copper metabolism. rAAV administration leading to prolonged ATP7B expression following a single injection is an attractive alternative to life-long daily maintenance therapy. Jackson toxic milk mice ($Atp7b^{Tx\text{-}J}$) have a G712D mutation in ATP7B resulting in loss of copper transport function. We evaluated $Atp7b^{Tx\text{-}J}$ mice over 26 weeks and confirmed that homozygous mutant mice showed an age-dependent increase in liver copper levels as measured by ICP-MS (inductively coupled plasma-mass spectrometry). Furthermore, at 4 weeks of age serum copper was lower in $Atp7b^{Tx\text{-}J}$ homozygotes relative to heterozygotes and remained low until 8 weeks when levels started increasing. Both serum and liver copper are therefore potential metrics for evaluation of new therapies. We demonstrated that introduction of a functional ATP7B gene using rAAV reversed the copper phenotype in the serum of $Atp7b^{Tx\text{-}J}$ mice. The ATP7B cDNA is 4.4 kb long which constrains the regulatory elements that can be packaged into AAV. Deletion of metal-binding domains 1-4 has been reported previously and we explored further deletions to provide even greater capacity. Several novel deletions retained copper transport activity in cellular

assays. We anticipate reporting their effect on the copper phenotype in Atp7b$^{Tx\text{-}J}$ mice after delivery with rAAV. Our studies demonstrate that Atp7b$^{Tx\text{-}J}$ is a useful model for evaluation of gene therapy for Wilson disease and that engineering ATP7B to allow use of regulatory elements that drive high levels of expression may be a feasible approach.

517. A Single-Vector Approach to Hypercholesterolemia Gene Therapy with AAV-Cas13d

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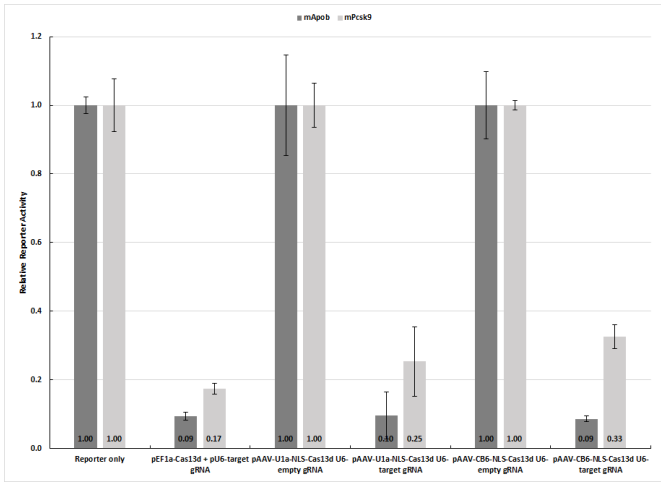
Primary or familial hypercholesterolemia, as well as secondary dyslipidemias, are frequently treated with statin therapy or other small molecule drugs to great clinical success. However, a significant portion of patients are resistant to treatment or develop intolerance to these compounds and are, thus, at elevated risk of cardiovascular disease-associated events. Several new drugs targeting serine protease proprotein convertase subtilisin/kexin type 9 (PCSK9) have recently demonstrated improved patient outcomes in such high-risk populations, yet therapeutic cost is prohibitively high for many given the chronic pathological nature of these diseases. However, PCSK9 inhibition as a means of increasing LDLR and reducing circulating lipids appears well suited for human gene therapy, offering a single dose with the potential for lifelong positive effect. Cas13d, specifically CasRX, has recently been described as an RNA-guided RNA nuclease with a robust capability for targeted RNAi. Here we describe initial efforts in adapting Cas13d for pre-clinical gene therapy *in vivo*, employing a single-vector AAV strategy that is capable of multiplexed knockdown of mRNA including PCSK9 and another potential therapeutic cholesterolemia target, APOB. Preliminary screenings have identified a number of potent Cas13d guide-RNA for each target, capable of reducing target expression by over 90% *in vitro*. Full-scale vector production is currently underway and results from initial delivery to mouse models to establish proof-of-concept for this exciting new therapeutic strategy will be discussed.



518. Development of a Novel Gene Therapy for Pompe Disease: Engineered Acid Alpha-Glucosidase Transgene for Improved Expression and Muscle Targeting

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Pompe disease (PD) is a rare autosomal recessive lysosomal disorder that results from a deficiency in acid α-glucosidase activity. PD is characterized by the accumulation of glycogen in lysosomes leading to dysregulation of normal cellular function, tissue damage in the heart, muscles and motor neurons. The current standard of care, enzyme replacement therapy (ERT), has limitations for improving muscle function, and cannot cross the blood-brain barrier, leading to progressive neurologic deterioration in long-term survivors of classic infantile PD. The most severe form results in death within 2-3 years if untreated. ERT using recombinant human GAA (rhGAA) delivered every other week via intravenous infusion is the only approved treatment available for PD. For ERT to be effective, rhGAA must be internalized in target muscle cells and delivered to lysosomes at clinically relevant doses. The vast majority of ERTs, including rhGAA, utilize the cation-independent mannose 6-phosphate receptor (CIMPR) pathway for cellular uptake. However, only a small proportion of the total rhGAA contains bis-phosphorylated high mannose glycans that have high binding affinity for CIMPR because it is poorly phosphorylated in cells. rhGAA production cell lines and manufacturing processes therefore have to be optimized to produce appreciable amounts of rhGAA with mannose 6-phosphate (M6P) for CIMPR binding and cellular uptake. Adeno-associated virus (AAV)-based gene therapy is a promising approach to achieving long-term expression of the enzyme in target organs. However, since carbohydrate processing cannot be controlled in transduced cells, inefficient GAA

phosphorylation may pose substantial challenges to cellular uptake. Many animal studies therefore utilize high gene therapy doses to produce sufficient amounts of M6P-containing hGAA to establish proof of principle. However, producing comparable amounts of hGAA in higher organisms would likely require substantial gene therapy doses that have been associated with significant safety signals in non-human primates and humans. Using such high doses would also exacerbate already challenging manufacturing processes for PD gene therapies. It is therefore beneficial to develop gene therapies that produce high levels of hGAA that are efficiently targeted to muscles using moderate doses. We have engineered the human *GAA* transgene to replace the native signal sequence with a more efficient sequence to enable higher protein expression and secretion of hGAA (5- to 10-fold higher than native signal sequence). Further, the engineered transgene was designed to produce hGAA containing a targeting motif that enables high-affinity binding to a cell surface receptor for efficient uptake and delivery to lysosomes. Once in cells, our hGAA product is indistinguishable from wild type GAA by western blotting indicating that the targeting motif is properly removed and the enzyme is normally processed in lysosomes. We believe that the combination of greater expression and secretion of hGAA with efficient muscle targeting may ultimately translate into a more effective gene therapy for PD. Studies are currently underway in *Gaa* KO mice using AAV-mediated gene delivery to assess the effectiveness of our engineered hGAA relative to wildtype GAA. Preliminary data will be presented from the animal studies as available.

519. Development of an Encapsulated Stem Sell Bioengineered Brown Adipose Transplant to Improve Metabolic Disorders

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The prevalence of obesity and metabolic disorders has increased dramatically over the past decades and has become a pandemic. By 2030, more than 50% of Americans will suffer from obesity, resulting in over 500 billion dollar loss in economic productivity. Obesity is a major risk factor for type II diabetes mellitus, hypertension, cardiovascular disease, osteoarthritis, and certain forms of cancer. Current therapeutic approaches, such as caloric restriction and exercise, which rely mainly on patient's will power to reduce energy intake and/or increase energy expenditure, are generally of limited effectiveness in obese patients. Bariatric surgery is the only clinically proven therapy in terms of weight loss and decreased morbidity and mortality in patients with a body mass index (BMI) over 40; however it has associated risks, high costs and requires proper management of patient's nutrition and physical activity. Alternative ways to increase energy expenditure could augment the current therapeutic options for treating obese patients and ultimately lead to successful clinical outcomes. Targeting brown adipose tissue (BAT) in humans, in order to increase BAT mass and/or activity, has emerged as a potential way to increase energy expenditure by energy wasting. BAT is a densely innervated and highly perfused tissue consisting of brown adipocytes, a cell type characterized by small multilocular lipid droplets, and a high number of mitochondria. Brown adipocytes are unique in their expression of the uncoupling protein 1 (UCP1) at the inner mitochondrial membrane. UCP1 short-circuits the proton gradient from ATP generation, thereby dissipating the energy stored in the gradient as heat. Largely studied in rodents, the existence of metabolically active BAT in healthy human adults has only recently been identified by 18-fluorodeoxy-glucose positron emission tomography combined with computed tomography (PET-CT). Recent studies indicate that the activity of BAT in humans is negatively correlated with weight, percent body fat and age. Importantly, BAT metabolic activity can be increased by the administration of a beta-3 adrenergic receptor agonist to healthy human adults, and BAT activity correlates with resting metabolic rates (RMR). Transplantation of brown fat in obese mice models has demonstrated multiple beneficial metabolic outcomes including regulating glucose homeostasis, insulin sensitivity and even reversing obesity. These findings provide a rationale for the therapeutic expansion of BAT for the treatment of metabolic disorders in humans. Due to the lack of transplantable BAT, tissue engineering of metabolically active transplantable BAT has recently emerged. We have previously described the isolation of human brown adipose derived mesenchymal stem cells (BADSCs) from mediastinal adipose tissue. We have demonstrated that these cells can be expanded in culture and differentiated into functional brown adipocytes *in vitro. Additionally, we demonstrated* that *in vivo* transplantation of differentiating BADSCs in a biological scaffold significantly reduced blood glucose levels and body weight in obese mice. In the present study, we have characterized multiple BADSCs populations isolated from mediastinal and cervical anatomical locations. We have also evaluated *in vitro* the use of an FDA approved immune-protecting encapsulation medical device as a delivery system for bioengineered BAT transplantation.

520. Adeno Associated Vector-Based Gene Therapy for Glycogen Storage Disease Type 3

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Glycogen storage disease type III (GSDIII) is a recessive disorder due to mutations in the glycogen debranching enzyme (GDE), the enzyme involved in the linearization of cytosolic glycogen. The lack of GDE leads to glycogen accumulation in all tissues. During childhood, GSDIII is mainly a metabolic disease characterized by hepatomegaly and fasting hypoglycemia. During adolescence, the metabolic manifestation of the disease becomes less prominent and a degenerative muscle weakness appears. At present, no curative treatment exists for GSDIII. A major limitation in the development of a gene therapy for GSDIII is the transgene size that exceed the AAV packaging size. We showed partial correction of the GSDIII phenotype in a mouse model of the disease using dual AAV vectors (*Vidal, Mol. Ther. 2018*). Here, we explored dual vector strategies based on recombination of AAV vectors, described as highly efficacious to deliver large transgenes. We compared different dual vector strategies to express GDE simultaneously in liver and muscle of GSDIII mice. In parallel, single vector strategies were also explored. Optimization of AAV capsid and transgene expression

cassette aimed at the safe and efficient expression of GDE in both muscle and liver by single AAV vectors supports this strategy for clinical translation.

521. Development of a Universal Circulating Biomarker of a Nuclease-Free Genomic Integration Technology, GeneRide™

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GeneRide™ is a novel AAV-based genome editing technology that leverages the natural process of homologous recombination to insert a therapeutic transgene into the genome. For liver-directed targets, the transgene is integrated into the *Albumin* locus. The integrated transgene can thus "hitch a ride" on the highly active *Albumin* endogenous promoter and results in high transgene expression selectively in hepatocytes. The transgene is precisely inserted in-frame between the penultimate and the stop codons of *Albumin*, and utilizes a P2A sequence that induces ribosomal skipping. This results in the production of a C-terminal tagged ALBUMIN (ALB-2A) and the therapeutic transgene as two separate proteins. Using ELISA and qPCR-based assays to quantitatively determine the level of ALB-2A in circulation and the percentage of modified *Albumin* allele in liver respectively, we demonstrate that plasma levels of ALB-2A are highly correlated with the percentage of modified hepatocytes and the level of transgene mRNA and protein expression. In neonatal mice, GeneRide™ activity can be detected as early as 1 week after a single intravenous administration, and transgene expression reaches steady state at approximately 3 weeks post injection. Most importantly, GeneRide™ is effective across developmental stages as similar levels of transgene integration and protein expression are observed in mice treated either at neonatal (PND 1) or juvenile (PND 21) stage. In conclusion, ALBUMIN-2A can be used as a robust circulating biomarker for GeneRide™ treatment in preclinical studies and is being developed to monitor GeneRide™ activity in the clinical setting.

522. Engineered Human iPSC-Derived Skeletal Muscles to Model Pompe Disease

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Current clinical investigation on muscle wasting disorders is limited by the availability of appropriate cell models that recapitulate the same cellular parameters as the organ of origin. Several transgene-free procedures can be used to generate muscle cells from patient iPSCs when muscle cell extraction from biopsies is not preferred. However, most of these cells are limited by their expansion and myogenic potential for disease modelling and cell-based therapies. In addition, muscle cells generated from pluripotent stem cells are immature and do not show appropriate cellular and structural organization as in the adult muscle. Here, we describe a directed differentiation protocol including FACS-purification to generate myogenic progenitor cells (MPCs) with robust expansion and myogenic potential *in vitro* and *in vivo*. In addition, we have created a 3D cell culture platform which allows MPCs to organize into arrays of terminally differentiated muscle fibers, termed muscle bundles. We have used CRISPR-Cas9 technology to generate isogenic cell lines from two Pompe disease patients, a metabolic myopathy caused by disease associated variants in the acid alpha glucosidase (*GAA*) gene which results in intralysosomal accumulation of glycogen. Insertion of the *GAA* cDNA into the *AAVS1* locus in iPSCs prevented glycogen accumulation in myotubes. We are currently exploring these isogenic muscle bundles for disease modelling and cell-based therapeutics.

523. Effects of PhK Deficiency in a Mouse Model of Glycogen Storage Disease Type IX

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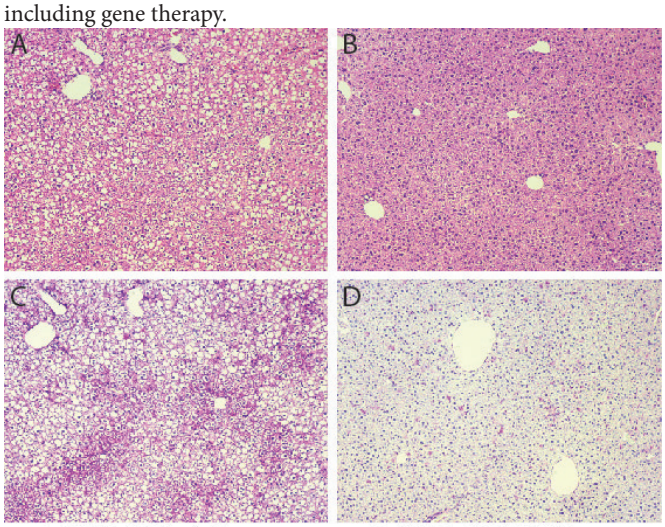
Mutations in the phosphorylase kinase (PhK) gene are associated with the pathogenesis of glycogen storage disease type IX (GSD-IX). An autosomal recessive disease, PhK deficiency causes glycogen accumulation and can lead to liver complications in pediatric cases with prolonged fasting. The breakdown of glycogen in the liver (glycogenolysis) requires the step-wise activation of two cytosolic liver enzymes; PhK phosphorylates the liver glycogen phosphorylase (pygl) which triggers a confirmation switch from phosphorylase b (inactive form) to the phosphorylase a (active form) converting glycogen into glucose-1-phosphate monomers. Mutations in one of four homotetramer subunits (α, β, γ, δ) of PhK have been shown to negatively affect PhK catalytic activities and regulatory functions during glycogenolysis. The most common autosomal recessive types of GSD-IX arise from mutations linked to the human PHKG2 (γ) and PHKB (β) genes. To understand the role of the GSD-IX β subunit and characterize the metabolic abnormalities reported in pediatric GSD-IX patients, we examined liver histology and hepatic glycogen content, fasting glucose/ketone levels, and serum biochemistry in a mouse model of GSD-IX β deficiency (*Phkb* $^{-/-}$). Examination of *Phkb* $^{-/-}$ livers in young (4-8 weeks) mice revealed hepatomegaly, excessive hepatic glycogen accumulation and reduced hepatic glucose. *Phkb* $^{-/-}$ mice exhibited elevated ketone levels over the course of the prolonged fast (2-6 hrs.) compared to wild-type mice. Analysis of hepatic liver glycogen revealed more than a two-fold change in glycogen levels (μg/mg of tissue) in *Phkb* $^{-/-}$ mice compared to wild-type mice. The increased glycogen accumulation was supported by significantly increased liver weight/ body weight percentages in *Phkb* $^{-/-}$ mice. Histological analyses by H&E confirmed extensive hepatocellular vacuolar change consistent with glycogen accumulation in *Phkb* $^{-/-}$ mice compared to wild-type mice (Panel A, B). Additionally, Periodic-acid Schiff (PAS) staining confirmed excessive glycogen deposition in *Phkb* $^{-/-}$ livers compared to wild-type mice (Panel C, D). Despite significantly elevated levels of glycogen retained in hepatocytes, serum biochemistry revealed slightly elevated triglycerides, however cholesterol, lactate, and uric acid levels (mg/dl) remained unchanged in *Phkb* $^{-/-}$ compared to wild-type mice. Altogether, *Phkb*-deficient mice may engage secondary energy sources including gluconeogenesis and fatty acid oxidation to stabilize blood glucose levels and prevent hypoglycemia. Since *Phkb*

deficiency impairs the breakdown of glycogen during glycogenolysis, elevated levels of ketones in *Phkb* $^{-/-}$ mice supported normalized blood glucose levels through the 24-hour fasting challenge. Therefore, these mice accurately mimic the mild symptoms such as elevated ketones and hepatomegaly reported in GSD-IX patients. This model will help to understand the metabolic abnormalities associated with other forms of GSD-IX, energy-metabolism studies and help to address management of GSD-IX in patient care and wellness. Due to the mildness of symptoms in this disease, potential metabolic complications later in life have not been fully elucidated. We will follow up this model with age-related studies and investigate potential preventive therapies including gene therapy.



A- H&E *Phkb* -/- liver
B- H&E *Phkb* WT liver
C- PAS *Phkb* -/- liver
D- PAS *Phkb* WT liver

524. A 5-Year Retrospective Study of Individuals with Phenylketonuria (PKU) Treated at Two Specialized Clinics

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HMI-100-001 is a retrospective study conducted at two specialized U.S. clinics that describes two cohorts of patients with hyperphenylalaninemia (HPA) over a 5-year period ending in November 2017. Phenylalanine hydroxylase (PAH) deficiency is an inborn error of metabolism due to mutations in the *PAH* gene, which results in phenylketonuria (PKU). The mutations result in the absence or deficiency of PAH, an enzyme that catalyzes the formation of tyrosine (Tyr) from dietary phenylalanine (Phe), leading to excess Phe. PAH deficiency is an autosomal recessive, monogenic defect, making it a suitable condition for potential AAV-based gene therapy. PAH deficiency manifests as a continuum of HPA phenotypes characterized by elevated blood Phe concentrations. Diagnostic sub-categories of PKU range from mild HPA (Phe levels 120-360 μmol/L) to the most common and severe form, "classic PKU," defined as Phe over 1200 μmol/L. Current U.S. treatment guidelines indicate that treatment is not required for mild HPA . With the advent of newborn screening in 1963, managing the disease by dietary restriction of Phe in infants before clinical symptoms appear became the standard of care. Untreated PKU in children results in progressive irreversible neurological impairment; however, even early-treated PKU adults present with higher rates of neuropsychiatric comorbidities. Maintaining the life-long diet requires the use of unpalatable protein substitutes, has side effects, and is difficult to adhere to, especially for adolescents and adults. There are no current treatments that address the underlying genetic defect. A total of 152 patients (10-40 years old) were enrolled in this study. The primary objective was to describe blood Phe control in these patients over a five-year period. The results showed that most patients were diagnosed with classic PKU (64.3% in the 10 to 18 age group and 67.1% in the over 18 to 40 age group). The number of patients with consecutive lab values decreased as the Phe threshold was lowered (Phe levels below 600 µmol/L, 360 µmol/L, 120 µmol/L, and 30 µmol/L). The data confirmed prior research demonstrating a relationship between increased age and decreasing control of Phe concentrations, where mean Phe was 456.8 $\pm$ 27.0 µmol/L (mean $\pm$ standard error) for patients 10 to 18 compared to 694.7 $\pm$ 36.7 µmol/L for patients over 18 to 40. Patients with classic PKU had higher mean Phe relative to the remaining diagnostic sub-categories of mild HPA, mild PKU, and moderate PKU. 62.5% of patients were reported as having a history of at least one neuropsychiatric condition, and 44.1% of patients were recorded as having more than one. 98 *PAH* genotypes were collected, with approximately 90 distinct mutations and no clear pattern between *PAH* genotype and HPA diagnosis (p-value = 0.21). Despite use of protein restriction, Phe concentrations over 360 μmol/L were observed, particularly in classic PKU patients. Among the classic PKU patients, 73.3% in the 10 to 18 age group and 92.7% in the over 18 to 40 age group had a 5-year mean Phe over 360 µmol/L. Overall the demographics and clinical data were consistent across both sites. Collectively, these real-world data show that Phe levels were elevated, even when a patient was on a Phe-restricted diet, and above the threshold (360 µmol/L) considered well-controlled based on current treatment guidelines. There remains an unmet need for therapies to control Phe concentrations without a Phe-restricted diet, particularly in patients diagnosed with classic PKU and those over 18 years of age.

525. Normalization of Phenylalanine Levels in a Phenylketonuria Mouse Model Using Recombinant AAV

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Phenylketonuria (PKU) is an autosomal recessive disease caused by loss of function of phenylalanine hydroxylase (PAH), leading to the accumulation of excess levels of phenylalanine (Phe) in the blood and in brain. Untreated, excess Phe can lead to significant developmental defects. A Phe restricted diet is the standard of care for the majority of PKU patients, but compliance is challenging with the majority of

adolescents and adults having Phe levels above the recommended range, which can result in intellectual, emotional and behavior problems. Moreover, patients can have difficulties recognizing these symptoms resulting in continued non-compliance. Gene therapy for PKU has the potential to lower Phe levels in PKU patients absent compliance issues, allow patients to eliminate the protein restricted diet and significantly improve quality of life. An evaluation of rAAV-PAH targeted to deliver a transgene encoding a functional copy of PAH to the liver providing sufficient activity to reduce Phe levels in PKU patients to the recommended range is underway. Multiple workstreams are in progress to: 1) generate reagents/assays to differentiate the human PAH transgene protein product from the native murine and macaque proteins, 2) develop a cell based potency assay for PAH activity to screen through optimized codon sequences, modified PAH alleles and expression cassettes, and 3) screen constructs packaged into AAV capsids, and dosed in the PAHenu2 animal model. Multiple vectors demonstrate stable lowering of the Phe levels from >2 mM to the recommended range (60 - 360 uM). These same vectors demonstrate a reciprocal increase in Tyr levels after rAAV-PAH administration, as well as darkening of coat color due to increased melanin synthesis. Ongoing animal studies will further characterize the biological effects of the rAAV vectors and optimize vector potency. These results show progress toward a rAAV-based gene therapy for the treatment of PKU.

Cardiovascular and Pulmonary Diseases

526. Targeting Lung Cell Population for Gene Supplementation and Gene Correction Therapy of Cystic Fibrosis

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Objectives: The advances in the field of chemical modifications along with codon optimization of mRNA and nano carriers have made mRNA based therapies suitable candidates for diseases associated with lack of functional protein. Cystic Fibrosis, a common genetic disorder among Caucasians is caused due to loss of functional CFTR protein which results in high rate of mortality and reduced life quality. Our group previously showed strong improvement of lung function (Forced Expired Volume in 1 sec ($FEV_{0.1}$)) up to 89% of normal values of wild type mouse model and expression of functional CFTR in CF knockout mouse model by delivering chemically modified h*CFTR* mRNA (cmRNA^{hCFTR})[1]. However, to optimize this further this project aims to specifically target major CFTR expressing cells: Alveolar Type II cells (ATII), endothelial cells, Club cells and epithelial cells (e.g. ionocytes[2]) to provide a precise therapy. Along with identification of Lung stem cells, Myoepithelial cells and Bronchio Alveolar Stem Cells (BASC), will prove useful for gene correction via CRISPR/Cas system for CF. **Method:** Lung cell populations related to CF pathology and stem-cell based therapy was identified by specific cell markers using flow cytometry (BD LSRFortessa X-20). Chemically modified reporter mRNA (mKate2) was delivered in lung cells using nano carriers via intratracheal instillation and intravenous injection in a CF knockout mouse model. Distribution of reporter protein (mKate2) in previously identified lung cell populations was compared between two nano carriers (Chitosan-coated PLGA and cationic nano liposomes) used to deliver cmRNA of the reporter protein. Similarly, cmRNA^{hCFTR} was used to compare the impact of the two nano carriers on functional improvement of lung parameters ($FEV_{0.1}$ and compliance) by FlexiVent®. **Results and Conclusion:** Chitosan-PLGA nano-carriers have shown deep lung cell delivery (Endothelial cells) of reporter protein (mKate2). In CF knockout mouse model a significant improvement of $FEV_{0.1}$ (~30%) and compliance has been observed with cmRNA^{hCFTR} only when delivered with nano carriers compared to naked delivery. Based on the analysis of distribution pattern of various nanocarriers a targeted delivery of cmRNA^{hCFTR} and CRISPR/Cas can be implemented for CF therapy. **Conflict of Interest:** M.S.D.K. holds a patent on RNA modification (EP2459231B1). M.S.D.K. and AKM A.H. hold a European patent on delivery of cmRNA^{hCFTR} complexed with nanoparticles (17169561.2-1401). **References:** 1 Haque, A. K. M. A. *et al.* Chemically modified hCFTR mRNAs recuperate lung function in a mouse model of cystic fibrosis. Sci Rep. doi: 10.1038/s41598-018-34960-0 (2018). 2 Montoro, D. T. *et al.* A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 560, 319-324, doi:10.1038/s41586-018-0393-7 (2018).

527. Codon Optimization Enhances Functional Cystic Fibrosis Transmembrane Conductance Regulator Expression

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Cystic fibrosis (CF) is a common genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes an anion channel. While *CFTR* is expressed in many tissues, the most severe symptoms are due to chronic pulmonary disease caused by persistent bacterial infection and inflammation. Recent advances in small molecule therapies that partially correct CFTR function are improving clinical outcomes for many people with CF. However, the benefits provided by these drugs are mutation-dependent and are ineffective for some patients. One treatment that could benefit all *CFTR* mutation classes is gene addition. Several clinical gene therapy trials for CF have taken place, but none have proven effective to date. Efficient delivery of a *CFTR* cDNA expression cassette to the airways requires a vector with sufficient carrying capacity that can transduce human airway epithelial cells (HAE) from the apical surface, produce high vector titers, and provide long-term transgene expression. Finding a vector that meets all of these criteria has proven difficult and led us to investigate strategies to increase transgene expression as another means to achieve therapeutic levels. Codon optimization has been used to increase transgene expression of other

therapeutic genes, such as factor VIII and factor IX, for the treatment of hemophilia. Vectors using these codon optimized genes are currently in clinical trials. Additionally, the recent discovery of the pulmonary ionocyte, an epithelial cell type that makes up approximately 1% of the HAE population, but produces ~50% of the *CFTR* mRNA transcripts in the large airways, suggests that high *CFTR* expression in a small proportion of epithelia could be therapeutic. There are many strategies to codon optimize a gene. A feature of most strategies substitutes native codons with those used by highly expressed genes for a particular species. This is thought to increase mRNA stability and leads to more efficient translation without changing the amino acid sequence. To test the therapeutic potential of *CFTR* codon optimization, we compared four different *CFTR* cDNA sequences in a model cell line. We cloned each codon optimized *CFTR* (co*CFTR*) sequence into the pcDNA3.1 expression vector. We then electroporated 4 µg of plasmid containing wildtype (WT) *CFTR*, one of the *coCFTR* sequences, or GFP into Fischer rat thyroid (FRT) cells. We used FRT cells because they express no endogenous *CFTR* and are able to form a polarized epithelium when grown at an air-liquid interphase (ALI). Seven days after ALI culture, we mounted the cells in Ussing chambers and measured transepithelial Cl^- transport. We also investigated protein production in human cells by transfecting HEK293 cells and isolating protein for western blot analysis. Each codon optimized sequence provided a different degree of improved *CFTR* expression and function, as evidenced by functional transepithelial Cl^- transport. While all co*CFTR* sequences increased protein and transepithelial Cl^- transport, one sequence, co*CFTR4*, yielded >20 times greater transepithelial Cl^- transport compared to WT *CFTR*. In future studies we will quantify mRNA levels and express these sequences in HAE using lentiviral vectors. Our findings suggest that codon optimization of *CFTR* could be used to increase protein expression possibly conferring therapeutic levels of CFTR. This may provide a useful reagent for developing a gene therapy vector that would benefit all *CFTR* mutations.

528. Identification of AAV Developed for Cystic Fibrosis (CF) Gene Therapy that Restores CFTR Function in Human CF Patient Cells

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Despite significant progress in our understanding of CFTR function, lung biology, and gene therapy vector delivery in other disease models, successful gene therapy for CF remains on the horizon because of challenges in lung delivery efficiency and efficacious CFTR transgene expression. We have developed a novel CF gene therapy, ABO-401, containing a regulatable human mini-CFTR (hCFTR) expression cassette that is efficiently packaged into our AIM™ capsid library vector AAV204. Transduction of ABO-401 into 293 and Lec2 cells yielded hCFTR membrane-localized protein production that can be detected by western blot and immunofluorescence. ABO-401 hCFTR channel function was confirmed by stimulation with forskolin and inhibition by $CFTR_{inh}$-172 in a membrane potential assay in a dose dependent manner. In Lec2 cells, stimulation with forskolin increased signal 3.5-fold compared to baseline, whereas preincubation with $CFTR_{inh}$-172 prevented any membrane potential changes. We tested the ability of natural AAV capsids and AIM™ vectors to transduce gut-corrected CF mouse lungs following administration using luciferase as a marker for gene expression. We found that AIM™ vector AAV204 demonstrated increased delivery over wild-type AAV6. Whole-body bioluminescence imaging (BLI), *ex vivo* BLI, RTqPCR, and immunofluorescent staining of lung sections demonstrates robust expression of AAV204 in the lungs. AAV204 also transduced human CF donor nasal and bronchial epithelial (HNE and HBE) cells maintained in air-liquid interface cultures following application to both the apical and basolateral compartments. Following these observations, we tested whether ABO-401 could restore CFTR function in human CF(ΔF508) nasal and bronchial cells by measuring changes in transmembrane conductance via Ussing chamber. As shown in Figure 1, forskolin-stimulated, $CFTR_{inh}$-172-inhibited current is restored to 6-7 µA as compared to vehicle. We then tested efficacy *in vivo* following nasal administration by measuring nasal potential difference (NPD). As shown in Figure 2, in treated mice ABO-401 corrected forskolin-stimulated current which is entirely absent in mice treated with vector containing a luciferase transgene. In summary, our results suggest ABO-401 is a capsid able to efficiently deliver a highly-expressed, functional copy of hCFTR to the CF lung that can correct chloride transport in human CF airway cells.

Figure 1: Transepithelial Current

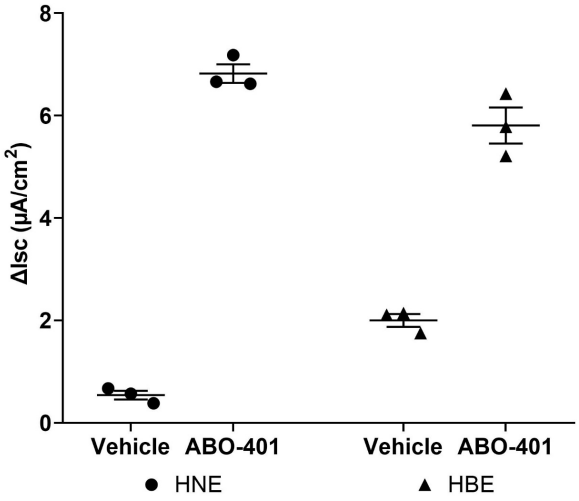
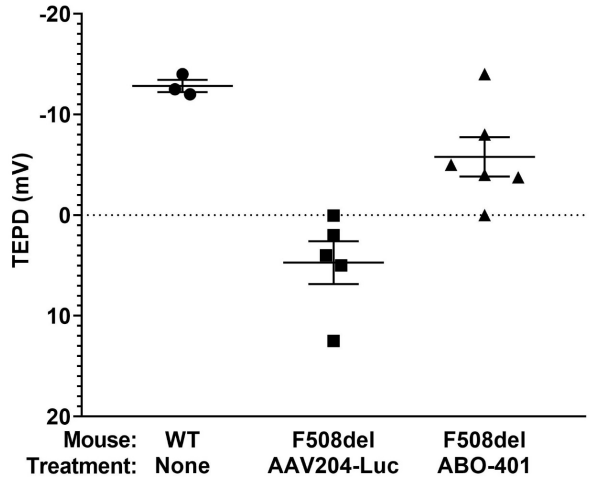


Figure 2: Nasal Potential Difference



529. Gene and Base Edited Cell Models of Cystic Fibrosis-Causing Mutations

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Cystic Fibrosis (CF) is the most common chronic and life-threatening genetic disease affecting the lungs. Although considered as a rare disease, CF affects over 30 000 individuals in the United States, one of the countries with higher incidence of this disease. According to the CFTR2 database (http://cftr2.org) there are more than 300 different mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene that can cause CF. Even though three CFTR modulators have been approved for clinical use, they only target roughly 10% of the mutations. Therefore, there are still hundreds of other disease-causing mutations in need of a targeted therapy. The use of cellular models provides insights into disease mechanisms that may contribute to the definition of novel therapeutic approaches. Although there are cellular models available, most of them are non-human cell lines overexpressing mutant versions of CFTR. However, in a recent study the endogenous CFTR gene was edited to develop homozygous cell lines for three relatively common CF-causing variants using a human bronchial epithelial cell line 16HBE14o$^-$. Here, we compare data from two gene editing methods for making isogenic cellular models homozygous for three rarer CF-mutations, using 16HBE14o$^-$ cell line. The first is a highly efficient method that uses Cas9/gRNA ribonucleoproteins (RNPs) and single strand DNA (ssDNA) oligonucleotide donor, with reduced off-target effects. Different RNA molecules were designed close to I507del and I1234V mutation sites; these were assembled with high fidelity Cas9 to form the RNPs, delivered into 16HBE14o$^-$ cells and successful validated by ICE (Inference of CRISPR Edits) webtool. The gRNA that showed highest double stranded break (DSB) formation was selected for the design and co-transfection with a ssDNA donor. For each of the mutations, co-transfection of Cas9 RNP and donor showed an efficiency of HDR and an indel formation both higher than 20% in the pool of edited cells. The second method, called A to G base editing, allows the direct and irreversible conversion of an adenosine into a guanosine in a programmable manner, without requirement of DSB or a donor DNA template. In this case, one gRNA has been designed for each of the mutations M1V and I1234V according to the base editing window 4 to 7 nt (counting PAM sequence 21-23). As a proof of principle experiment, both gRNA and base editor (ABE 7.10) plasmids were co-transfected into Calu-3 cells and successful validated using EditR (Edit Deconvolution by Inference of Traces in R) and BE-Analyser webtools. These results showed an editing efficiency comparable with the one achieved by HDR, without any previous selection, but importantly the indel formation was nearly 1%. Consequently, this method is being used to produce the desired mutations in 16HBE14o$^-$ cells. In summary, we showed that the two different genome editing methods have been established for the generation of isogenic homozygous mutant CF cell lines. The next step will be isolation of single cell colonies which then are going to be genotyped and the off-target effects evaluated for the selected clones to guarantee specificity. Ultimately these cells will be characterized at the cellular and molecular level in order to find disease signatures for CF that may result in novel therapeutic outcomes. *Work supported by UID/MULTI/04046/2013 center grant from FCT, Portugal (to BioISI) and Cystic Fibrosis Foundation grant HARRIS17G0. LS is the recipient of a fellowship from BioSys PhD programme (Ref. PD/BD/130969/2017) from FCT (Portugal).*

530. An Update Progress Report of Clinical Safety and Efficacy for Gene Therapy with Sendai Virus Vector Expressing the Human FGF-2 Gene (DVC1-0101) to Treat Peripheral Arterial Disease

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Background:A new gene product, namely DVC1-0101, has been developed in clinical phase in Japan. DVC1-0101 is a RNA virus vector based on a non-transmissible recombinant Sendai virus vector expressing the human fibroblast growth factor gene (SeV/dF-hFGF2). The first-in-men phase I/IIa clinical trial was carried out for 12 patients with no-optional critical limb ischemia staged by Fontaine III (n=9) and IV (n=3). The investigation period was 6 months and follow up period was up to 5 years after the gene transfer. This phase I/IIa clinical trial was completed in 2010 and finalized follow-up period in 2015. Subsequently, a phase IIb, randomized, double-blinded clinical trial began in 2014, and has completed interim analysis in 2018. This phase IIb clinical trial was carried out for 15 patients with intermittent claudication staged by Fontaine IIb (n=13) and III (n=2) at the point of interim analysis, and the investigation period was 6 months. **Objective:** The aim of this study is to update progress report of clinical safety and efficacy of DVC1-0101. **Methods:** Safety parameter was included rates of death, limb salvage, and serious adverse events with cardiovascular problems, cancer, renal dysfunctions, and inflammatory disease. Efficacy parameter was the rate of improvement in walking. DVC1-0101 was administrated in a four dose-escalation manner up to 5×10^9ciu/60 kg to one limb per

patient in the phase I/IIa clinical trial, and 1 and 5 × 10^9ciu/leg of DVC1-0101 or placebo-controlled was administrated in the phase IIb clinical trial. DVC1-0101 was administrated at 30 sites intramuscularly in both clinical trials.**Results:** Overall all 27 patients were administrated DVC1-0101 included placebo-controlled in this progress report. Two (16.7%) of the patients in the phase I/IIa died during this investigation period and no death in the phase IIb trial. These are acute-on-chronic progression of preexisting interstitial pneumonitis (2 years and 1 month after gene transfer) and heart failure (4 years and 8 months after gene transfer). Fifteen serious adverse events occurred in ten patients with no cardiovascular events or inflammatory disease that could be definitively attributed to gene transfer. There were also no cancer and renal dysfunctions newly observed in this investigation period. The rate of change in absolute claudication distance (ACD) improved after administration of DVC1-0101 in phase I/IIa was 254.2% in 6 month. There are 4 in 15 patients, in the phase IIb trial, were improved more than 200% of the rate change in ACD by the geometric mean value of 4 to 6 month. Conclusions: In conclusion, it must be noted that these results are extracted from a phase I/IIa with no randomization and no-placebo control, and the interim report from double-blinded trial. Thus, the findings are preliminary and must be interpreted with caution; however, the results are suggestive of DVC1-0101 possibly showing safety use in patients with peripheral arterial disease to compare natural course of death and limb salvation. The presented data may be valuable concerning rates in cancer, cardiovascular events, and inflammatory diseases following angiogenesis gene therapy in the absence of any long-term data. There is a possible tendency toward improving walking performance in DVC1-0101.

531. Gene Therapy for Surfactant Protein B Deficiency Using Recombinant AAV

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Surfactant Protein B (SP-B) deficiency is a rare, autosomal, recessive genetic disease of the lung that causes alveolar collapse and severe respiratory distress in neonates. The disease is invariably fatal during the first months of life. Surfactant replacement therapy in these full-term infants has been shown to be ineffective. The only treatment option is lung transplantation, which is rarely attempted as the mean age of death is 3 months. At an estimate, 1 in 1 million live births in North America and Europe are affected. To improve prognosis, we wish to develop gene therapy for SP-B deficiency using recombinant adeno-associated viral (rAAV) vectors. Effective gene therapy for SP-B deficiency needs to target alveolar type II (ATII) cells in the lung parenchyma as they are the only cells that can produce mature SP-B. We selected AAV serotypes 5, 6.2 and 9 to compare transduction of ATII cells using eGFP as a reporter transgene. Mice (n=2-4) were dosed intranasally with 1E11 genome copies (GC). After 28 days, fixed-frozen sections of the treated lungs were analysed for cell transduction using confocal microscopy and automated imaging of whole lung sections. Immunostaining (with anti-pro-surfactant protein C antibody) was used to identify ATII cells. Recombinant AAV2/5 achieved the highest transduction rate of total murine lung cells (mean 3.06%, SD=0.96) which was significantly different from naive (Kruskal-Wallis testwith Dunn's post-hoc test, P= 0.0234). Mean transduction rates with serotypes 9 and 6.2 were lower (0.32%, SD=0.16 and 0.34%, SD=0.20, respectively). The rAAV2/5 vector also achieved the highest mean transduction rate in the target ATII cells which was significantly different from naive (19.4%, SD=4.6, p=0.014 with Kruskal-Wallis testwith Dunn's post-hoc test, P=0.0141) compared with rAAV2/6.2 and rAAV2/9 (4.0%, SD=1.8 and 4.5%, SD= 1.0 respectively). Furthermore, serotype 5 was the most specific for transduction of ATII cells with no other transduced cell types observed. Serotypes were further investigated in preliminary studies using human precision cut lung slices (PCLS) of surgical lung resections. Fresh human lung tissue was cut in 500 µm sections and treated ex-vivo with 1E11 GC of rAAV2/5 expressing eGFP as reporter transgene. On day 12, PCLS (n=4) were processed in fixed-frozen sections and analysed for native eGFP fluorescence using confocal microscopy. Although serotype 5 showed the highest transduction rate in the murine lung, transduction was not observed in any of the 2-3 analysed sections per human lung slice. This stresses the importance of choosing appropriate model systems for the investigation of gene therapies. Further studies are ongoing to compare rAAV2/5 with other serotypes in human models of the lung.

532. Development of a Barth Syndrome Gene Therapy Program

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Barth syndrome (BTHS) is caused by mutations in the gene encoding tafazzin (*TAZ*). Tafazzin is important for remodeling cardiolipin in the inner mitochondrial membrane in cardiac and skeletal muscles. In BTHS, tafazzin loss-of-function results in abnormal mitochondrial cristae structures which causes destabilization of electron transport chain supercomplexes and a reduced ability to consume oxygen and generate energy in cardiac and skeletal muscle. BTHS patients experience increased muscle fatigue and heart failure. Currently there are no effective therapies for BTHS other than supportive cardiac care. The goal of this project is to develop a clinically relevant adeno-associated virus (AAV) mediated gene delivery approach to treat BTHS patients. Thus far our program has: 1) identified relevant clinical and physiologic outcome variables in BTHS patients, and 2) identified the optimal AAV-*TAZ* expressing vector through testing and comparisons in BTHS mice and human induced pluripotent stem cell (iPSC)-derived cardiomyocytes and myotubes. We are now preparing to: 3) test the safety of AAV-*TAZ* expressing vectors through toxicology studies, and 4) test the efficacy and safety of AAV-*TAZ* gene therapy in patients with BTHS. Aim 1: In 29 BTHS and 28 unaffected human subjects, exercise studies have identified impaired exercise tolerance (VO_{2peak}), lower cardiac strain (echocardiography), altered cardio-skeletal energetics (^{31}P magnetic resonance spectroscopy), and blunted exercise fatty acid metabolism and elevated glucose metabolism (stable isotope tracer methodology) in BTHS vs. controls. Aim 2: Our preliminary vector comparison data have demonstrated that administration of 1x10^{13}

vector genomes/kg of dsAAV9-Des-*TAZ* (desmin promoter, cardio-skeletal distribution) to neonates or adults corrects disease phenotypes in a BTHS mouse model. Global tandem mass tagging (TMT) proteomics analyses of cardiac tissues shows dramatic improvement in treated groups. Administration to BTHS patient derived cells (iPSCs differentiated into cardiomyocytes or skeletal myotubes) representing a variety of different mutations in the *TAZ* locus improves mitochondrial structure and function regardless of the underlying mutation. Aims 3, 4: IND-enabling and human studies are planned and *in vivo* testing of a human codon optimized (AAV-Des-co*TAZ*) vector has begun. Our current data have identified relevant cardio-skeletal muscle functional and physiologic outcomes in human subjects and have determined that the Des promoter is optimal for treatment of BTHS.

533. Evaluation and Optimization of Gene Correction Using an All-RNA-Based CRISPR/Cas Approach in Genetic Lung Diseases

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Objective: Cystic Fibrosis (CF) is the most common life-limiting genetic disease among Caucasians affecting not only the lung but also other organs like pancreas and intestine, leading to reduced life expectancy and quality of life. In contrast, Surfactant Protein B (SP-B) deficiency is a rare congenital disease currently only treatable by lung transplantation. Newborns with this disorder usually do not survive more than a few months. Both diseases can be caused by point mutations in the CFTR (CF) and SFTPB (SP-B deficiency) genes in the genome. Gene correction using CRISPR/Cas systems offers a new approach to correct the underlying mutation and give rise to new opportunities for therapy. The aim of the project is to optimize the usage of *Cas9* mRNA and single guide RNA (sgRNA) in cellular *in vitro* models to achieve homology-directed repair (HDR) of mutations in *CFTR* and *SFTPB*. **Methods**: Chemically modified mRNA (cmRNA) encoding for humanized *S. pyogenes* Cas9 protein (hSpCas9) and sgRNAs targeting ΔF508 or 262_263delTT mutations of the CFTR gene and 121ins2 mutation of the SFTPB gene were transfected into Cystic fibrosis bronchiolar epithelial (CFBE41o-), HEK293 and A549 cells. Genomic DNA was isolated 3 days post transfection and regions in close proximity to targeted mutations were PCR-amplified for analysis of induction of insertions and deletions (indels) by T7 Endonuclease I assay and TIDE analysis. In a following experiment single-stranded oligodeoxynucleotides (ssODNs) as a repair template were transfected together with *Cas9* cmRNA and sgRNA and HDR was measured using Restriction fragment length polymorphism (RFLP) assay and TIDER analysis. Off-target effects were evaluated by GUIDE-seq and followed by whole genome sequencing. **Results:** *Cas9* mRNA yielded a clear peak with a size of just beneath 4 kb when tested for RNA integrity by Agilent Bioanalyzer 2100. We analyzed multiple sgRNAs targeting all three mutations sites for optimization of indel efficiency. After optimization T7 Endonuclease I assay showed indel efficiencies of 82% for the 262_263delTT target region, 65% for the ΔF508 target region and 75% for the 121ins2 target region, respectively. The TIDE analysis displayed stable indel efficiency around 50% for all tested mutations. Furthermore, ssODNs varying in size were tested for HDR induction with the selected sgRNA and *Cas9* cmRNA for all mutations. So far HDR levels reached less than 10%. **Conclusion:** The delivery of CRISPR/Cas systems in an all-RNA approach together with ssODNs to achieve homology-directed repair of point mutations *in vitro* provide an option to transfer the findings *in vivo* for CF and SP-B deficiency en route towards CRISPR/Cas-based therapies.

534. Correction of the Cystic Fibrosis 3849+10kb C>T Mutation Using a CRISPR-Cas9 NHEJ Strategy Delivered by Receptor-Targeted Nanocomplexes

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Background: Cystic Fibrosis is an autosomal recessive disorder caused by mutations in the CFTR gene. The 10th most common mutation, 3849+10kb C>T, generates a cryptic splice site, resulting in the formation of a pseudoexon containing a PTC, producing a truncated version of the protein. CRISPR/Cas9 allows for precise targeting of mutations by a guide RNA targeting molecule followed by double strand DNA cleavage by Cas9 nuclease. However, delivery of the CRISPR components into cells and target organs remains a challenge. **Aims:** Here, we aim to use non-viral, receptor-targeted nanocomplexes to deliver CRISPR/Cas9 technology. Collaborators designed pairs of Cas9 guide RNAs to create targeted double-stranded breaks in CFTR either side of the mutation, resulting in high efficiency excision via non-homologous end-joining repair, when tested in a mini-gene assay in HEK293T cells. We wished to repeat these experiments in bronchial cells isolated from a patient homozygous for this mutation to confirm functional restoration of the CFTR protein channel. **Methods:** Primary CFBE cells were lentivirally transduced with the *BMI-1[[Unsupported Character - Codename]]* proto-oncogene to expand proliferative potential for the course of experiments. Pairs of gRNAs were complexed with Cas9 protein and formulated with our lipid-based nanoparticles for transfection. 48 h post-transfection, genomic DNA was isolated, and cells were re-seeded for repeat transfections. Cells were then expanded for ALI culture. After 5 weeks differentiation on ALI, CFTR mRNA was analysed via qRT-PCR, and protein expression analysed by Ussing Chamber. **Results:** After one nanoparticle transfection, a DSB efficiency of 26% was achieved in primary CFBE cells, as measured by Inference of CRISPR Edits (ICE) software, and T7 endonuclease assay. Of this, 10% of sequences had the expected 179 bp excision, indicative of successful cutting by both guides. After four repeat transfections, DSB efficiency increased to 82%, with expected excision occurring in 61% of sequences (Figure 1). This was able to restore normal mRNA splicing and, importantly, CFTR channel function as measured by Ussing Chamber. **Conclusion:** This approach could be used to correct aberrant splicing signals in several other CF mutations, and indicates that correction of splice mutations leads to functional restoration of the

CFTR protein. It also shows that repeat dosing strategies are possible with our non-immunogenic nanocomplexes. Moreover, this targeted gene excision strategy may also be applicable in the study many other genetic disorders where deep-intronic mutations have been identified as a disease cause.

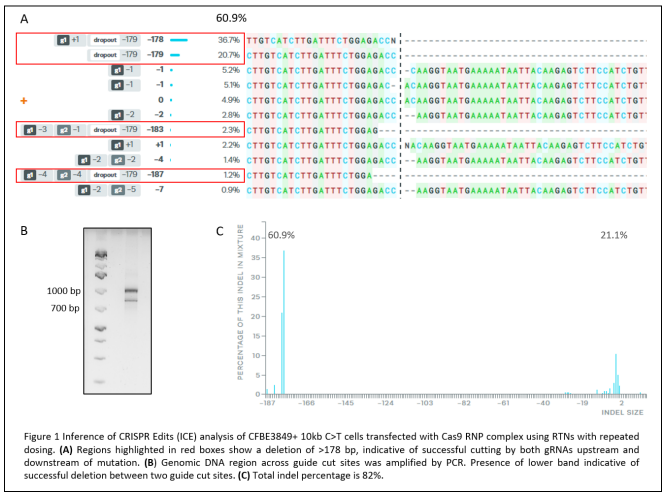

Figure 1 Inference of CRISPR Edits (ICE) analysis of CFBE3849+ 10kb C>T cells transfected with Cas9 RNP complex using RTNs with repeated dosing. **(A)** Regions highlighted in red boxes show a deletion of >178 bp, indicative of successful cutting by both gRNAs upstream and downstream of mutation. **(B)** Genomic DNA region across guide cut sites was amplified by PCR. Presence of lower band indicative of successful deletion between two guide cut sites. **(C)** Total indel percentage is 82%.

535. Abstract Withdrawn

536. Optimizing the Utilization of Chemically Modified Cas9 mRNA / sgRNA Against the Underlying Genetic Defect in Surfactant Protein B Deficiency and Cystic Fibrosis Based on Expression Kinetics

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Objective: Chemical modification of mRNA nucleosides to reduce immune response made mRNA-based therapy a viable option for protein supplementation or gene correction. Based on published comparison studies of various chemical modifications we have conducted a study focusing on comprehensive expression patterns and understanding of *in vitro* and *in vivo* kinetics. On the basis of these kinetics, we strive to optimize the utilization of *Cas9* mRNA and sgRNA in various monogenetic diseases. Surfactant Protein B (SP-B) deficiency, an orphan disease that results in progressive, hypoxemic respiratory failure in infants and Cystic fibrosis (CF), the most common life-limiting autosomal-recessive disease in the Caucasian population were investigated. CRISPR/Cas has become a powerful tool for engineering genomes in diverse organisms and showed promise for correcting disease-causing mutations. However, off-target effect still poses a potential risk for CRISPR/Cas based therapy and a regulated expression of Cas9 protein can increase the chance of higher correction with limited adverse effects. **Method:** In this study, the kinetics of six commonly used and published chemically modified mRNAs (cmRNAs) have been evaluated. DsRed and mKate2 have been used as marker proteins and mRNA of these Red Fluorescent Protein (RFP) variants have been chemically modified and transfected into A549 and HEK293 cells. Expression at different time points (Eight time points from three hours to seven days) was measured using flow cytometry. Quantitative RT-PCR was used to determine the amount of mRNA in the cells at those time points. Based on expression kinetics of marker proteins chemically modified *Cas9* mRNA and sgRNA mediated gene correction approaches have been conducted in HEK293 cells against 121ins2 (SP-B) and 262_263delTT (CFTR) mutations. T7 endonuclease I assay and TIDE have been used to evaluate insertion and deletion (Indel). Off-target effects were evaluated by GUIDE-seq and followed by whole genome sequencing.**Results:** Certain chemical modified mRNA reached maximum protein expression (mKate2 and DsRed) as early as 12 hours (by flow cytometry) while being almost undetectable after 3 days, whereas others had a delayed onset of expression (48 hours), but could be detected even after seven days. These expression patterns are also reflected in a functional study of CRISPR/Cas based indel induction. One chemical modified *Cas9* mRNA can achieve indel induction efficiency around 60% (by both TIDE analysis and T7 assay) within 24 to 48 hours for 121ins2 and 262_263delTT mutations. Other chemically modified *Cas9* mRNA took 72 hours to 5 days to reach a similar level of indel induction. mRNA level and related protein expression in cells (by RT-qPCR) also showed a direct correlation to expression pattern based on used modification.**Conclusion:** Using *Cas9* cmRNA for a controlled expression profile of the Cas9 protein will help to correct the underlying 121ins2 (SP-B) and 262_263delTT (CFTR) frame shift mutation, thus leading to an efficient and specific gene correction, while exhibiting very limited to no detectable off-target effects at the same time.

537. Strategies for Enhancing Endothelium-Specific Transgene Expression for Gene Therapy of Atherosclerosis

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Background: Gene therapy of atherosclerosis requires high-level transgene expression in order to: 1) increase therapeutic efficacy; and 2) allow use of lower vector doses, thereby minimizing vector-related toxicity. We previously showed that infusion of a helper-dependent adenoviral (HDAd) vector including a CMV promoter-driven apolipoprotein A-I transgene (HDAdapoAI) into carotid arteries of fat-fed rabbits can prevent or reverse atherosclerosis. However, the effects on atherosclerosis were partial and will need to be improved before clinical application. Here we attempt to increase expression of apo A-I from HDAdapoAI by adding transcriptionally active sequences to our apo A-I expression cassettes. We identified these transcriptionally active sequences using both a candidate-sequence approach (i.e., addition of known transcriptionally active sequences) and a novel unbiased bioinformatics-based approach (to discover new transcriptionally active sequences). The bioinformatics-based approach is a modification of methods used previously to identify enhancers of transcription in hepatocytes and cardiomyocytes. **Methods:** We identified transcriptionally active sequences both by searching the biomedical literature for sequences known to increase transcription in

endothelial cells and with a bioinformatics-aided search of the human genome, designed to identify new cis-acting sequences that enhance transcription of nearby genes. Because our gene-therapy approach relies on transgene expression in vascular endothelium, we focused on identification of transcriptionally active sequences located within 100 kb of the transcription start sites (TSS) of genes that are highly and preferentially expressed in endothelial cells. Both known and putatively novel transcriptionally active sequences were cloned, then inserted (as monomers or, in 1 case, as multimers) upstream of our existing apo A-I expression cassettes. The new expression cassettes were screened *in vitro* by plasmid transfection into bovine aortic endothelial cells. Some of the cassettes were also incorporated into HDAd vectors and tested *in vivo* in rabbit carotid arteries. **Results:** Addition of a known shear-stress-response element did not increase apo A-I expression either *in vitro* or *in vivo*. Addition of 1-5 copies of a *Mef2c* enhancer sequence increased apo A-I expression from a cassette containing a minimal promoter sequence; however, adding even one copy of the *Mef2c* enhancer to an expression cassette that already contained a highly active promoter/enhancer sequence decreased apo A-I expression. The bioinformatics-based strategy identified 11 potential cis-regulatory modules (55 - 352 bp in length) near the TSS of four genes that are highly expressed in endothelial cells (1 - 4 per gene, located both 5′ and 3′ of the TSS). Cloning of these sequences into two existing endothelial-selective expression cassettes (containing either the minimal promoter sequence or the highly active promoter/enhancer sequence) was confirmed by DNA sequencing. Data obtained with these cassettes will be presented. **Conclusions:** Trial-and-error addition of known transcriptionally active sequences did not reliably increase apo A-I expression. An unbiased bioinformatics-based approach identified sequences (termed cis-regulatory modules) associated with high transcriptional activity in endothelial cells. Incorporation of these cis-regulatory modules into our expression cassettes may increase apo A-I expression. This novel bioinformatics-based approach may also provide a general method for designing cell-type-selective gene therapy vectors.

538. Nrf2/Keap1/ARE Signaling-Mediated Antioxidative Protection of Human Placental Mesenchymal Stem Cells of Fetal Origin in Alveolar Epithelial Cells

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The imbalance between oxidant and antioxidant may result in oxidative stress that mainly caused by an increased production of reactive oxygen species (ROS). Subsequently, the oxidative stress leads to cell necrosis and apoptosis, which ultimately causes systemic inflammatory responses and diseases. In this regard, oxidative injury has been recognized to play an important role in pulmonary injury, and is associated with the development and progression of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), two clinical syndromes of acute respiratory failure with substantial morbidity and mortality. Despite huge progresses have been made in treatments of these diseases in the past few decades, the mortality rate of ALI/ARDS remains approximately 40%. Therefore, new effective therapeutic agents and/or strategies are urgently required. Mesenchymal stem cells (MSCs) have potencies of proliferation, differentiation and self-renewal, and have shown a promise in treatment of many human diseases in clinical trials, mainly owing to their roles in immunoregulation and their anti-oxidative capacity. Our previous works have demonstrated a strong immunoregulatory role of human placental MSCs of fetal origin (hfPMSCs), the antioxidant activity of hfPMSCs however has not been interrogated. In the present study, the anti-oxidative activity of hfPMSCs was examined by accessing their ability to scavenge oxidants and radicals, and protection of alveolar epithelial cells from anti-oxidative injury using cell culture model and conditioned culture medium (CM) of hfPMSCs. Results showed a comparable anti-oxidative capacity of the CM with 100 μM of vitamin C (Vc) in terms of the total antioxidant capacity (T-AOC), scavenging ability of free radicals DPPH, hydroxyl radical (·OH) and superoxide anion radical (O_2^-), as well as activities of antioxidant enzymes of SOD and GSH-PX. Importantly, both of co-cultures of hfPMSCs and hfPMSC-CM could significantly protect A549 epithelial cells for H2O2-induced cell apoptosis ($p<0.01$). Mechanistically, both hfPMSCs and hfPMSC-CM were able to increase the expression of anti-apoptotic proteins and inhibit pro-apoptotic proteins in A549 cells ($p<0.01$), implying hfPMSCs and the hfPMSC-CM could protect or recover the injured A549 cells through inhibiting cell apoptosis. In addition, Nrf2/Keap1/ARE signaling pathway related proteins Nrf2 increased but Keap1 decreased in hfPMSC-CM treated cells compared to the injury group. These data clearly suggested that hfPMSCs protected the H_2O_2-induced cell oxidative injury at least in part by regulating the Nrf2-Keap1-ARE signaling-mediated cell apoptosis. Our study thus provided a new insight into the anti-oxidative mechanism and novel functions of hfPMSCs as antioxidants in disease treatments, which is warranted for further investigations.

Neurologic Diseases II

539. Improved Disease Amelioration with Combination Therapy for Niemann-Pick C1 Disease

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Niemann-Pick C (NPC) disease is a fatal, inherited metabolic disorder resulting from defects in one of two genes: *NPC1* or *NPC2*. Both of these proteins are believed central to a cell's ability to traffic cholesterol out of the lysosome to other cellular locations. A defect in either the NPC1 or NPC2 protein results in significant lipid storage within late endosomal/lysosomal compartments, impeding normal cell function and eventually leading to cell death. Particularly hard hit are neurons within the central nervous system. Clinically, the CNS involvement translates as a progressive loss of intellectual and motor function of NPC patients, who eventually succumb to NPC disease. At present, the NPC community has 3 distinct clinical trials in different phases of development. While very exciting, the expected

approval of one or even two compounds begs the question of whether combination therapy will provide even greater benefit. To address this important question, a combination therapy study was carried out in NPC1 mice: VTS-270 (2-hydroxypropyl-beta-cyclodextrin) + gene therapy (AAV9.EF1a(s).hNPC1). Individually, each of the therapies have shown significant disease amelioration in animal studies. Mice were enrolled in the study and efficacy of combination treatment was evaluated. Assays included behavioral testing, weight monitoring, survival, disease pathology, and gene copy number. Key data suggested greater disease amelioration with combination therapy as compared to individual mono-therapies. These results are particularly enticing given the recent surge of companies expressing interest in development of therapies for NPC disease and even more so with respect to improving the quality of life for NPC patients.

540. Correction of Spinal Muscular Atrophy in Mice with Single Stranded rAAV-hSMN1 Vectors

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Spinal muscular atrophy (SMA) is the most frequent genetic cause of infant death. It is an autosomal recessive neuromuscular disorder due to mutations in the *SMN1* gene, leading to a selective loss of spinal cord α-motor neurons and proximal muscle weakness. Remarkable progress has been made in the last years on the development of therapeutic approaches for SMA, including gene therapy. In particular, intravenous administration of self-complementary (sc) recombinant AAV9-*hSMN1* vectors have shown efficacy in several animal models and a clinical trial in type 1 SMA patients. Since scAAV vectors display some limitations, such as limited packaging capacity, lower yield production and higher immunogenicity than single-stranded (ss) AAV vectors, we evaluated the therapeutic efficacy of four single stranded (ss) rAAV9 vectors expressing the human *SMN1* coding sequence under various regulatory sequences in the *Smn2B/-* mouse model. Vectors were administrated into the cerebrospinal fluid (CSF) of newborn mice and compared for phenotype rescue. These ssAAV9-*hSMN1* vectors were able to prolong the lifespan of *Smn2B/-* mice with differences in the median survival rate. This treatment ameliorated the behavioral and motor functions of mutant mice, increased the number of spinal motor neurons and improved the morphology of neuromuscular junctions. We identified an expression cassette that outperformed the other constructs. These results demonstrate the importance of regulatory sequences in vector design and the feasibility of SMA gene therapy by administration of ssAAV-*hSMN1* vectors.

541. Leveraging the Human Genome to Engineer GABAergic Interneuron Selective Regulatory Elements for Targeted AAV Expression and GABAergic Circuit Modulation

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Numerous central nervous system (CNS) disorders, ranging from rare genetic epilepsies to more prevalent diseases such as autism spectrum disorders and schizophrenia, are thought to be directly or indirectly affected by GABAergic interneuron hypofunction (GIH). Supportive of this hypothesis, several FDA-approved small molecule therapies modulate GABAergic tone by targeting GABA receptors or GABA reuptake throughout the brain. However, many of these therapies have dose-limiting toxicity and/or side effects, limiting their clinical utility. Recently, a convergence of research has implicated impairment in GABAergic cellular function and neurotransmitter release as a key contributor to numerous CNS diseases. However, there are currently no approved therapies that modulate the release of GABA in a cell type selective, activity-dependent manner. Gene therapy holds the potential to achieve circuit-specific modulation of target gene pathways to address a variety of severe neurodevelopmental/neuropsychiatric disorders, but has thus far failed to achieve the selectivity necessary for this type of precision medicine. Using deep sequencing, bioinformatics, and machine learning we have developed a platform technology capable of generating cell type selective regulatory elements (REs) comprised of human genomic sequences. Our initial efforts have focused on engineering elements capable of driving potent and selective expression within GABAergic interneurons to address unmet need in indications with GIH as a core underlying mechanism. Neuronal subtype selectivity was initially evaluated in human iPSC-derived GABAergic neurons in vitro, and then moved into animal models for in vivo verification using transgenic reporter lines and histological markers to confirm neuronal subtype selectivity. When compared constitutive regulatory elements, such as CBA, engineered GABA-selective REs exhibited a high degree of selectivity for various GABAergic markers, and captured a higher percentage of the GABAergic population. This pattern of expression was maintained across various AAV capsids, brain regions, and species, including non-human primate. Selective targeting of GABAergic interneurons creates the opportunity to address several GIH indications that have heretofore been difficult to target using standard AAV approaches.

542. Developing CRISPRa Tools to Treat Alzheimer's Disease

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There is no cure for Alzheimer's Disease (AD). With the estimated increase of AD prevalence, there is a great urgency to find an effective therapy. CRISPRa was used to develop a novel therapeutic strategy. In order to counter Aβ and tau pathologies simultaneously, we focused on activating genes related to endogenous autophagy/lysosomal pathway. Transcription factor EB (TFEB) was selected as an initial therapeutic target as it can target Aβ and tau pathologies . To determine if CRISPRa could be used to activate TFEB, primary neuronal cultures were transduced with lentiviral vectors expressing Sp deactivated Cas9 with VPR activator (dCas9-VPR) or expressing dCas9 with VPR activator together with sgRNAS for TFEB (dCas9VPR gTFEB). Quantitative PCR analysis indicated a significant 2-fold increase of TFEB mRNA expression in the dCas9VPR gTFEB group. In addition, Western Blot analysis of TFEB protein expression indicated a significant 2-fold increase in TFEB protein expression in the dCas9VPR gTFEB. Interestingly, confocal imaging of TFEB immunofluorescence of dCas9VPR gTFEB showed TFEB located in the soma as expected, suggesting that the increased in TFEB expression does not lead to increased autophagy in physiological conditions. Next, we tested if activated TFEB could degrade Aβ. Primary neuronal cultures transduced with dCas9VPR or dCas9VPR gTFEB were treated with 10 μM of Aβ. Immunofluorescence performed 48 hours after treatment showed an increased number of LAMP1 vesicles in the dCas9VPR gTFEB group, suggesting an increased autophagic response against intracellular Aβ. After proof of principle that CRISPRa could be used to activate TFEB in primary cells, we decided to switch to a CRISPRa system that was compatible with AAV for efficient brain delivery. We focused on the dSA-VPR mini fusion protein. When AAV8 expressing the dSA-VPR mini was delivered to the striatum of mice, it was possible to observe that the Cas9 signal was mostly located outside the nucleus. To improve the dSA-VPR mini, we redesigned it to have a hemagglutinin tag to facilitate detection and 2 synthetic NLS that have been shown to drastically improve nuclear localization of Cas9. Preliminary testing using AAV8 expressing the dSA-VPR mini V2 showed that the dSA-VPR mini V2 had a very efficient nuclear localization in the hippocampus of mice. Due to the different PAM requirements of the SaCas9 system, we needed to screen for novel sgRNA suitable for TFEB activation. We developed an assay where 4 plasmids were be transfected into 293T. One plasmid expressed the dSA-VPR mini V2. One plasmid used the mouse TFEB promoter, the genomic sequence where the sgRNA will bind, to drive the expression of the TdTomato reporter gene. One plasmid expressed the sgRNA. Lastly one plasmid expressed blue fluorescent protein was used as transfection control. The assay was done in 96 well plates and allowed the screening and identification of single sgRNA or double sgRNA combinations that activated TdTomato robustly. Our results suggest that CRISPRa can be used to target therapeutic genes in primary neurons and has the potential to develop novel therapies for AD.

543. Developing Gene Therapies for Inherited Retinal Dystrophies 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 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, hence guiding precision medicine. **Results and Discussion:** Due to the differing mechanisms of action of different disease-causing mutations in the eye across multiple genes, different editing approaches should be used. We find that for some autosomal dominant mutations, gene knockout via allele-specific editing is efficacious - while for some autosomal recessive mutations, gene augmentation or 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 polymeric nonviral carriers of protein based CRISPR machinery (e.g. ribonucleoprotein or s1mplexes) can edit as efficiently 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. 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.

544. 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 older people, caused by abnormal blood vessel leakage and bleeds leading to progressive degeneration of the central retina (macula). Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that is known to be up regulated in retinal tissue under hypoxic conditions and VEGF over-expression has a key role in the development of wet AMD. A number of anti-VEGF therapeutic strategies have been developed and are the current standard of care for wet AMD, including aflibercept, ranibizumab and bevacizumab. However, development of new treatment paradigms are necessary because these treatments are only effective for 1-2 months and as such require frequent injections for an extended period of time causing a significant burden to patients, and are associated with ocular side effects such as inflammation, haemorrhage and retinal detachment. We have therefore developed an alternative treatment that could avoid the complications, discomfort and healthcare burden associated with repeat injections in which an Equine Infectious Anaemia Virus (EIAV) lentiviral vector is engineered to express the anti-VEGF protein, aflibercept directly in the eye following a single subretinal injection (OXB-203). Clinical data from our Retinostat® (EIAV-endostatin and angiostatin) program (Campochiaro, 2017) following a single subretinal injection in three US clinical sites has demonstrated stable long term expression of the transgenes out to 6 years (so far) providing proof-of-principle for this type of single injection gene therapy approach. An EIAV lentiviral vector (OXB-203) was constructed encoding the anti-VEGF protein aflibercept with a signal sequence inserted to allow extracellular secretion of the protein. Anti-VEGF ELISAs and binding analysis of EIAV-aflibercept transduced cell supernatants demonstrated comparable binding characteristics to that of recombinant aflibercept. *In vitro* angiogenesis assays in HUVEC cells demonstrated that both cell proliferation and matrigel tubule formation were significantly inhibited with vector derived aflibercept similar to that of recombinant aflibercept. Preclinical studies using a rat choroidal neovascularization model following a subretinal administration of EIAV-aflibercept vector are ongoing.

545. Eye-Targeted Hematopoietic Stem Cell Transplantation for Ocular Cystinosis

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Cystinosis is caused by a deficiency in the lysosomal cystine transporter, cystinosin (*CTNS* gene), resulting in cystine crystal accumulation in tissues. In the eyes, crystals accumulate in the cornea causing photophobia and eventually blindness. We previously showed that systemic hematopoietic stem progenitor cell (HSPC) transplantation rescue the corneal defects in a mouse model of cystinosis. We found that HSPC transplantationled to substantial decreases in corneal cystine crystals, restored normal corneal thickness, and lowered intraocular pressure (IOP) in $Ctns^{-/-}$mice. We also demonstrated that HSPC-derived progeny differentiated into macrophages, which displayed tunneling nanotubes capable of transferring cystinosin-bearing lysosomes to diseased cells in the cornea. Because there is an ocular non-nephropathic form of cystinosis, we wanted to test if a local injection of HSPCs would have the same impact on the corneal defects. We thus performed intracameral and intravitreal HSPC injection in 2-month-old animals. We are comparing these two groups with mice systematically transplanted with HSPCs as well as non-transplanted control mice. At 6-months post transplantation all the mice have been subjected to in vivo confocal microscopy to quantity corneal cystine crystals. We found that systemic and intracameral injections led to significant reduction of the number of crystals compared to controls, but not intravitreal injection. These findings are confirmed by mass spectrometry with a reduction of cystine content in those 2 groups. We are now investigating by confocal microscopy the fate and phenotype of the HSPC progeny transplanted locally and if they also differentiate in macrophages that are capable to provide functional cystinosin to the disease adjacent cells. This work is a proof of concept for ocular cystinosis but also other inherited eye disorders.

546. Miniaturizing Usher Syndrome Type 2A Gene for Gene Therapy via Cilia Formation Evaluation

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Usher Syndrome (USH), an inherited disorder, is the leading cause of deaf-blindness. Patients exhibit progressive degeneration of retinal photoreceptors and sensorineural hearing defects. Mutations in USH2A (USH Type 2A) gene, which codes for the protein Usherin (600kDa) account for about 70% of all USH cases. Currently the most expedited path to clinical application for photoreceptor degeneration is subretinal AAV-mediated gene augmentation. However, due to AAV volumetric constraints, using the full length 15.6 kb coding sequence of USH2A is not feasible. For this reason, our study aims to develop a functional minigene of USH2A to fit into AAV packaging for gene augmentation therapy. Usherin contains a large number of repetitive motifs: 35 Fibronectin type III motifs (FN3), 10 Laminin EGF motifs (EGF_Lam), and 2 Laminin G domains. These motifs are often associated with protein bindings in the extracellular matrix. Although several key positions have been shown to affect fibronectin and collagen binding, the role of most motifs are yet to be examined. Usherin localizes to homologous membrane microdomains, the stereocilia of hair cells in the ear and the periciliary region of photoreceptor connecting cilia in the retina. The majority of the protein projects into the extracellular matrix and a small intracellular region containing PDZ binding motif forms complexes with other Usher proteins. Such

localization and protein structure suggest a possible role of Usherin in maintaining interactions between cilia and the periciliary structures. Thus, we hypothesize that USH2A dysfunction causes ciliopathy. As it is currently unclear how many, and which, of these repetitive motifs are required for effective protein binding in the periciliary region, we use the coding sequence of the short non-repetitive region of USH2A as a backbone and the repetitive motifs as building blocks to design minigenes with variant combinatory motif repeats. To examine the function of these minigenes, we conducted in vitro screening of cilia formation with USH2A Null Oc-k1 cell line, which demonstrates compromised ciliogenesis in comparison to WT Oc-k1 cell line. Oc-k1 cell line is derived from the organ of Corti of a P14 Immortomouse and expresses inner ear cells' protein marker OCP2. Our results show that minigene-1, a proof of concept construct that lacks FN3 motifs #6,7 and 8, significantly improved cilia formation in USH2A Null Oc-k1 cell line. The expression of full-length human USH2A gene also yielded similar results. Our analysis was done through detection of Arl13B, a marker for functional cilia. Fluorescence microscopy and automated batch image analysis was performed for all experiment groups. We also found that change in localization of Usherin accompanies the ciliogenesis process. Oc-k1 WT cells demonstrate enriched localization in the periciliary region after serum starvation whereas serum-fed condition lacks such enrichment in the periciliary region. We are currently examining the localization of minigene-1 protein in USH2A Null Oc-k1 cells, under serum fed and starvation conditions. Localization in periciliary region of the minigenes will also provide further support for its cilia rescue function. Due to the large number of motif repeats, we expect that screening a combinatorial library with variant motifs will be necessary in optimizing minigene design. Six more exploratory minigenes are under development, which will guide us to design such combinatorial library. Our future direction also includes further validation of the therapeutic effect in USH2A-/- mouse model.

547. Gene Therapy for Infantile Parkinsonism

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Most inherited neurodegenerative disorders are incurable, and often only palliative treatment is available. Gene therapy has great potential to address this unmet clinical need. We developed gene therapy for Dopamine Transporter Deficiency Syndrome (DTDS), caused by bi-allelic mutations in *SLC6A3*, which encodes the dopamine transporter (DAT). Patients present with early infantile hyperkinesia, severe progressive childhood parkinsonism and raised cerebrospinal fluid dopamine metabolites. The absence of effective treatments and relentless disease course frequently leads to death in childhood. Using patient-derived induced pluripotent stem cells (iPSCs), we generated a midbrain dopaminergic (mDA) neuronal model of DTDS. Lentiviral gene transfer restored DAT activity and prevented neurodegeneration in all patient-derived mDA lines. To progress towards clinical translation, we utilized the knockout (KO) mouse model of DTDS, which recapitulates human disease, with reduced survival and parkinsonism features, including tremor and bradykinesia. Neonatal intracerebroventricular injection of adeno-associated virus (AAV) vector provided neuronal expression of human DAT which rescued motor phenotype, lifespan and neuronal survival in the substantia nigra (SNc) and striatum.

548. Efficient Delivery of Clinically-Validated Adeno-Associated Viral (AAV) Vectors to the Central Nervous System: A Systematic Evaluation of Multiple Routes of Administration and Viral Serotypes in Cynomolgus Macaques

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Gene therapy using adeno associated viral (AAV) vectors has transformational potential to treat disorders affecting the central nervous system. Studies in small animal models have shown that delivery of AAV vectors into the cerebrospinal fluid (CSF) can successfully result in gene transfer to cells throughout the brain and spinal cord, making neurological diseases amenable to gene therapy approaches. Essential to the translation of this approach into the clinic is the identification of safe and effective routes for AAV delivery into the CSF of large animal models. Intracerebroventricular (ICV) is a particularly attractive route of delivery due to its proximity to forebrain structures and thus its potential for broad cortical distribution. In clinical practice, administration of drugs via ICV is a relatively

common surgical procedure that is routinely performed in adults and young children alike. In this study, we systematically quantified biodistribution and transduction efficiency of clinically-validated AAV serotypes, including AAV serotype 9 (AAV9), AAV serotype 5 (AAV5) and AAV serotype 1 (AAV1) via ICV administration in juvenile neutralizing antibody (NAb) negative male cynomolgus macaques (Macaca fascicularis). We prepared AAV vectors expressing green fluorescent protein (eGFP) driven by a chicken beta actin promoter (CBA) via triple transfection of HEK293 cells. Vectors were titered via digital droplet PCR (ddPCR) and delivered at a controlled dose via unilateral ICV administration. Biodistribution was evaluated across CNS tissues and peripheral organs. Additional routes that are typically explored for the delivery of AAV to CSF include bilateral ICV, intrathecal lumbar (IT-lumbar), and intracisterna magna (ICM). Thus, along with characterization of unilateral ICV route, we directly compared the biodistribution and transduction efficiency of AAV9 across these 4 main routes of CSF delivery (unilateral ICV, bilateral ICV, IT-L, ICM) at a controlled dose. Intra-CSF routes were additionally compared to intravenous (IV) injection at a similar dose. Thus, in this multi layered study, we demonstrate how the combination of route of administration and AAV serotype offers a tailored approach to target CNS biodistribution to various brain structures. Our findings inform the selection of an intra-CSF route of administration and AAV capsid serotype selection for clinical translation of a CNS-directed gene therapy.

549. Hematopoietic Stem and Progenitor Cell Gene Therapy for 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 intron 1 of the frataxin gene (*FXN*).This mutation leads to the reduction of frataxin expression, a mitochondrial protein involved in iron metabolism. FRDA is characterized by ataxia, areflexia, sensory loss, muscle weakness, and cardiomyopathy. Symptoms typically begin between 5 to 15 years of age and patients will be in wheelchair within 10-15 years of onset. Currently, there is no treatment for FRDA. In 2017, we showed that transplantation of mouse wild-type hematopoietic stem and progenitor cells (HSPCs) prevents the development of the locomotor deficits, the neuronal degeneration in the dorsal root ganglia and the oxidative damages in brain and muscle in the YG8R mouse model of FRDA. We also showed that the mechanism of rescue is mediated by the transfer of frataxin from the HSPC-derived microglia/macrophages to neurons/myocytes. Our goal is to develop an autologous transplantation of gene-corrects HSPCs to move toward a future clinical trial. We first optimized the conditions of our gene-correction approach in lymphoblasts isolated from FRDA patients and obtained up to 62% of gene correction. At the mRNA and protein levels, frataxin expression reached the same level than their carrier parents' cell lines. We thus moved towards the manufacturing development of the human product using first CD34$^+$cells isolated from healthy donor peripheral blood. We successfully gene-corrected 24 to 50% of the CD34$^+$cells. The capacity of the gene-modified CD34$^+$cells to differentiate into the different hematopoietic lineage cells was tested in vitro by Colony Forming Unit assays and in vivo in NOD *scid* gammaimmunodeficientmice. Our protocol will be now tested in CD34$^+$cells isolated from FRDA patients. With this study, we are laying the foundations for a future clinical trial for autologous HSPC transplantation for FRDA.

550. CRISPR-Based Allele-Specific Editing for the Treatment of Autosomal Dominant Retinitis Pigmentosa

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Retinitis pigmentosa is a group of inherited diseases causing retinal degeneration and can result from defects in more than 60 genes. Rhodopsin is a G-protein-coupled receptor, and a pigment found in rod photoreceptor cells. A point mutation in codon 23 of the rhodopsin (*RHO*) gene (g.129528801C>A) leads to the substitution of proline with histidine (P23H). Accumulation of the misfolded mutant rhodopsin in rod photoreceptor cells results in primary rods degradation and progressive visual loss. This mutation accounts for 10% of all advanced retinitis pigmentosa cases. Gene therapy has been challenging because the dominant effect of the mutant protein needs to be suppressed or disrupted. CRISPR/Cas9 technology allows for *in vivo* targeted gene disruption by introducing site-specific double strand breaks and could potentially offer treatment for diseases associated with dominant mutations in human *RHO* gene. Here, we have developed a method for specific inactivation of the human *RHO*-P23H mutant allele using Cas9 from *Staphylococcus aureus* (SaCas9) and a single guide RNA (sgRNA) that is highly selective for the P23H mutation to preserve the function of the wild type allele. To identify appropriate guides, a K562 cell line with a homozygous g.129528801C>A mutation (*RHO*-P23H cell line) was generated and used to examine the allele specificity of sgRNAs carrying spacers ranging from 18-24 nt in length. Multiple guides were identified that efficiently discriminated between the WT and the P23H alleles. No "off target" genetic modifications were detected for any of the tested guides using either directed (bio-informatic) or undirected approaches. We are currently testing the top Cas9/sgRNAs *in vivo* using both constitutive and self-inactivating AAV vector-based delivery systems in *knock-in* mouse models carrying the normal human *RHO* or mutant *RHO*-P23H genes. Taken together, our results suggest that specific inactivation of the P23H allele using CRISPR/Cas9 holds promise as a potential treatment for patients with ADRP due to the *RHO*-P23H mutation.

551. Gene Therapy to Provide Systemic Protection from Oxidant Stress

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Oxidative stress derived from environmental factors, including pollution, and smoking, is a common cause of tissue damage with concomitant initiation and acceleration of diseases such as chronic obstructive pulmonary disease, atherosclerosis, and cancer. To provide a persistent extracellular anti-oxidant enzyme shield that will inactivate the common oxidative stress mediators superoxide and H_2O_2, we have developed a gene therapy approach that mediates expression of secreted anti-oxidant enzymes. To provide a front line defense against extracellular reactive oxidants, our strategy is to mediate long-term expression by adeno-associated virus (AAV) of a novel, monomeric, secreted, functional catalase and a modified extracellular superoxide dismutase. Catalase is a tetrameric intracellular enzyme that even if secreted is too large to diffuse across organs. A therapeutic monomeric version of catalase was created by removal of the wrapping loop domain that mediates tetramer formation. With the addition of a secretory signal, the human catalase monomer (hCatWL$^-$) is secreted and remains capable of functioning to catalyze extracellular H_2O_2 to H_2O. Superoxide dismutase 3 (SOD3) is secreted, but it is a large tetramer and has a heparin-binding domain that attaches it to cell surfaces. We modified a loop critical for tetramer formation and removed the heparin-binding domain to produce an effective antioxidant enzyme monomer (hSOD3hd$^-$) that does not bind to cell surfaces. *In vitro* analysis of 293T cells transfected with the monomer plasmids mediated expression of hCatWL$^-$ and hSOD3hd$^-$, both of which were secreted into the supernatant demonstrating activity of catalase and SOD, respectively. Human large airway epithelial cells transfected with plasmid expressing hSOD3hd$^-$ were protected from cytotoxicity (assayed by lactate dehydrogenase release) following exposure to the oxidative stress from either cigarette smoke extract or xanthine oxidase (both $p<0.02$, compared to control cells treated with an irrelevant plasmid). Transfection with the hCatWL$^-$ plasmid protected these cells from cigarette smoke extract ($p<0.05$). Based on this *in vitro* data, both modified enzymes were encoded into the expression cassettes of serotype rh.10 AAV gene transfer vectors to genetically modify the liver to express and secrete the monomers. Mice administered 10^{11} genome copies of the AAVrh.10hCatWL$^-$ or AAVrh.10hSOD3hd$^-$ via the tail vein had significantly greater catalase or superoxide dismutase activity in the sera than controls. Together, these studies suggest that AAV-directed expression of catalase and SOD monomers alone and/or together, may be an effective means of protecting vulnerable organs from extracellular oxidative stress.

552. Evidence of Intercellular Transmission of Frataxin in Patient-Derived Cells via Two Distinct Mechanisms

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Friedreich's ataxia (FRDA) is the most frequent inherited autosomal recessive neurodegenerative disease. This lethal disease, currently untreatable, occurs due to reduced expression of frataxin (*FXN* gene), a nuclear encoded mitochondrial protein essential for iron metabolism. Recently, our group has revealed that hematopoietic stem and progenitor (HSPC) cell transplantation prevents development of the disease phenotype in a mouse model of FRDA, providing an evidenced-based approach for treatment of FRDA patients. In vitro, we observed transfer of frataxin-eGFP from donor fibroblasts to FXN-deficient fibroblasts. In vivo, we also observed transfer of frataxin-eGFP in disease neurons from HSPC-derived microglia in brain, spinal cord and dorsal root ganglia. However, the mechanism of frataxin transfer and their functional implications are still matter of debate. Here, we examined the intercellular transmission of frataxin using human fibroblasts from healthy and FRDA patients. We have preliminary results suggesting that frataxin could be transfer via two distinct mechanisms. First, frataxin could be mobilized through long actin-based membranous extensions termed tunneling nanotubes (TNTs), which directly connects the cytoplasm of two cells. Second, frataxin can be shuttled via small extracellular nano-size vesicles with multivesicular bodies origin known as exosomes. Whether or not there is a preferential mechanism of transfer still remains to be determined. Furthermore, we are currently examining whether frataxin is transfer alone or associated to mitochondria. Overall, the results of this study have the potential to mechanistically explain our previous findings using cell therapy in a mouse model of FRDA.

553. AAV9 Bio-Distribution with Different Routes of CNS Administration in Rodents

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Introduction: Improved strategies for adeno-associated virus (AAV)-mediated gene transfer have enabled genetic correction of inherited neuromuscular disease (NMD). Recent studies in NHPs and piglets have demonstrated that high doses of AAV injected systemically can lead to toxicities with acute systemic inflammation, coagulation defects, hepatic toxicity and sensory neuron degeneration. Similar to several other neuromuscular diseases, Friedreich's Ataxia (FA) manifest with both systemic and neurological symptoms. In FA, high AAV doses would be required to penetrate the CNS and treat the neurological manifestations by systemic delivery. This approach would likely increase toxicity and immune reactions to the vector

capsid. Therefore, successful clinical translation of these therapeutic approaches depends upon the safety and efficacy of more targeted CNS routes of vector administration, at significantly lower doses. **Objective:** The purpose of this study was to determine the optimal CNS route of AAV9 administration at lower doses to achieve widespread bio-distribution and transgene expression needed in FA. **Methods:** Adult wild type C57BL6 mice (n=10/group) & Sprague Dawley rats (n=3/group) were administered AAV9 vector expressing human *FXN* gene (rAAV9-CBA-hFXN or GFP). Assuming the average mouse brain weighs 0.004g, we delivered 6E10 vg/mice (1.5E14 vg/kg brain weight) via either stereotaxic deep cerebellar nucleus (DCN), or intra cerebra ventricular (ICV), or cisterna magna (ICM), or free hand lumbar intrathecal (IT) injection. Animals were sacrificed at 1-month post injection for molecular assays (n=5/group, vector genome copy and FXN mRNA expression). The remaining animals were trans-cardially perfused, with 4% paraformaldehyde, for immuno-fluorescence (GFAP, IBA), and histochemistry (H & E). Due to high mortality in the ICM treated group (n=4/10) due to vector related toxicity, repeat ICM injections (n=3) were performed with a vector dose of 5E13 vg/kg brain weight. Statistical analysis was performed using one-way ANOVA with multiple comparisons. **Results:** Preliminary analysis shows no difference (p=0.6) in cerebellar bio-distribution for direct DCN injections and ICM injections, even though ICM injections were performed at 1 log lower doses. Cerebellar distribution was significantly superior with ICM as compared to both ICV ($p<0.01$) and IT ($p<0.001$) injections. Similarly, cervical, thoracic and lumbar spinal cord bio-distribution was superior for ICM injections as compared to DCN ($p<0.0001$), ICV ($p<0.0001$), and IT ($p<0.05$) injections. All approaches were significantly better than DCN injections for dorsal root ganglion bio-distribution. However, no group difference in dorsal root ganglion bio-distribution was observed with ICV, ICM or IT injections. Significantly, high amount of vector was detected in systemic circulation with IT injection as measured in heart ($p<0.001$) and liver ($p<0.01$). Small amount of vector was also observed in the heart and liver of ICM and ICV treated mice. Parallel studies in rats using rAAV9-GFP as a fluorescent tracker was technically challenging but feasible for ICV and ICM injections. The GFP expression in ICV and ICM injected rats were consistent with the vector bio-distribution profile observed in mice. Further analysis for this study is still ongoing. **Conclusion:** Preliminary results shows vector CNS infusion via ICM injections, at carefully titrated lower doses, may be the most suitable approach to target all the affected regions of CNS in FA.

554. Intrathecal Delivery of AAV9-mfge8 Cause Increased Synapse Loss in ALD Mice

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Mutations in ABCD1 cause the neurodegenerative disease adrenoleukodystrophy, which manifests as the spinal cord axonopathy in nearly all males surviving into adulthood. Microglial dysfunction has long been implicated in pathogenesis of brain disease. Our previous study demonstrated upregulation of several phagocytosis-related markers such as MFGE8 and TREM2 in the spinal cord of humans and mice with AMN, preceding complement activation and synapse loss. Subsequent study in ALD (Abcd1-/-) mouse plasma and human serum shows increased MFGE8 expression, indicating certain role of MFGE8 in AMN. To determine the detailed function of MFGE8 in AMN disease progression, an AAV9 vector carrying mfge8 gene was generated and then delivered into ALD mouse spinal cord by intrathecal injection. To test the function of AAV9-mfge8, we first performed mixed glial culture from Abcd1-/- mice and then transduced the cells with different doses of AAV9-mfge8 ($4.3X10^4$gc/cell and $4.3X10^5$gc/cell). MFGE8 protein (around 60KD) was detected by western blot after $4.3X10^5$gc/cell transduction. More sensitive ELISA (enzyme-linked immunosorbent assay) detected a dose-dependent increase of MFGE8 in cell culture medium. Treatment with AAV9-MFGE8 didn't cause observable changes on cell morphology. After verifying the function of AAV9-mfge8 in vitro, we then performed intrathecal delivery of AAV9-mfge8 into Abcd1-/- mice. 3 weeks after delivery, over-expression of MFGE8 can be right detected in spinal cord, DRG and spleen tissue but not in AAV9-empty vector injected group, indicating the success of MFGE8 overexpression by AAV9 vector in vivo. To investigate the impact of MFGE8 upon AMN disease progression, we further performed intrathecal injection in large cohort of mice. Mouse body weight and behavior were monitored until 11month of age. Afterwards, mice were euthanized and tissues were harvested for further analysis. AAV9- mfge8 delivery didn't cause significant changes on body weight. Mechanical sensitivity which was increased in Abcd1-/- mice didn't show significant change after AAV9- mfge8 delivery. Detailed analysis in spinal cord tissue showed further reduction (although not significant) of synaptic proteins including synaptophysin and PSD95 after AAV9-mfge8 delivery compared to AAV9-empty vector. While no increase in microglia activation was detected, there was certain increase of GFAP expression after AAV9-mfge8 delivery. In conclusion, increased MFGE8 expression may enhance the spinal cord neurodegeneration in AMN. Hence, MFGE8 blocking may provide potential therapeutic effect for AMN.

555. Selective Repression of C9ORF72 Repeat Expansion-Containing Transcripts for the Treatment of ALS

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Amyotrophic lateral sclerosis (ALS) is characterized by the loss of motor neurons in the CNS, with progressive paralysis and early death. The most frequent genetic cause of ALS is the expansion of hexanucleotide (GGGGCC) repeats in the non-coding region of C9ORF72 gene. Analysis of disease-affected patient tissue shows accumulation of repeat expansion-containing transcripts as well as repeat-derived dipeptide translation products suggesting a gain-of-function pathologic mechanism. To decrease the levels of repeat expansion-containing transcripts in cells, we designed a library of engineered transcription factors comprised of a zinc finger protein (ZFP) targeting the repeat region fused to a DNA-binding repressor

protein. We assessed the activity of the ZFP transcription factors (ZFP-TFs) by transfecting patient-derived fibroblasts (850 repeats on the expanded allele and 5 repeats on the normal allele) and quantifying transcripts using various techniques. We identified ZFP-TFs that can selectively repress the expression of pathogenic transcripts from the expanded allele over a wide dose range, while preserving expression of transcripts lacking repeat expansions. These findings illustrate the potential use of ZFP-TFs for the treatment of ALS.

556. Dissection the Role of Extra-Cerebellar Regions in Spinocerebellar Ataxia Type 1 Pathogenesis by RNA-Seq

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The most commonly occurring Spinocerebellar ataxia's (SCA1, SCA2, SCA3, SCA6, SCA7 and SCA17) are caused by the unstable expansion of the CAG trinucleotide repeat in the coding region of respective proteins. Gain of toxic function of these proteins with an expanded polyglutamine tract is believed to be the primary driver in these diseases. In preclinical models, strategies such as RNAi and antisense oligonucleotides (ASO) that reduce the overall expression of the mutant protein have shown therapeutic promise. The unanswered question is which regions of the brain need to be targeted to sufficiently alleviate the disease symptoms since clinical manifestations in most SCA's extend beyond the primary site of damage in each disease. Keeping this in mind we sought to study the involvement of the striatum and cortex, brain regions that are known to be less affected in SCA1 pathogenesis. SCA1 is caused by polyglutamine expansion in Ataxin1 and mainly affects the cerebellum and brainstem. Ataxin1 is primarily involved in the gene regulatory pathways and interacts with many transcriptional factors, mainly a transcriptional repressor called Capicua (CIC). Gain of function of mutant Ataxin1-CIC complex leads to transcriptional de-regulation and lies at the crux of cerebellar pathology in SCA1 but its role in other brain regions remains to be evaluated. Further, our analysis of the cortex and striatum of SCA1 Knock-in-mice revealed inclusion positive for p62, a multifactor protein involved in pathways such as autophagy, inflammation and triggering protein polyubiquitination to name a few. Thus, to understand the degree of involvement of these brain regions in SCA1 pathogenesis and the possible involvement of P62 inclusions, we performed transcriptomic analysis of these brain regions in SCA1 knock-in mice. This study will assist better design of RNAi or ASO therapeutic approaches for this complex fatal disease with no present cure.

557. An Outer Retina Change by Optical Coherence Tomography (OCT) Imaging is a Common Hallmark of Early Retinal Degeneration and Can be Used to Assess Gene Therapy Outcomes

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Gene therapy holds promise for treatment of retinal degenerative diseases. To assess treatment outcomes in animal models, a long waiting period following vector administration is often needed especially when using animals with slow retinal degeneration. To shorten this period, it is critical to identify early disease hallmarks using non-invasive methods. Spectral-domain optical coherence tomography (SD-OCT) is a widely used method for monitoring retinal structure changes *in vivo*. Using SD-OCT imaging, we collected retinal optical sections from four mouse lines with mutations in different disease-causing genes. These mouse lines included *Rpgr-/-*, *Ttll5-/-*, *Reep6-/-* and a human mutant rhodopsin transgenic line *RHO P347S*, mimicking retinitis pigmentosa with different inheritance patterns and disease mechanisms. Imaging was conducted on each mouse line prior to obvious photoreceptor cell death, and C57/BL6 wild-type (WT) mice were used as controls. While images from WT mice revealed four well-separated hyperreflective bands in the outer retina (designated as outer retina reflective bands, ORRB), which is consistent with a number of previous studies, the second (ORRB2) and the third (ORRB3) bands were merged in retinas of all four mutant mouse lines. A detailed OCT monitoring indicated that in *Rpgr-/-* mice, this ORRB change occurred between postnatal days 25 (P25) and 30 (P30). Once ORRB2 and ORRB3 had merged, they did not seem to separate again spontaneously. However, at this stage, no obvious changes were identified on either hematoxylin and eosin stained sections or electron microscopic ultra-sections. Merging of ORRB2 and ORRB3 was not observed in WT retina at any age, suggesting the pathological nature of the phenomenon. Additionally, it appears to be rod photoreceptor-related, as it was not observed in *Rp2-/-* retina in which the mutation mainly affects cone photoreceptors. To evaluate whether gene replacement therapy could restore this optical change, we subretinally injected adeno-associated viral (AAV) vectors encoding human REEP6 and RPGR into 2-month-old *Reep6-/-* and 1 month-old *Rpgr-/-* mice, respectively. At six weeks post-injection for *Reep6-/-* and four months post-injection for *Rpgr-/-* mice, the ORRB2 and ORRB3 bands were partially separated in the vector-treated eyes but not in the buffer-treated control eyes. The restoration was even more apparent in the *Reep6-/-* mice at three months post-injection. The ORRB2 and ORRB3 were almost completely separated, similar to those observed in WT retina. Our data suggest that the ORRB change could be a common hallmark of early retinal degeneration, and its restoration could be used as a rapid assessment of therapeutic effects following gene therapy or other treatment interventions.

558. Development of Novel Promoters for Neurological Gene Therapy

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The developments in gene therapy provide great therapeutic potential for previously untreatable neurological diseases. However, neurological diseases are diverse with different brain regions or central nervous system (CNS) cell types affected. It is clear that the use of ubiquitous promoters may not be suitable for all neurological gene therapies and delivery of therapeutic genes needs to be specific to the neurological disease. We describe the design of novel CNS selective promoters using a bioinformatics-based promoter construction platform. Data derived from large-scale functional genomics datasets and machine learning algorithms were applied to identify functional gene regulatory elements that we used as constituent parts to construct novel CNS promoters. Here we present results obtained from these novel synthetic CNS promoters compared to human Synapsin 1 promoter and evaluated *in vivo*. All promoters were cloned into AAV expression cassettes containing a GFP reporter gene and packaged into AAV9 vectors. Neonatal CD1 mice received titre matched viral vector by intracerebroventricular or intravenous delivery and euthanised at P35 for GFP expression by free floating immunohistochemistry for whole brain biodistribution and immunofluorescence. Systemic organs were collected for off-target expression analysis. We demonstrate that our genomics-based platform can successfully be used to produce novel CNS promoters by rational design with validated selective expression in the CNS *in vivo.* This platform will support the development of the next generation CNS gene therapies.

559. CRISPR-Based Transcriptional Control of Dopaminergic Gene Programs

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Drugs of abuse increase dopamine concentrations in the nucleus accumbens, a key reward structure that integrates contextual and cue-related information and regulates motivated behavior. This surge of dopamine triggers cell signaling cascades that converge in the nucleus to cause changes in gene expression, which are thought to lead to the observed functional and structural alterations in the reward circuit after exposure to drugs of abuse. Here, we used both *in vitro* striatal neuron culture models and *in vivo* cocaine administration to map acute gene expression changes following dopamine receptor activation in the rat genome. RNA-seq analysis confirmed similar increases in immediate early genes in both *in vitro* and *in vivo* datasets, with an enrichment of genes that contain binding motifs for the transcription factor CREB. To target dopamine-regulated genes, we optimized a dual lentivirus CRISPR system for targeted neuronal gene modulation via fusion of a transcriptional activator (VPR) (CRISPRa) to a catalytically dead Cas9 (dCas9). This system effectively drives expression at many gene targets, provides titratable gene expression, and enables robust gene targeting *in vivo*. Using multiplexed CRISPR sgRNA delivery to recreate a dopamine-induced gene expression program in cultured striatal neurons, we integrated this approach with high-throughput electrophysiological assays to identify how dopamine-regulated gene programs influence neuronal physiology and function. Ongoing work is centered around understanding how this gene expression program impacts neuronal function and associative reward learning. Our results indicate that this neuron-optimized CRISPR-based transcriptional modulation system will enable specific and large-scale control of gene expression profiles within the CNS to elucidate the role of gene expression in neuronal function, behavior, and neuropsychiatric disorders.

560. Further Refinement of the Gene Therapy Treatment and Vector for AB-Variant GM2 Gangliosidoses in a Mouse Model Using Self-Complimentary Adeno-Associated Virus Serotype 9

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GM2 Gangliosidoses is a group of autosomal recessive neurodegenerative, lysosomal storage disorders. It results in rapid neurological decline and death by age 4, in its human infantile form; there is currently no cure. These disorders are characterized by the excessive accumulation of GM2 ganglioside within the cell's lysosome, eventually leading to cell death. In a properly functioning cell, the metabolism of GM2 ganglioside into GM3 ganglioside is mediated by the β-Hexosaminidase A (HexA) enzyme, and its essential cofactor, the GM2 activator (GM2A) protein. GM2 Gangliosidoses manifests as three forms: Tay-Sachs, Sandhoff and AB-Variant. AB-Variant, the rarest of the three forms, is characterized by a mutation in the *Gm2a* gene, resulting in a deficiency of GM2A protein. Being a monogenic disease, AB-Variant is an exceptional candidate for gene therapy treatment, which is a promising method for producing a one-time treatment strategy. Previously a single stranded Adeno-associated virus serotype 9 expressing *GM2A* showed efficacy in decreasing GM2 accumulation in short-term and long-term using intravenous route of delivery in our lab. This study uses a self-complementary (sc)AAV9-*GM2A* vector delivered via intrathecal route into a *Gm2a*$^{-/-}$ mouse model (which lives normal life) and assess the short-term therapeutic efficacy. A dose of $1.0x10^{11}$vg/kg or $0.5x10^{11}$vg/kg was administered via a lumbar puncture, at six weeks of age. Starting at eight weeks of age, mice underwent monthly behavioural testing to assess coordination, activity and strength. All mice were euthanized at twenty weeks of age, where blood and various organs were collected for further biochemical and molecular analysis. We hypothesized that (1) scAAV9 will have a

better efficacy than the previously trialed single stranded vector; and (2) the higher dose will prove to be the most efficacious in reducing GM2 accumulation and having a wide vector biodistribution. The behavioural data does not show any statistical differences between any of the cohorts (treated vs. untreated and between doses), as brain damage is expectedly less due to modest build-up of GM2 noted at twenty weeks of age, thus, likely not having a large effect on behavioural phenotype. Ganglioside extraction assays showed a significant decrease in accumulation within the midbrain of mice that received the medium dose treatment. Consistent with this data was the vector biodistribution, which displayed the highest quantity of vector genomes per mouse genome within mice that received the higher dose, in both the liver and midbrain. It is promising to see a wide vector distribution and a reduction in GM2 accumulation which lays a solid foundation towards clinical gene therapy of AB-Variant GM2 Gangliosidosis.

561. CRISPR Cas9/gRNAs Selective Targeting of the GUCY2D Mutant Allele for Autosomal Dominant Cone-Rod Retinal Dystrophy

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Autosomal dominant cone-rod dystrophy type 6 (CORD6) is an early-onset retinal degeneration disease caused by mutations in *GUCY2D*, the gene encoding retinal guanylate cyclase-1 (retGC-1). It is characterized by infantile-onset of poor vision, abnormal color vision, photophobia, loss of visual field, and macular atrophy. There are currently no therapies available to treat CORD6. One of the common mutations found in CORD6 patients is c.2513G>A in codon 838 (R838H). Selective disruption of such a pathogenic GUCY2D mutant could potentially be an effective treatment of CORD6. To test this hypothesis, we investigated several different CRISPR/Cas9 systems to identify guides that selectively targeted the R838H mutation. Using "in vitro" guanylate cyclase functional assays, knock-in reporter cell lines, and therapeutically relevant human patient fibroblasts with the R838H mutation, we have identified gRNAs able to efficiently disrupt the GUCY2D R838H mutant allele without targeting the wild type allele. In order to reduce long-term in vivo expression of Cas9, we are currently evaluating self-inactivating AAV vectors carrying Cas9 and gRNA in rodent retina. Screening is ongoing for gRNAs able to efficiently target other CORD6-associated GUCY2D mutations.

562. AAV-Mediated Ribozyme Gene Therapy for Ocular Herpes

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Herpes simplex virus 1 (HSV-1) causes herpes stromal keratitis (HSK), the leading cause of infectious blindness in the United States with an estimated 35,000 people affected each year. Therapies are limited to topical antivirals and steroids, and ultimately corneal transplants, but these are incompletely effective because they do not treat the latent virus genome reservoir. Since heterochromatin formation on latent genomes makes them less than ideal targets for endonucleases, our approach targets viral noncoding-RNAs expressed during latency that have been shown to facilitate the ability of the virus to reactivate. In a recent study, we utilized hammerhead ribozymes delivered by AAV8-Y733F vectors to knockdown the HSV-1 latency-associated transcript (LAT) and observed a 50% reduction in reactivation *in vivo*. The design and *in vitro* testing of a new generation of ribozymes targeting the LATs and related viral noncoding-RNAs that facilitate reactivation will be presented. These ribozymes are being packaged into AAV capsids singularly and multiplexed in order to test their ability to reduce or prevent reactivation *in vivo*. The goals of this approach include: 1) characterizing the specific roles of overlapping and antisense viral transcripts in reactivation, 2) determining the combinatorial effects of knocking these transcripts down, and 3) evaluating therapeutic efficiency in reducing or preventing reactivation as a potential new treatment for HSK.

563. Efficient Targeted Epigenetic Gene Regulation in Mouse Brain by Purified Protein-, Viral-, and Cell-Mediated Delivery Methods

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The advent of targetable gene and epigenetic editing has opened up new possibilities for treatment of genetic disorders. Such new methods are surely needed since it estimated that only 14% of genes in human are "druggable". In disorders due to imprinting, haploinsufficiency, dominant negative alleles, and enhancer mutations, epigenetic editing of endogenous genes presents a therapeutic path that offers several advantages over gene therapy and gene editing. However, this is only true if the epigenetic effects can persist to produce a long-term effect on gene expression, similar to gene therapy and editing. We present several paradigms in which epigenetic editing proteins can be efficiently delivered throughout the brain in a mouse model of the neurologic

disease. We demonstrate how long-term persistence of effect allows short-term treatment, thus avoiding long-term expression of our gene regulator and consequent concerns of immune responses. We demonstrate that such "hit-and-run" epigenetic editing in the brain is safer than nuclease-mediated gene editing in terms of p53 responses and potential vector integration. We directly compare protein-based and cell-based "hit-and-run" epigenetic editing with virus-based delivery. While protein-based approaches accommodate minimally invasive routes of administration, treatments restricted to the central nervous system reduce total therapeutic protein load and avoid peripheral immune responses to the therapy. Importantly, our data demonstrate that such treatments can rescue several disease phenotypes in a mouse mode of Angelman Syndrome. These data support the further development of epigenetic editing *in vivo* for the study and treatment of other common and rare neurologic disorders.

564. A Novel In Vitro Modeling System for the Study of MeCP2 Pathology and Treatment Response

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Confidential: Rett syndrome is an X linked neurodevelopmental disorder affecting approximately 1 in 10,000 girls. Patients exhibit a loss of previously achieved developmental milestones and progressive loss of motor function with most adult patients requiring 24/7 supportive care. Pathology at the cellular level is characterized by global compaction of neural somas with shortened and fewer dendrites. The majority of cases of Rett syndrome result from loss of function mutations of the transcription factor Methyl-CpG binding protein 2 (MeCP2). We previously developed an Adeno-associated virus serotype 9 (AAV9) vector expressing human MeCP2 cDNA under the control of a synthetic promoter. We have performed extensive dosing safety and efficacy studies with this vector, demonstrating efficacy in a MeCP2 knockout mouse model of the disease and safety in their wild type cohorts. However, Rett patients have a variety of MeCP2 mutations, many of which produce residual mutated protein. Little is known about the impact of remaining misfolded or truncated MeCP2 protein isoforms on pathology or treatment response in Rett patients. In order to further investigate the role of various MeCP2 mutations on Rett syndrome pathology, we have developed a novel *in vitro* modeling system utilizing induced neural progenitor cells (iNPCs) directly converted from Rett patient fibroblasts. These iNPCs can be differentiated into a variety of central nervous system cell types and examined for indications of pathology including changes in downstream markers, gene expression profiles, and morphological features. Furthermore, we have also developed an astrocyte/neuron co-culture assay in which Rett patient astrocytes differentiated from iNPCs can be co-cultured with green fluorescent protein positive mouse neurons and monitored over several days for indications of pathology stemming from contact with affected astrocytes. We find that Rett patient cell lines show a range of mutation specific pathological phenotypes including altered expression of downstream markers and morphological changes when compared to those of unaffected controls. We have also successfully transduced our cells with AAV9 vectors *in vitro* with a robust expression pattern. We are utilizing this modeling system to further the study of mutation specific variation in pathology and help us to determine optimal patient responsiveness to future drug and gene therapy treatments.

565. Retinal Pigment Epithelum 65 RPE65

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Aim: To rectify the Retinitis Pigmentosa (Loss of sight) by using DNA activator. **Principle:** Retinal pigment epithelium- specific 65 kDa protein, also known as retinoid isomerohydrolase is an enzyme of the veterbate visual cycle that is encoded in humans by the RPE65 gene. It is expressed in the retinal pigment epithelium and is responsible for the conversion of all trans-retinyl-ester to 11 cis-retinol during phototransduction. Visual phototransduction: It is the sensory transduction of the visual system. It is process by which light is converted into electrical signals in the rod cells, cone cells and photosensitive ganglion cells of the retina of the eye. The visual cycle is the biological conversion of a photon into an electrical signal in the retina. This process occurs via G-protein coupied receptors called opsin which contain the chromophore 11-cis-retinal is covalently linked to the opsn receptor via Schiff base forming retinylidene protein. The isomerase activity of RPE65 has been shown, it is still uncertain whether it also acts as hydrolase. Finally, it is oxidized to 11-cis retinal before travelling back to the rod outer segment where it is again conjugated to an opsin to form new, functional visual pigment (rhodopsin). The protein encoded by this gene is a component of the Vitamin A visual cycle of the retina which supplies the 11-cis-retinal chromophore of the photoreceptors opsins visual pigements. The protein encoded by this gene has acquired a divergent function that involves the concentrated O-aklyl ester cleavage of its all-trans retinyl ester substrate and all trans to 11-cis double bond isomerization of the retinyl moiety. It is essential enzymatic isomerization step in the synthesis of 11-cis-retinal. Mutations in this gene are associated with early onset severe blinding disorders such as Leber Congential Mutations found in patients with Retinitis Pigmentosa were Arg91Trp (CGG to TGG), Ala132Thr (GCC to ACC), Leu341Ser (TTA to TCA), Glu404 (4bps ins) (CAG to GCTGGAG), and Val452Gly (GTC to CGC) **Methods:** Patients received a subretinal injection of DNA activator-RPE65 (by taking patients one drop of their own blood) in the poorer-seeing eye, at either of 2 close levels/day, and were followed up for 2 years after treatment **Main Outcome Measures:** The primary safety measures were ocular and non ocular adverse events Exploratory efficacy measures included changes in best-corrected visual acuity (BCVA), static perimetry and total visual field hill of vision (VTOT), kinetic perimetry visual field area, and responses to a quality-of -life questionnaire. **Statistical Analysis:** All patients tolerated subretinal injections and there were no treatment-related serious adverse events. Common adverse events were those associated with the surgical procedure with control group includes subconjunctival hemorrhage in 8 patients and ocular hyperemia in 5 patients. In the treated eye. BCVA increased in 5 patients, V30 increased in 6 patients. One subjected showed a decrease in BCVA and 2 patients showed a decrease in kinetic visual field area. **Conclusions:** Treatment with DNA activator RPE65 was

not associated with serious adverse events and improvement in 1 or more measures of visual function was observed in 9 of 12 patients. The greatest improvements in visual acuity were observed in younger patients with better baseline visual acuity. Evaluation of more patients and a longer duration of follow-up will be needed to determine the rate of uncommon or rare side effects or safety concerns

566. Economic Burden of Infant-Onset (Type 1) Spinal Muscular Atrophy (SMA1): A Retrospective Claims Database Analysis

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Background: SMA1 is a rapidly progressing, debilitating neurodegenerative disease resulting from bi-allelic survival motor neuron 1 (*SMN1*) gene deletion/mutation and subsequent motor neuron loss, muscle weakness, respiratory failure, and early death. The data on the cost burden of SMA1 for United States health plans are limited. **Objectives:** This retrospective analysis estimated the economic burden of SMA1 using QuintilesIMS's PharMetrics Plus Health Plan Claims Database. **Approach:** Infants with ICD-9 codes for SMA ≤1 year old were classified as SMA1 (N=119) and matched (1:1) with a random sample of infants by age, gender, index year, and Charlson Comorbidity Index. Healthcare resource utilization (HCRU) and costs (pharmacy, outpatient, and inpatient/hospitalization) incurred between February 2011 and November 2016 during the post-index/follow-up period (≥30 days up to 360 days) were compared. **Results:** Significantly more SMA1 patients had ≥1 all-cause HCRU claim vs. matched patients (98.3% vs. 54.6%, $P<0.0001$). Mean per-patient-per-month (PPPM) all-cause HCRU was higher for SMA1 infants: pharmacy (1.43 vs. 0.37 prescriptions); outpatient (14.10 vs. 2.17 services); and inpatient (0.23 vs. 0.003 admissions) (all, $P<0.0001$). Mean PPPM hospital admissions (0.23 vs. 0.003), length of hospital stay (6.93 vs. 0.09 days), procedures per admission (1.49 vs. 0.03), and readmissions (0.04 vs. 0.00) were also significantly greater for SMA1 infants (all, $P<0.0001$). Pharmacy, outpatient, and inpatient costs PPPM were greater in SMA1 infants ($371 vs. $20; $4,192 vs. $232; and $22,500 vs. $22, respectively [all, $P<0.0001$]), resulting in extrapolated all-cause total annual costs of $324,751 (SMA1 cohort) vs. $3,294 (matched cohort). **Conclusions:** The economic burden of SMA1 is substantial; treatments that alter the early natural course of the disease may improve long-term cost.

Cancer-Immunotherapy, Cancer Vaccines

567. Multi-Functional Engineered Natural Killer Cells Derived from Induced Pluripotent Stem Cells for Immunotherapy of Solid Tumors

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Despite effort and resources being devoted to understanding the molecular mechanisms of cancer biology and to developing treatments for cancer patients, cancer remains a major cause of mortality throughout the world. This is in large part because the pathogenesis of solid cancers causes severe immunosuppression of natural killer (NK) cells, through mechanisms that include the generation of extracellular adenosine by the cancer-associated enzyme CD73, as well as the expression of CD155. NK cells modified with gene fragments can be mobilized to combat the immunosuppressive mechanisms encountered in the tumor microenvironment. However, NK cells are often difficult to source and exhibit a low ex vivo expansion, which can slow the development of immunotherapies for solid tumors. Therefore, induced pluripotent stem cells (iPSCs), a source of allogenic NK cells, prove promising in overcoming these challenges. We have generated "off-the-shelf" NK cells, differentiated from iPSCs, using a novel, streamlined, and efficient feeder-free cell culture protocol. This protocol differs from other NK cell differentiation protocols, as it utilizes well defined medium, cytokines, and reagents, in a system free of feeder cells, thus providing a reproducible method of generating NK cells *in vitro*. Due to the high *in vitro* expansion rate of iPSCs, differentiated NK cells can act as a continuous source of allogeneic NK cells which can be genetically engineered, before or after differentiation, for use in cancer immunotherapy. Additionally, we are genetically engineering NK cells with a responsive genetic construct that is based on the synthetic Notch (synNotch) system and utilizes extracellular TIGIT and intracellular signaling elements to redirect the signal induced by the typically inhibitory CD155/TIGIT interaction to trigger release of anti-CD73 scFv. Engineered NK cells are generated via transfection using mRNA electroporation and are assessed for cytotoxic function against $CD155^+/CD73^+$ solid tumor targets. Following the formation of embryoid body structures and eleven days of hematopoietic progenitor cell differentiation, we differentiated iPSCs into hematopoietic progenitor cells. These cells were characterized via flow cytometry for markers expressed on hematopoietic progenitors - CD34, CD43, and CD45 - yielding results consistent with feeder-based differentiation protocols. Following four weeks of NK cell differentiation, utilizing a defined, commercially-available medium, we observed high expression of several NK cell maturation markers (CD56, CD94, NKp46) yielding mature, phenotypically-functional NK cells. In parallel, peripheral blood-derived primary NK cells transfected with our multi-functional CD155-CD73-targeting construct through mRNA electroporation, were characterized functionally against solid tumor targets, including patient-derived GMB43 cells. As a platform for allogeneic NK cell therapy, we have shown that iPSCs can be differentiated to yield functional NK cells, using an efficient feeder-free protocol. We also showed that it is possible to redirect TIGIT-induced inhibition of

NK cell function while triggering release of therapeutic anti-CD73 scFv via a multi-functional genetic construct. This genetic construct is also being characterized pre-clinically for the ability to inhibit immunometabolic suppression and enhance cytotoxicity of NK cells against solid tumor targets, improving cancer targeting over traditional chimeric antigen receptor-NK therapies.

568. Regulation of CD40L Transgene Expression in Human CAR-T Cells Using FDA Approved Ligands

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Chimeric antigen receptor modified T cells (CAR-T) have shown clinical efficacy in the treatment of B cell malignancies and multiple myeloma. Several challenges restrict their application across hematologic malignancies and solid tumors, including: limited CAR-T cell expansion and persistence, tumor microenvironment-induced immunosuppression, and antigen negative tumor escape. Cluster of Differentiation 40 Ligand (CD40L), a tumor necrosis factor superfamily member transiently expressed on activated CD4 T cells, promotes dendritic cell (DC) licensing and activation through interaction with the CD40 receptor. Co-expression of engineered CD40L in CAR-T cells has the potential to reduce antigen-negative tumor escape even when native CD40L is downregulated, thereby increasing antitumor efficacy. The cytokine program associated with CD40L-mediated DC activation could also improve T cell expansion and activity. However, activation of the CD40 pathway using agonistic antibodies causes systemic immune activation that has been associated with adverse clinical events, thus limiting therapeutic application. We therefore hypothesized that precise and titratable regulation of CD40L would allow for its safe inclusion in CAR-T cell therapy, thus empowering the next generation of potent cellular immunotherapies.To enable regulation of CD40L, we applied destabilizing domain (DD) technology which utilizes human protein domains that are inherently unstable in the cell but are reversibly stabilized when bound to FDA-approved small molecule ligands. Fusion of transgenes to a DD confers ligand-dependent, reversible regulation to any protein of interest. We therefore fused human CD40L to a DD derived from the *E. coli* dihydrofolate reductase (ecDHFR) which can be regulated by the clinically-approved antibiotic trimethoprim (TMP). We evaluated CD40L expression and in the absence of ligand, the CD40L-DD fusion was expressed at very low levels in transduced T cells. Exposure to TMP increased CD40L expression in T cells in a dose-dependent manner. To test the activity of CD40L-DD, we incubated transduced Jurkat T cells with a reporter cell line that reads out CD40 receptor activation. Addition of TMP increased CD40 activation to similar levels seen in cells constitutively expressing CD40L. To evaluate the effect of regulated CD40L expression on DC activation, monocyte derived human DCs were exposed to control or CD40L-DD expressing T cells. After TMP treatment, the levels of inflammatory cytokines IL12, TNFα, and IFNγ were elevated in co-cultures of DC and CD40L-DD T cells compared to co-cultures of DCs and control T cells.To determine the effect of CD40L on CAR-T activity in vivo, T cells expressing CD40L and CD19-targeting CAR were infused into $CD19^+$ Nalm6 tumor-bearing mice. Increased tumor regression was seen in mice that received T cells co-expressing CD40L with CD19-targeting CAR compared to CAR alone. Studies are underway to evaluate the effect of regulated CD40L expression on CAR-T cell anti-tumor efficacy in vivo. These findings indicate that CD40L can be regulated using DDs and FDA approved small molecule ligands, and that regulated CD40L transgene expression by human T cells promotes DC activation and increases CAR-T antitumor activity. Regulated CD40L can be applied to CAR-T therapy to enhance immunotherapy potency by increasing T cell expansion, promoting DC activation, and inducing further epitope spreading.

569. Highly Efficient Multiplex Human T Cell Engineering without Double-Strand Breaks Using Cas9 Base Editors

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Chimeric antigen receptor engineered T cell (CAR-T) immunotherapy has shown efficacy against a subset of hematological malignancies, yet its autologous nature and ineffectiveness against epithelial and solid cancers limit widespread application. To overcome these limitations, targeted nucleases have been used to disrupt checkpoint inhibitors and genes involved in alloreactivity. However, the production of allogeneic, "off-the-shelf" T cells with enhanced function requires multiplex genome editing strategies that risk off-target effects, chromosomal rearrangements, and genotoxicity due to simultaneous double-strand break (DSB) induction at multiple loci. Moreover, it has been well documented that DSBs are toxic lesions that can drive genetic instability. Alternatively, CRISPR/Cas9 base editors afford programmable enzymatic nucleotide conversion at targeted loci without induction of DSBs. We reasoned this technology could be used to knockout (KO) gene function in human T cells while minimizing safety concerns associated with current nuclease platforms. In preliminary work we demonstrated that first-generation BE3 and BE4 mRNA delivered with sgRNA targeting canonical splice sites was able to mediate efficient (>70%) editing and protein knockout at *TRAC*, *B2M*, and *PDCD1*. However, when editing all three loci in multiplex, editing efficiency decreased substantially, with only 21.9± 1.1% of endpoint cells being triple-null. Through systematic reagent and dose optimization involving the use of codon optimized BE4 (coBE4) mRNA, we achieved C to T editing and protein KO at rates exceeding 90% for *TRAC*, *B2M*, and *PDCD1*. This enhanced efficiency remained consistent in multiplex, where the frequency

of triple-null cells reached 89.6± 4.2% using the optimal dose of coBE4 mRNA. Compared to controls, multiplex BE4 edited T cells maintained a non-exhausted memory phenotype, expanded robustly, and retained cytokine polyfunctionality. When equipped with a CD19 CAR, multiplex BE4 engineered T cells exhibited enhanced ability to specifically kill CD19 target cells. This effect was further magnified when target cells were engineered to overexpress PD-L1. Importantly, DSB induction was dramatically reduced compared to SpCas9 nuclease, with coBE4 exhibiting the lowest rate of indel formation overall. At a single off-target site for the *PDCD1* sgRNA, SpCas9 nuclease generated indels at ~13%, while BE4 indels were below the level of detection for our NGS assay. Further, with spCas9 nuclease, we readily detected all 12 possible translocation outcomes in the multiplex setting, whereas no translocations were detected in BE4 engineered cells. Finally, we demonstrate simultaneous multiplex gene knockout and targeted integration of an rAAV donor by exploiting the nickase function of BE4 or incorporation of orthogonal Cas9 systems. This streamlined approach to genome engineering may be broadly applied for the development of safe and effective cell therapies.

570. Bispecific T Cell Engager (BiTE) Targeting IL13Rα2 Improves Survival in a Pre-Clinical Model of Glioblastoma. Bispecific T Cell Engager (BiTE) Targeting IL13Rα2 Improves Survival in a Pre-Clinical Model of Glioblastoma

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Background. Glioblastoma (GBM) is the most common and prognostically poor adult brain tumor. The outstanding efficacy of bi-specific T-cell engager (BiTE)-mediated T cells against hematological malignancies offers hope that they can similarly target solid tumors like GBM. We **hypothesized** that BiTE protein with specificity to the tumor associated antigen, IL13Rα2, will engage a host T cell immune response to promote anti-glioma activity in pre-clinical models of GBM. We have previously established several murine glioma models that express human IL13Rα2, enabling studies of our BiTE protein lacking cross-reactivity with murine antigen in fully immunocompetent host. **Methods.** BiTE molecule consisting of two single chain variable regions (scFv) of antibodies, scFv2C11 (against murine CD3ε) and scFv47 (against human IL13Rα2), connected through a 15aa Gly_4S flexible linker (named $BiTE^{IL13R\alpha2}$) were synthesized and sub-cloned in a lentiviral expression cassette. The BiTE molecule in which the CDR3 of the scFvIL13Rα2 light chain was replaced by the CDR3 domain of a non-specific antibody MOPC-21 (named $BiTE^{IL13R\alpha2off}$) resulted in a loss of interaction with scFvIL13Rα2. For production of soluble BiTE proteins, HEK293T cells were transduced with lentiviral particles. Proteins were isolated from the supernatants using His-Tag affinity chromatography and characterized for their size, purity, stability, and binding specificity against IL13Rα2 by SDS-PAGE, Western Blot, and ELISA. The ability of $BiTE^{IL13R\alpha2}$ to activate T cells were measured in (i) a ^{51}Cr release cytotoxicity assay against IL13Rα2-positive/negative GL261 and SMA560 murine glioma cell lines, (ii) by flow cytometry measuring for CD69 and CD25 T cell activation markers, and (iii) the production of cytokines, IFNγ and TNFα. For *in vivo* analysis, VmDk mice bearing intracranial SMA-560-IL13Rα2 glioma were treated systemically with recombinant proteins. The survival of the mice was recorded and analyzed according to long rank test. **Results.** The recombinant $BiTE^{IL13R\alpha2}$ protein was successfully generated and characterized. We determined that purified $BiTE^{IL13R\alpha2}$ protein specifically binded to IL13Rα2 but not to IL13Rα1, whereas $BiTE^{IL13R\alpha2off}$ had no binding activity to both proteins. BITE-LL protein shows good thermos stability for 48 hours of incubation at 37°C in media containing 10% serum. The co-culture of murine $CD3^+$ T cells with IL13Rα2-expressing GL261 or SMA560 glioma cells in the presence of $BiTE^{IL13R\alpha2}$ resulted in concentration dependent cytotoxicity in glioma cells. No cytotoxicity of $CD3^+$ T cells in the presence of $BiTE^{IL13R\alpha2off}$ or in co-culture with IL13Rα2-negative cells was observed. Flow cytometry analysis of $CD3^+CD8^+$ T cells for CD69, CD25 expression and production of IFNγ and TNFα, confirmed antigen-dependent activation of T cells by $BiTE^{IL13R\alpha2}$ protein. Finally, treatment of immunocompetent mice bearing SMA560-IL13Rα2 tumors with $BiTE^{IL13R\alpha2}$ resulted in about 50% improvement of survival over mice treated with a negative control protein, $BiTE^{IL13R\alpha2off}$, thus further supporting $BiTE^{IL13R\alpha2}$ therapeutic properties against GBMs *in vitro* and *in vivo*. **Conclusions**. Our data demonstrate that the activation of murine T cells by $BiTE^{IL13R\alpha2}$ is antigen-specific and that systemic treatment with $BiTE^{IL13R\alpha2}$ protein confers survival benefit in immunocompetent mice bearing intracranial glioma.

571. Chimeric Antigen Receptor T Cell Therapy for γδ T Cell Malignancies

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There are two main types of T Cell Receptor (TCR). 95% of T-cells express TCR consisting of αβ heterodimers. A much rarer T cell population express γδ TCR. T cell lymphomas may derive from either population. Typically, lymphomas which express γδ TCR, such as Hepatosplenic T cell Lymphoma (HSTL), are extremely aggressive and carry poor prognosis. They respond poorly to standard chemotherapy treatments, and alternative options are limited, with no available immunotherapies. We therefore decided to develop a Chimeric Antigen Receptor (CAR) T cell therapy for γδ TCR-expressing malignancies. We cloned a commercially available single chain variable fragment (scFv) specific for γδ TCR into a 2nd generation CAR format, including a spacer derived from CD8-stalk, CD28 transmembrane domain and 41BBζ endodomain, and expressed it αβ T cells by retroviral transduction. We demonstrated specific cytokine secretion and cytotoxicity in co-culture with γδ TCR-expressing cell lines (Loucy, BE13, Molt-13), compared to a control α-CD19 CAR. We developed a murine model of γδ TCR-expressing malignancy. NSG mice were intravenously injected with 4x10^6 Loucy cells, engineered to stably express Firefly luciferase. Tumour engraftment in bone marrow was confirmed at D12 following injection, and mice were treated with 0.8x10^6 α-γδ TCR or control α-CD19 CAR T-cells. Disease burden was monitored by bioluminescence imaging and flow cytometry of blood, bone marrow and spleen. Mice receiving α-γδ TCR CAR demonstrated substantial reduction of tumour burden and prolonged survival (HR =

14.7 for control, p = 0.0003) compared to control-CAR treated animals. In conclusion, we have developed a novel CAR T cell immunotherapy against γδ TCR expressing malignancies and demonstrated that our new therapy is highly functional in vitro and in a small animal model of γδ T-cell leukaemia. Given the very poor prognosis and lack of effective therapies for γδ TCR-positive malignancies, as well as the considerable efficacy of CAR T-cell therapy in analogous B-cell disorders, our approach could bring much needed benefit to patients suffering these conditions.

572. Short-Term Local Expression of a PD-L1 Blocking Antibody from a Self-Replicating RNA Vector Induces Potent Antitumor Responses

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Immune checkpoint blockade based on monoclonal antibodies (mAbs) has shown high efficacy against many cancer types. However, these therapies require repetitive systemic administration of high mAbs amounts, frequently leading to adverse effects. An alternative to avoid this toxicity could be expressing mAbs locally in tumors. To evaluate this possibility, we developed vectors derived from adeno-associated virus (AAV) and Semliki Forest virus (SFV) to express an anti-PD-L1 mAb and tested them in a murine colon adenocarcinoma model (MC38). Intratumoral administration of AAV-anti-PD-L1 and SFV-anti-PD-L1 led to similar mAb expression in tumors 24h after treatment, with very low serum levels. However, expression in SFV-anti-PD-L1-treated tumors diminished quickly, due to the transient nature of this vector. Despite short expression, SFV-anti-PD-L1 induced higher antitumor responses, resulting in >40% complete regressions, while AAV-antiPD-L1 only provided very modest effects. The antitumor efficacy of SFV-anti-PD-L1 was also observed in a melanoma model (B16-OVA). Interestingly, SFV-anti-PD-L1 antitumor effect was superior to that provided by anti-PD-L1 mAb given systemically, or locally in tumors. The higher antitumor activity of SFV-anti-PD-L1 could be related to the induction of type I interferon (IFN-I) responses, as a result of viral RNA replication. In fact, we detected strong upregulation of several IFN-stimulated genes in tumors treated with SFV vectors. Analysis of immune responses in treated mice revealed that SFV-anti-PD-L1 promoted tumor infiltration, increasing tumor-specific CD8 T cells with an effector phenotype, while AAV-anti-PD-L1 did not induce relevant immune changes. In addition, SFV-anti-PD-L1 upregulated co-stimulatory (CD137 and OX40) and co-inhibitory (LAG-3 and PD-1) markers in tumor CD8 T cells, suggesting that this therapy could benefit from combination with mAbs against those receptors. In fact, combination of SFV-anti-PD-L1 and anti-CD137 mAb showed higher antitumor effect than each single agent. These results indicate that local transient expression of immunomodulatory mAbs using vectors able to induce IFN-I responses could represent a potent and safe approach for cancer treatment.

573. A Novel MUC1-Specific CAR-T Therapy (P-MUC1C-101) for Treatment of Multiple Solid Tumors

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Background: MUC1 is a heterodimeric glycoprotein (MUC1 N'/C') that is expressed on the apical surface of normal epithelial cells in various tissues, forming a protective mucosal barrier on the luminal epithelial surface. However, during tumorigenesis, malignant cells lose polarity and over-express hypo-glycosylated MUC1. Moreover, the N-terminal (N') of MUC1 can be shed, leaving the MUC1-C-terminal (C') protein on the cell surface. MUC1-targeting vaccines and therapeutic antibody conjugates are in various stages of development, and chimeric antigen receptor (CAR) T cell therapies targeting N' MUC1 are currently being investigated. Here we generated a novel MUC1C-specific CAR T cell product that targets a MUC1-C terminus epitope with efficacy against a wide variety of blood and epithelial-derived primary tumor cells, while not targeting normal epithelial or fibroblast cells. **Methods:** We first tested the newly generated MUC1C-specific CAR in an RNA platform to test degranulation against multiple blood and solid tumor cells, as well as normal cells. We also examined reactivity against murine tumor cells to assess binder cross reactivity. We next tested P-MUC1C-101 created using the piggyBac® DNA Modification System, a non-viral CAR-T manufacturing method that produces CAR T products with a high percentage of a highly desirable stem cell memory (T_{SCM}) phenotype. We assessed the *in vitro* functionality of P-MUC1C-101 via tumor cell killing, cytokine production, and proliferation against various human tumor cells. For comparability, we tested a previously generated single chain variable fragment (scFv)-based CAR (Tn CAR) that is reported to target glycosylation neoantigens that are present on N' MUC1. Lastly, we determined P-MUC1C-101 anti-tumor efficacy, as well as *in vivo* safety in a MCF7 orthotopic breast cancer murine model. We are currently investigating efficacy at stress doses in established triple negative breast cancer and ovarian cancer models. **Results:** The MUC1C-specific CAR specifically degranulated against a wide array of MUC1N'/C'$^{+}$ tumor types, including breast (including triple-negative), pancreatic, colorectal, prostate, cervical, ovarian, non-small cell lung, chronic myelogenous leukemia (CML), and multiple myeloma tumors. The degree of CAR T activity correlated with MUC1 expression, even in the presence of shed MUC1 N' protein, suggesting that CAR potency is not abrogated by N' MUC1 shedding. MUC1C-specific CAR also degranulated against murine MUC1+ leukemic macrophages, establishing cross-reactivity of the product and the opportunity to confirm on-tumor specificity in preclinical mouse models. Using piggyBac®, >95% of P-MUC1C-101 cells were CAR+, with >60% of cells exhibiting a T_{SCM} (CD45RA+CD62L+) phenotype. P-MUC1C-101 mediated potent and specific *in vitro* killing, IFN-γ production, and expansion against lung and breast tumors expressing endogenous levels of MUC1-C. *In vivo*, P-MUC1C-101 significantly reduced established, orthotopic MCF7 tumors (assessed via caliper measurement) and increased survival (>75 days) in comparison to control CAR-treated (50 days) and Tn CAR-treated (70 days) immune deficient mice without any signs of adverse effects. **Conclusions:** Our novel MUC1-C specific

CAR demonstrated marked and specific potency against tumor cells representing nine different indications *in vitro*, including both solid and hematologic malignancies. P-MUC1C-101 displayed efficacy *in vivo,* without any apparent toxicity in an orthotopic breast tumor model. Future efforts will focus on moving P-MUC1C-101 into the clinic.

574. Multi-Antigen Specific Chimeric Antigen Receptor T Cell Therapy for Acute Myeloid Leukemia

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A major advance for adoptive T cell therapy is the chimeric antigen receptor (CAR), which is a single chain variable fragment (scFv) fused to the CD3ζ and/or co-stimulatory domains. However, relapse of leukemic cells that do not express the antigen targeted by CAR is still a risk. As is the potential for targeting hematopoietic stem cells (HSCs) that share the same antigen expression, off-tumor on-target toxicity. Multi-antigen specific CARs have been shown to be a possible solution to mitigate such risks. Therefore, we set out to develop a multi-antigen CAR T cell therapy that targets well described antigens for Acute myeloid leukemia (AML), including CD33 and CD123. To generate scFv sequences for these targets, mice were immunized with these antigens; spleens were collected and fused with myeloma cell lines. The antibodies of fused hybridomas were screened for binding and activation against antigens by high throughput flow cytometry. After screening, we derived multiple de novo CD33, and CD123 scFvs by sequencing the IgH and IgL rearrangements associated with the selected hybridomas. We incorporated CD33 and CD123 scFvs into standard mono-specific CARs utilizing a 41BB co-stimulatory domain to validate their antigen-specificity. These constructs were transduced into T cells using SFG gamma retrovirus following our established methods. Gene transfer assessment of CAR T cells demonstrated about 50-80% transduction efficiency for CD33 and CD123 scFvs. There were no differences in CD4 and CD8 proportions in these CAR T cells. We next examined the CARs for their cytotoxic ability using a Real-Time Cell Analysis (RTCA) system. For the CD33 CARs, 2 (6A11-1 and 27A3-1) out of 5, and for the CD123 CAR, 2 (15A12-11 and 15 A12-12) out of 8, scFv sequences transduced into T cells were highly efficacious at killing target cells and generated significant amounts of cytokines such as IFN-γ, TNF- α, and IL-6. CAR T cells with these same scFv sequences were able to proliferate better in response to targeted antigen. Furthermore, we discovered that the percentage of CAR positive CD8 T effector memory cells on day 14 after antigen stimulation was higher for the same two CD33 scFv sequences. In addition, these CD33 CAR T cells did not show high exhaustion phenotype (PD-1) compared to the other CAR T cells. In order to test the dual antigen target efficacy we double transduced the T cells with CD33 or CD123 scFvs to select for the best possible combination of bi specific CARs. Clear differences in cytotoxic ability and cytokine production were observed with dual transduction of some combination of CD33 and CD123 scFvs. We have subsequently generated CD33/123 CARs. We will utilize these sequences to generate bispecific CARs to evaluate their *in vitro* and *in vivo* safety and function.

575. Highly Multiplexed, Single-Cell Functional Profiling of CAR-T Cells Enables More Predictive Product Characterization, Cell Manufacturing Analysis, and Cellular Biomarkers across Product Types

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Collecting and using a patients' own immune cells is a rapidly emerging immunotherapy approach. Genetically reprograming T cells to express a chimeric antigen receptor (CAR) has already paved the way for successful immunotherapies to fight against leukemia and lymphoma, and research into solid-tumor CAR-T cells are also underway. Nevertheless, a lot is still unknown in terms of exactly how these re-engineered cells will behave once reinfused into a patient, including efficacy and potential side-effects. Moreover, there is a need to effectively characterize the antigen-specific response of these cell products for manufacturing analysis and optimization. In this poster, we review single-cell polyfunctional profiling results obtained from several different sets of pre-infusion CAR-T samples, including anti-CD19 CAR-T samples from Novartis Pharmaceuticals [1] and Kite Pharma (Gilead) [3], GoCAR-T cell products from Bellicum Pharmaceuticals [2], and Bispecific CD19/22 CAR-T cells from the NIH [4]. In each case, CD4+ and CD8+ CAR-T cells were stimulated (details in [1], [2], and [3]) and subsequently analyzed at a single-cell level using IsoPlexis' IsoCode chip. A 16-plex cytokine panel was used with samples in study [2], while samples in studies [1], [3], and [4] used a 32-plex cytokine panel. Single-cell profiling revealed highly polyfunctional and heterogeneous responses across each patient cohorts. In study [1], an association was determined between the polyfunctional strength index (PSI) of the CAR-T samples and the clinical outcome of the patients after receiving the treatment ($p = 0.0119$). Similarly, an association was seen between pre-infusion PSI and post-infusion grade 3+ CRS in the patients ($p = 0.174$). In all studies, the CAR-T cells secreted a wide range of effector, stimulatory, regulatory cytokines; the CD4+ samples of [1] and [3] also secreted inflammatory cytokines. The secretions were highly specific to antigen-stimulation, and a significant portion of the CAR-T cells were polyfunctional (secreted multiple cytokines). In study [4], a consistently higher polyfunctionality was observed in CAR-T samples produced using a modified manufacturing method. The results of these studies demonstrate the potential benefits of single-cell profiling as a way to better understand how these CAR-T products behave in response to antigen-specific stimulation. Analyzing the single-cell polyfunctionality of CAR-T profiles may potentially provide a valuable quality check of the pre-infusion product, may provide key characterization data for optimizing the manufacturing process of the product, and may also help in developing biomarkers to predict eventual patient outcome in response to the therapy. The promise of more predictive product characterization lays the groundwork for greater potential clarity in product release and also potentially more proactive administration in the future.

576. Computational Modeling of Interactions between Engineered and Wildtype T-Cells Quantifies Therapeutic Success of Anti-CD19 CAR T-Cell Therapy

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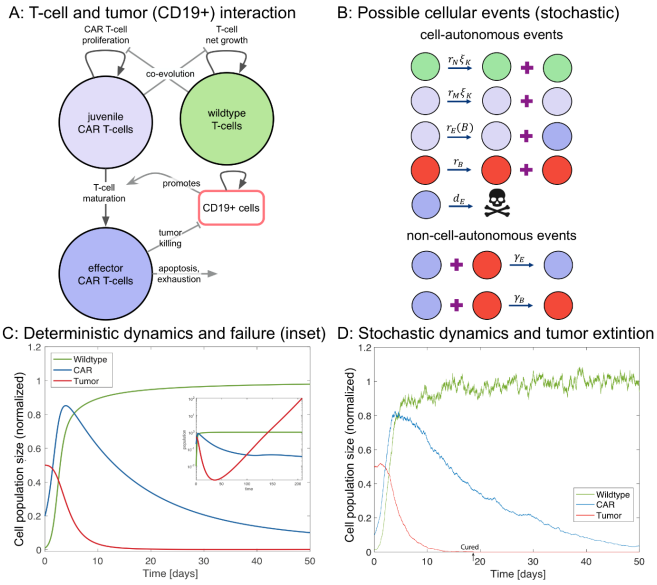
Recent clinical advances have led to multiple new cancer immunotherapies that involve CAR T-cells targeting specific antigens, for example second generation Chimeric Antigen Receptor (CAR) T-cell that target CD19+ lymphoma cells. Non-Hodgkin Lymphoma (NHL) is the most common hematologic malignancy in the United States with an estimated 72,000 new cases (4.3% of all cancer cases) and 20,000 deaths (3.4% of all cancer deaths) in 2017; the median 5-year survival rate is 71%. Despite a possible cure for aggressive lymphomas with front-line chemotherapy, there exist patients that do not response or relapse and develop refractory disease. These patients have a median overall survival of less than seven months. CAR T-cell therapy for refractory aggressive NHL relies on expansion of engineered T-cells in order to kill targeted lymphoma cells. We here develop a mathematical modeling approach to gain comprehensive quantitative understanding of the T- and B-cell population dynamics after patient conditioning and CAR T-cell infusion. We combine theoretical-ecology modeling with statistical data-analysis based on clinical data to recapitulate CAR T-cell and tumor cell density over time. The modeling approach is based on the assumptions that CD8+ CAR T-cells, although in advantage at administration due to lymphodepletion, compete with wildtype T-cells, and that the maturation of juvenile (CCR7+, naïve and central memory) T-cells into effector (CCR7-, effector memory and effector) T-cells is affected by the antigen CD19 that is present on tumor cells (Fig. 1A, B). With this approach we are able to predict possible kinetic scenarios deterministically (Fig. 1C) and stochastically (Fig. 1D), which leads to statistical statements of the most likely time and probability of cure (extinction of malignant CD19+ cells), and patient specific cure rates depending on the clinically achievable CAR to wildtype T-cell ratio or the initial refractory tumor growth rate. Our modeling approach can thus be used to recapitulate the response rates as observed in recent clinical trials (ZUMA-1), and to propose testable treatment alterations specifically tailored to the patient. For example, we predict that determination of the rate of change in tumor burden prior to treatment, in combination with adjusting the infused product composition of juvenile and effector CAR T-cells, could improve patient outcomes.

FIGURE 1



577. HVJ-E in Combination with CXCL-2 Suppressed Murine Melanoma through the Accumulation of Anti-Tumorigenic Neutrophils in Tumor Microenvironment

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Inactivated Sendai virus particles (hemagglutinating virus of Japan envelope; HVJ-E) activate antitumor immunity mainly through RIG-I/MAVS signaling pathway and Toll-like receptor (TLR) signals independent. We attempted to examine whether RIG-I agonist and TLR agonist synergistically activate anti-tumor activity. To investigate this aim, we injected HVJ-E with or without poly I:C (TLR3 agonist) into B16-F10 melanoma tumor models. Tumor growth was significantly suppressed by combination treatment of HVJ-E and poly I:C. Poly I:C treatment recruited more neutrophils than HVJ-E treatment. To measure the reason of neutrophil infiltration, we used cytokine array to check the treatment of each groups and the data showed neutrophil chemotactic CXCL2 produced from tumor bed by poly I:C, but not HVJ-E. Tumor associated neutrophils (TANs) are traditionally considered to favor tumor development, while N1 phenotype of TANs can suppress tumor growth. For check the neutrophil phenotype in tumor, we used FACS to analysis the neutrophil activation marker Fas and ICAM-1 of TAN in each treatment groups. In the results, poly I:C not increased N1 phenotype ratio, but recruited neutrophils to tumor bed. HVJ-E treatment showed no increase of neutrophil number in tumor site but induces neutrophil to N1 phenotype. So when co- treatment of poly I:C and HVJ-E increased N1 phenotype of TANs. Both neutrophil depletion and neutrophil CXCL2 neutralization decrease synergistic anti-tumor effect of HVJ-E and poly I:C combination treatment. Our results indicated that poly I:C and

HVJ-E can activate anti-tumor effect via the conversion of recruited neutrophils to N1 phenotype. Because of high toxicity of poly I:C treatment, we considered to combine HVJ-E with CXCL2 rather than poly I:C. And when combination of HVJ-E with recombinant CXCL2 protein or CXCL2 pDNA, both of them suppressed mouse melanoma. In our research, we showed the polarized neutrophil to N1 type is a new function of HVJ-E in antitumor effect and also showed the potential of HVJ-E vector containing CXCL2 gene may be a novel cancer gene therapy strategy.

578. Cargocytes: A Novel Cell Therapy Platform to Drive Anti-Tumor Immunity

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Adoptive cell therapy has emerged as a powerful new tool to treat cancer and other diseases. However, the clinical utility of many cell-based therapies is limited by inadequate production of therapeutic factors, production of unwanted factors, unwanted engraftment into the body, and poor controllability resulting in poor pharmacokinetics. Genetic and bioengineering strategies can overcome many limitations, but modification of genomic DNA or the introduction of new genetic material into therapeutic cells raises substantial safety concerns, ultimately making FDA approval difficult. To address these limitations, we developed a novel platform for cell-based therapies in which any cell type, including immortalized cells, can be extensively engineered and loaded with therapeutic cargo, and then rendered safe for patient administration through removal of the nucleus. Enucleated cells (Cargocytes) retain many desired biologic functions (endogenous and engineered), such as viability up to 72 hours, retention of cell surface markers/proteins, secretion of bioactive molecules, robust *in vitro* and *in vivo* chemotaxis, and delivery of a wide range of cancer-fighting cargos. Using this technology, we demonstrate that Cargocytes derived from hTERT-immortalized mesenchymal stem cells (hTERT MSCs) can be engineered to express therapeutic levels of IL-12 that potently activate an anti-tumor immune response in a preclinical mouse model of triple negative breast cancer. hTERT MSC-derived Cargocytes robustly delivered IL-12 to the tumor with definable pharmacokinetics, activated known IL-12 biomarkers, and induced infiltration of tumor-fighting immune cells that inhibited tumor growth and improved animal survival. In addition, co-administration of Cargocytes secreting IL-12 and injection of anti-PD-L1 antibodies further improved animal survival without notable adverse events. Collectively, our findings indicate that Cargocytes provide a new means to deliver powerful immunomodulatory biologics to malignant tumors in a safe and controllable manner. The genetic tractability and excellent safety profile of the Cargoycte platform enables bioengineering of a wide range of designer therapeutics derived from genetically modified cells, immortalized stem cells, and even cancer cells, each with improved clinically relevant functions.

579. A Replicating Single-Cycle Adenovirus Vector for Oncolytic Immunotherapy of Cancer

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Most adenovirus (Ad) vaccines are E1-deleted replication-defective Ad (RD-Ad) vectors. Delivery of one RD-Ad to a cell delivers one copy of a transgene gene to express "1X" protein. In contrast, an oncolytic replication-competent Ad (RC-Ad), kills cancer cells and also amplifies transgenes thousands of times in each cell to amplify protein production and immune responses. While oncolytic RC-Ads are more potent, they risk causing uncontrolled Ad infections in immunocompromised patients. To harness oncolysis, transgene replication, and amplified immune responses, but avoid the risk of Ad infections, we developed single-cycle adenoviruses (SC-Ads). SC-Ads still replicate their DNA and transgenes and kill cancer cells like an RC-Ad, but they cannot produce infectious virions. We armed SC-Ads with the T cell immune checkpoint stimulator 4-1BBL and with the antigen presenting cell stimulator granulocyte macrophage colony stimulating factor (GMCSF). Both cytokines recruited tumor infiltrating lymphocytes (TILs) into tumors with markedly higher activity by SC-Ad-4-1BBL. Both mediated significant reductions in tumor growth and extended survival after single injection virus in immunocompetent mouse models of A20 lymphoma. Co-injection of these vectors with an Ad expressing the cognate A20 antigen did not improve anticancer efficacy, These data suggest that SC-Ads may be a safer platform for oncolytic immunotherapy against lymphomas and other cancers.

580. Optimizing CD123-CAR T Cells for the Adoptive Immunotherapy of AML

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Background: The outcome of recurrent/refractory acute myeloid leukemia (AML) remains poor, and the adoptive transfer of T cells expressing chimeric antigen receptors (CAR T cells) has the potential to improve outcomes. CAR T cells targeting the AML antigen CD123 have shown promise in preclinical models, and early Phase clinical testing is in progress. However, CD123 is expressed at low levels in normal hematopoietic progenitor cells, raising safety concerns. The goal of this project was to generate and compare T cells expressing different CD123-CARs and CD20 as a suicide switch (CD123-CAR^{CD20}T cells). **Methods:** We generated five bicistronic lentiviruses (LVs) encoding CD20, a 2A sequence, and CD123-CARs. For four CD123-CARs we used the CD123-specific single chain variable sequence (scFv) 26292 (292) and for one the CD123-specific scFv 26716 (716). Two different hinge/transmembrane (TM) domains (CD28 or CD8a), and costimulatory domains (CD28, 41BB, and CD28.41BB) in combination with the zeta (z) signaling domain were

evaluated. CAR T cells were generated by LV transduction, and their effector function was evaluated in vitro and in a xenograft AML model. **Results:**CD123-CAR^{CD20}T cells were successfully generated with all LVs. There was no significant difference in CD20 expression (positive cell range: 83-97.7%), and CD123-CAR^{CD20}T cells were readily killed in the presence of rituximab and complement (80% killing within 1 hour incubation). CAR expression varied between 71 and 95%, and the % of CAR+ T cells was significantly lower post transduction with the LV encoding the CD8aTM.CD28z CAR ($p<0.05$). Expansion, viability and phenotype (CD3, CD4, CD8, CCR7, CD45RO) of CAR T cells showed no significant differences. However, CD8a.41BBz CAR T cells produced significant amounts of IFN-g (200-600 pg/ml) at baseline in comparison to other CAR constructs ($p<0.001$). In coculture assays, CD123-CAR^{CD20}T cells recognized CD123+ AML cells (MOLM13) as judged by IL-2 and IFN-g secretion in contrast to non-transduced (NT) T cells ($N=5$, $p<0.0001$) with no significant differences between CARs. The *in vivo* activity of CD123-CAR^{CD20}T cells was evaluated in the NSG/MOLM13-firefly luciferase AML model. On Day 7 post AML cell injection, mice received a single dose of $3x10^6$or $1x10^7$CD123-CAR^{CD20}T cells, and anti-AML activity was monitored by bioluminescence imaging (n=5 per group). At both cell doses CD123-CAR^{CD20}T cells had significant antitumor activity in comparison to untreated controls resulting in a survival advantage. At the lower cell dose, T cells expressing CARs with a 716 scFv had inferior ($p<0.01$) antitumor activity. Addition of the 41BB signaling domain to 292.CD28TM.CD28.z CARs did not improve anti-AML activity. **Conclusion**: Here we compared five LVs encoding CD20 and CD123-CARs. Our results revealed significant differences regarding CAR expression, baseline signaling, and anti-AML activity. Based on our findings, we selected T cells expressing CD28TM for future clinical testing.

581. An Ex-Vivo Patient Derived Immunotherapy Platform to Investigate CAR T-Cell Efficacy against Lung Cancer

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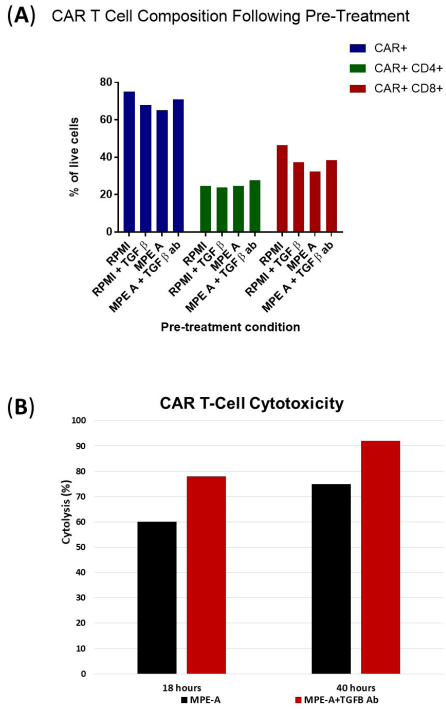
Background: Adapting chimeric antigen receptor (CAR) T-cell therapy for use in solid malignancies faces many obstacles, such as overcoming an immunosuppressive environment containing PDL-1/PD-1, TGF-β and other T-cell suppressive cytokines. Current in vitro methodologies allow testing of CAR T-cell efficacy in the presence of these factors in isolation, typically added to culture media. One potential source of a combination of immunosuppressive factors is malignant pleural effusion (MPE), which is a tumor-associated accumulation of fluid between the membranes lining the lungs containing a variety of immune inhibitory and inflammatory cells and factors, such as TGF-β. We aimed to develop, optimize and translate a culture system that is derived from patient tumor environment to investigate CAR T-cell efficacy. Methods: MPEs were collected from lung cancer and mesothelioma patients and the concentration of TGF-β and other soluble factors was measured by ELISA and Luminex assays. Mesothelin-targeted CAR T cells were pre-treated via co-culture with tumor cells in either complete RPMI (RPMI + 10% FCS), complete RPMI with recombinant TGF-β protein (2 ng/mL), or MPE known to be high in TGF-β1 and TGF-β2 with and without TGF-β blocking antibodies. Following pre-treatment, CAR T-cell phenotype was assessed by flow cytometry, and long-term cytotoxicity (up to 72 hours) against mesothelin-expressing tumor cells was determined using a impedance-based assay. Results: In 24 MPEs taken from lung cancer and mesothelioma patients, mean concentrations of TGF-β1, TGF-β2, and TGF-β3 were 1.25 ng/mL (range 0.28 - 13 ng/mL), 245 pg/mL (range 23 - 1855 pg/mL), and 16 pg/mL (range <9.8 - 67 pg/mL), respectively. MPE A (high in TGF-β1, TGF-β2, and a combination of other cytokines) was selected for further analysis for TGF-β-mediated inhibition on CAR-T cells. Following pre-treatment, CAR T-cell transduction and proportion of CD4 and CD8 cells were within 10% standard deviation for all conditions (**Fig A**). Pre-treatment of CAR T-cells in complete RPMI with recombinant TGF-β protein resulted in inhibition of cytotoxicity against tumor cells compared to complete RPMI alone. In addition, pre-treatment of CAR T-cells in MPE A with TGF-β blocking antibodies rescued inhibition, evidenced by an increase cytotoxicity against tumor cells compared to CAR T-cells pre-treated with MPE A alone (**Fig B**). Conclusion: Pre-treatment of CAR T-cells with either recombinant TGF-β or an MPE high in TGF-β inhibits CAR T-cell cytotoxicity. In MPE, addition of TGF-β blocking antibodies during pre-treatment and co-culture with tumor cells rescues CAR T-cells from TGF-β-mediated inhibition even under the influence of other T-cell inhibitory cytokines. The use of MPE as means to study CAR T-cell efficacy provides a translationally relevant model of tumor immune suppression, which is more comprehensive than testing agents in RPMI media alone.



582. Nano-Emulsion Based WT1 DNA Vaccine Elicits Significant Anti-Tumor Response in a Murine Model of Melanoma

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DNA based vaccination using an antigen specific gene-transfer construct is an attractive approach in oncologyas it offers a range of humoral as well as cellular interference that can target and eradicate cancer cells. Wilms' tumor protein encoding gene (WT1) expression is up-regulated in several malignancies (e.g. myeloid leukemia and melanoma) but is maintained at lower levels in normal cells/adult tissues. WT1 is thus a promising tumor antigen for the development of a universal cancer vaccine. The clinical translation of this approach has been impeded by its relatively low efficacy when tested *in vivo*. Thus, methods to improve the potency of the gene construct so as to generate adequate concentrations of WT1 antigen and the enhanced adjuvanic effect of the vaccine formulation may be beneficial.To overcome some of the current limitations, we reasoned that a DNA vaccination strategy to deliver WT1 plasmid in a water-oil-water (W/O/W) double emulsion adjuvant, may further improve its immune potency. We thus formulated a W/O/W- murine(m)WT1 emulsion and studied its characteristics *in vitro*and *in vivo*. Co-culture of WT1emulsion complex with HeLa cells demonstrated an increase in expression of proinflammatory cytokines, TNF alpha (TNF-α) and IL-6 in comparison to control cells. To further test its potency *in vivo*, we administered the mWT1 emulsion complex into subcutaneous region of C57BL/6J mice. Animals immunized with mWT1 emulsion complex had three weeks later, demonstrated significantly higher expression of serum IFN-γ, IL-6 and TNF-α, IL-12 in thymus and IL-6, and IL-12 in spleen of immunized mice. Furthermore, this complex elicited a significant increase in $CD4^+$(34.7% vs 16%), $CD8^+$(21.5% vs 8.4%) cells in splenocytes of immunized animals in comparison to control group, respectively. To test if the vaccination protocol is efficacious *in vivo*, we developed a syngeneic murine melanoma tumor model by administration of B16F10 mouse melanoma cells into C57BL/6J mice. Animals vaccinated with the mWT1 emulsion complex demonstrated considerably attenuated the tumor growth (1.4±1.05 $cm^3$for immunized mice tumor vs 12.84±1.25 $cm^3$for tumor control group). Furthermore, 4 out of 6 (33.33% survival) mice in the control group died before day 35 due to the tumor burden, but all mice (n=6/6, n=100% survival) in pre-immunized groups survived up to 35 days after the implantation of tumor. Taken together, our data highlights the immunogenic potential of nanosized W/O/W emulsion WT1 DNA vaccine and its therapeutic role for eliciting significant anti-tumor activity.

583. Baboon Retrovirus Envelope Pseudotyped Lentivectors Permit Robust Transduction of NK Cells and Represent a New Superior Tool for CAR NK-Cell Cancer Immunotherapy

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NK-cell resistance to transduction is a major technical hurdle for developing NK-cell immunotherapy. By using baboon envelope pseudotyped lentiviral vectors (BaEV-LVs) encoding eGFP, we obtained a transduction rate of 30±2,2% in freshly isolated NK-cells and 87±6.7% in NK-cells obtained from the NK cell Activation and Expansion System (NKAES), even at low MOI, with a sustained transgene expression for at least 21 days. BaEV-LVs outperformed Vesicular Stomatitis Virus type-G (VSV-G)-, RD114- and Measles Virus (MV)- pseudotyped LVs ($p<0.001$). mRNA expression of BaEV receptors, ASCT1 and ASCT2, was detected in freshly isolated NK cells and NKAES, with a higher ASCT1 expression in NKAES. Transduction with BaEV-LVs encoding for CAR-CD22 induced a robust CAR-expression on 43% of NKAES cells, which allowed the specific killing of NK-resistant pre-B-ALL-RS4;11 cell line. Remarkably, by using a larger vector encoding a dual CD19/CD22-CAR, separated by T2A, we could, despite a low viral titer, transduce and re-expand dual-CAR-expressing NKAES, which killed efficiently and specifically both $CD19^{KO}$or $CD22^{KO}$, RS4;11 cells. These dual CAR expressing NKs might thus overcome tumor evasion to single CAR-therapy. Our results suggested that BaEV-LVs could be the long-awaited tool that will help bringing NK-cell-based immunotherapy to the clinic and study NK-cell biology much more efficiently.

584. PD-1 RNA Interference Contained in a Mesothelin Chimeric Antigen Receptor T Cells Display Enhanced Anti-Tumor Activities

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Cancer immunotherapy using Chimeric Antigen Receptor adoptive transferred T cells(CAR-T) has made a fascinating progress during the last decade. It already showed therapeutic effect against B- cell malignancies, however, the solid malignancies still show resistance mainly because of the immune checkpoints and tumor microenvironment. Mesothelin (MSLN) is a tumor specific antigen comprehensively expresses on many tumor cells. Here in this study, we made a MLSN-CAR vector containing a PD-1 short hairpin RNA(shPD-1) based on the Lentiviral vector. Our results showed after T cell transduction, MSLN-CAR-shPD-1 demonstrate remarkable PD-1 expression knockdown compare with the wild type MSLN-CAR when the PD-1 got re-stimulation. After the bioluminescent based cytotoxicity assay, which clearly shows that MSLN-CAR-shPD-1 T cells have better tumor killing activity than the wild type MSLN CAR

T cells in both Panc-1 and H226 cells, especially when PD-L1 is stable expressed on these tumor cells. Next, we tested MSLN-CAR-shPD1 has better tumor killing consistency and proliferation for the long term both *in vitro* and *in vivo*, and the Tumor Infiltrating Lymphocytes(TIL) from MSLN-CAR-shPD1 injection have better cytotoxicity and viability ex vivo. MSLN-CAR-shPD1 inhibit the PD1/PD-L1 axis and enhance the lytic granule polarization at the cytotoxic Immune Synapse, the confocal microscopy result showed that after PD-1 knockdown, the MSLN-CAR T cells and tumor cells conjugation has faster lytic granule delivery and stronger immune synapse intensity.

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585. FVIII Expression by its Native Promoter Sustains Long Term Correction Avoiding Immune Response in Hemophilic Mice

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Herein we describe a successful gene therapy approach for hemophilia A (HA) using the natural F8 promoter (pF8) to direct gene replacement to FVIII secreting cells. The promoter sequence and the regulatory elements involved in the modulation of F8 expression are still poorly characterized and biased by the historical assumption that FVIII expression is mainly in hepatocytes. Bioinformatic analyses have highlighted an underestimated complexity in gene expression at this locus, suggesting an activation of pF8 in more cell types than those previously expected. We used FANTOM5 data resources to dissect and confirm TSSs involved in F8 expression and to accurately map alternative promoter regions across approximately 2000 samples of human origin. Using FANTOM5 Zenbu Genome Browser web tool, we found that five independent TSSs are associated to human F8. TSS1 corresponds to the region already described which promotes the transcription of the annotated reference sequence (NM_000132). TSS1 resulted in being the most frequently used in FANTOM5 samples. In human tissues, including those relevant to HA, expression of F8 could be detected in the liver, spleen, lymph nodes and heart. Very low to undetectable expression levels detected in the brain. To elucidate which TFs could be involved in FVIII expression regulation, we performed an in-silico analysis of the 1175 bp pF8 sequence. This analysis besides confirming the presence of TFBS belonging to HNF1, HNF3, C/EBPα and C/EBPβ previously described, also revealed the presence of several TFBS potentially recognized by endothelial and hematopoietic TFs. This prediction highlights the presence of a complex FVIII transcriptional regulation and supports the reported extrahepatic FVIII expression and production in endothelial and hematopoietic cells. C57Bl/6 mice injected with a lentiviral vector expressing GFP under the pF8 (LV.pF8.GFP) confirm the predominant GFP expression in liver sinusoidal endothelial cells, with a few positive cells detectable also in hematopoietic organs. To evaluate pF8 activity in hematopoietic cells, Lin- cells were isolated from total BM of C57Bl/6 mice, transduced with LV.pF8.GFP and transplanted into busulfan-conditioned C57Bl/6 mice. FACS analysis confirmed pF8 activity in BM-derived cells, with GFP expression detected in PB of transplanted mice that remained stable for up to 4 m after transplantation. The PB, BM and spleen of pF8-Lin- transplanted mice revealed a marked GFP expression in myeloid cells. Analysis of the liver and spleen from pF8-Lin- transplanted mice revealed a predominant GFP positivity in macrophages. Therapeutic gene delivery (LV.pF8.FVIII) in hemophilic C57/Bl6 and 129-Bl6 mice successfully corrected the bleeding phenotype, rescuing up to 25% of FVIII activity using a codon-optimized FVIII without the appearance of inhibitors, and sustained this activity for 1 year. Of note, LV.pF8.FVIII delivery in FVIII-immunized HA mice resulted in the complete reversion of inhibitor titer with the recovery of therapeutic FVIII activity. Depletion of regulatory T-cells (Tregs) in LV-treated mice allowed the formation of anti-FVIII antibodies, indicating a role for Tregs in immune tolerance induction. The significant blood loss reduction observed in all LV.pF8.FVIII-treated mice 1 year after injection confirmed the achievement of a long-term phenotypic correction. Altogether, our results highlight the potency of pF8-driven transgene expression to correct the bleeding phenotype in HA, and potentially in other diseases where an endothelial-specific expression is required.

586. Prenatal Therapy Leads to Corrective Plasma Levels of fVIII for 22 Months in the Absence of Therapy-Related Toxicity or Development of fVIII Immunity in a Large Animal Model

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Prenatal transplantation (PNTx) poses minimal risk to both the fetus and the mother, and has been successfully used to treat patients with hereditary immunodeficiency disorders. Since 75% of hemophilia A (HA) patients have a family history of HA, and prenatal diagnosis is available to pregnant HA carriers, PNTx constitutes a clinically viable approach to treat these patients. The goals of PNTx for HA are to: 1) provide long-term sustained factor VIII (fVIII) levels, thereby reducing/avoiding the need for postnatal treatments; and 2) induce immune tolerance to fVIII, eliminating the risk of the most serious adverse event in HA treatment, the development of fVIII inhibitors. We previously reported that transplanting sheep fetuses (n=11) with human placental cells (PLCs) transduced with a lentiviral vector encoding a bioengineered fVIII transgene (mcoET3), at a dose of 10^7-10^8/kg, increased plasma fVIII activity levels by 41±14% (n=6) for up to 22 months post-transplantation, when compared to control non-transplanted animals. Evaluation of the mcoET3-PLCs prior to PNTx demonstrated that they secreted 8.44IU fVIII/10^6cells/24h, with

a vector copy number of 0.35±0.05 per diploid genome equivalent. Here, we investigated whether PNTx-treated animals developed liver inflammation and/or mounted an immune response to mcoET3. At all time points post-transplant, hematological parameters and liver enzymes (AST, ALT, and alkaline phosphatase) were normal, demonstrating the absence of any liver toxicity. To investigate transplanted animals developed anti-mcoET3 IgG antibodies, an mcoET3-specific ELISA was performed at 3-6 and 10-16 months postnatally. At 3-6 months of age, all PNTx-treated animals were devoid of anti-mcoET3 IgG. At the later time point, one of the animals had a low anti-ET3 ELISA titer while maintaining normal fVIII activity levels. To determine whether these animals had developed memory T lymphocyte responses to mcoET3, ELISpot assays for INF-gamma (T_{h1}) and IL-4 (T_{h2}) were performed at 3 different time points. A positive response was defined the following criteria: 1) the ratio between the number of spot-forming units (SFU) in the presence and absence of ET3 $\geq$2 (Stimulation Index $\geq$2); and 2) the mean number of SFU differed by at least $10/2x10^5$ PBMC. No T_{h1}or T_{h2} mcoET3-specific cells were ever detected in any of the transplanted lambs. Challenge of transplanted animals with ET3 protein is currently underway. Because maternal safety is paramount for the clinical application of PNTx, birthing ewes were also evaluated for the potential development of immunity to mcoET3. The birthing ewes (*n*=6) harbored neither anti-mcoET3 IgG antibodies, nor T_{h1}or T_{h2} mcoET3-specific cells. In conclusion, PNTx of fetal sheep recipients at the equivalent of 16-17 gestational weeks in humans, with PLCs genetically modified to expressa bioengineered fVIII transgene (mcoET3) resulted in sustained fVIII plasma levels >40% for more than 1 year after birth with no evidence of therapy-related toxicity, nor the development of a fVIII immune response.

587. Vector Genome Processing of Valoctocogene Roxaparvovec in Mouse and Monkey Livers

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Hemophilia A (HA) is a congenital bleeding disorder caused by deficiency in clotting factor VIII (FVIII) activity. Valoctocogene roxaparvovec (BMN 270) is an AAV5-based gene therapy vector that encodes a B-domain-deleted (BDD) human FVIII (hFVIII-SQ) under the control of hybrid liver-specific promoter (HLP) and is currently being evaluated in phase 3 clinical studies for the treatment of severe HA. Previous studies in lung, liver, and muscle transduced by AAV gene therapy in mice and non-human primates (NHP) have shown that circularized monomeric and concatemeric episomes are the major DNA species associated with long-term, persistent expression of the gene product in the target cell. In order to understand the kinetics of valoctocogene roxaparvovec vector genome processing, long-term studies of up to 6 months post valoctocogene roxaparvovec administration have been conducted in bothmurine and nonhuman primate models. In the present study, we used drop-phase droplet digital PCR (ddPCR), quantitative PCR and Southern blotting following various nuclease treatments, to analyze, characterize and quantify the different molecular forms of valoctocogene roxaparvovec vector genome in the liver at several time points. These experiments demonstrated formation of monomeric and multimeric circular episomes in the livers as early as one week post dosing. Quantities of circular episomes (monomers and concatemers) increased over 6 months with predominantly head-to-tail configuration. In contrast, the non-circular DNA population became more fragmented, and decreased in total quantity. The levels of duplex, circular genomes significantly correlated with the levels ofFVIII transcripts. In conclusion, long-term (up to 6 months) FVIII expression from valoctocogene roxaparvovecvector genomes transduction is associated with formation of circular episomes in the liver.

588. A Novel Strategy of Lung-Specific Gene Therapy for the Treatment of Hemophilia A via Intranasal Delivery of Lentiviral Vectors Encoding Factor VIII

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Recently, emerging evidence showed that lung is a reservoir for haematopoietic progenitors/stem cells (HSCs) in addition to bone marrow. The new study reported that platelets can be produced from megakaryocytes located in the extravascular space inside the lung. Furthermore, our previous data demonstrated that external factor VIII (FVIII) gene driven by the specific promoter GP1bα (G) could express FVIII protein in megakaryocytes for long-term by transducing HSCs in mice. FVIII protein stored in platelets can be released to facilitate clotting upon platelets activation. Thus, intranasal (IN) delivery of lentiviral vectors (LVs) targeting FVIII gene expression in megakaryocytes in the lung have the potential to correct HemA mice phenotype. Firstly, G-GFP-LVs were used to test if IN delivery can produce GFP expression in murine platelets. We found that IN delivery of 24μl G-GFP-LVs produced up to 0.075% positive GFP expression in platelets after 7 days. Although the efficiency was low, GFP expression can be detected by flow cytometer. There was a significant difference compared with the untreated control group. To improve the transduction efficiency, we used higher titers/volume of LVs in mice pretreated with N-Acetyl Cysteine and Dexamethasone drugs.After IN delivery of increased dosages of G-FVIII-LVs into HemA mice under the optimized protocol, we examined the FVIII expression in platelets by intracellular staining and FVIII ELISA at day 7 post treatment. There was 0.23% FVIII positive platelets in circulating platelets and FVIII expression can be detected up to 0.3 $mU/10^8$ platelets. We also tested the functional clotting formation by Rotation thromboelastometry (ROTEM). The clotting parameters obtained from IN gene therapy mice were comparable to those of wild type mice, including Clotting Time (CT), Clotting Formation Time (CFT), α angle and Max of Clotting formation (MCF). Moreover, significant difference in the CT values was observed between HemA mice group and IN gene therapy group (2641±1437(s) vs 587±354(s); p=0.032) after 7 days treatment. These data indicated successful transduction of megakaryocytes in the lung following IN delivery, leading to therapeutic correction of HemA. Furthermore, FVIII positive platelets staining persisted at 0.23%

from day 7 to day 30 post treatment. The clotting parameters tested by ROTEM after day 30 treatments were not significantly different compared with the data after day 7 treatment, indicating potential transduction of hematopoietic stems cells by LVs. In addition, we did not detect formation of anti-FVIII inhibitors by Bethesda assay.Our data showed that we successfully produced FVIII expressing platelets to partially correct HemA phenotype in mice via IN delivery of G-FVIII-LVs into the lung. The benefit of FVIII expression and storage in platelet α-granules is to protect FVIII from naturalizing antibodies and to decrease the possibility of inducing inhibitory antibodies. IN delivery of LVs encoding FVIII gene targeting lung would be a promising option for therapeutic treatment of hemophilia patients.

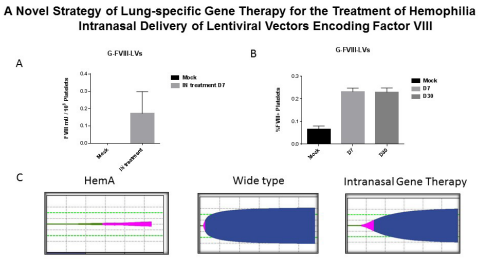

Fig A: FVIII expression was detected by ELISA assay in platelets on D7 after intranasal Lentivirus treatment (N=4).
Fig B: The FVIII intracellular staining in platelet were measured by flow cytometer on D7 and D30 after intranasal Lentivirus treatment (N=4).
Fig C: The representative schematic of the blood clotting in HemA mice group, wide type mice group and intranasal Gene Therapy mice group by ROTEM (N=4).

589. Bioengineering Coagulation Factor IX through Ancestral Sequence Reconstruction

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Vector potency remains a key optimization parameter in clinical gene therapy applications. In the field of liver-directed adeno-associated viral (AAV) vector gene therapy, significant advances have been made in capsid identification/engineering, synthetic transcriptional regulatory element development and codon optimization. The remaining aspect of vector design, transgene product optimization, is often ignored. One exception to this is the implementation of the Padua mutation (R338L) into blood coagulation factor IX (fIX). This mutation has been shown to confer a 7 - 8-fold benefit to human fIX specific activity in vitro as well as in vivo in clinical gene therapy trials. Recently, we described the utilization of ancestral sequence reconstruction (ASR) to improve the pharmaceutical properties of blood coagulation factor VIII (fVIII). Certain ASR variants of fVIII were shown to possess increased biosynthesis, specific activity, half-life and resistance to anti-human fVIII inhibitory antibodies. To expand on this platform approach, we performed ASR on fIX to infer an evolutionary tree, which turned out to be identical that of trees constructed by ASR of other coagulation factors including, V, VII, VIII, X, tissue factor and von Willibrand factor, while extending beyond the class Mammalia. For fIX ASR, we resurrected 8 ancestral (An) fIX variants spanning the ancient primate, rodent and ungulate lineages. *In vitro* recombinant expression experiments in HEK-293 and HepG2 cells identified two An-fIX variants (An96 and An97) that displayed 11-fold greater fIX activity production than normal human fIX. To determine the mechanistic relationship between the An96/97 and Padua functional residues, the R337L mutation was bioengineered into An96 that had been codon-optimized using an identical liver-directed algorithm. An96-Padua displayed fIX expression 4-fold better than human fIX-Padua suggesting that the functional mechanisms conferring increased activity are independent and additive. Commercial anti-human fIX antibodies appear to display decreased reactivity with An96 thus it was not possible to determine specific activity using this method. Therefore, we generated stable production HEK-293 cell lines and recombinant An96-Padua was purified using mixed-mode and ion exchange chromatography resulting in a >95% pure preparation displaying a specific activity of 6,450 IU/mg, which is 32-fold higher than commercial recombinant fIX utilized clinically and is predicted to be at least 4-fold higher than recombinant human fIX-Padua. In order to determine if this increased functionality translates to better gene therapy outcomes, AAV2/8-An96-Padua vector was generated and compared directly to an identical AAV2/8-hfIX-Padua vector using intravenous delivery to hemophilia B mice. Mice that received AAV2/8-An96-Padua displayed 4-fold greater plasma fIX activity than mice expressing AAV2/8-hfIX-Padua over the course of 12 weeks. In summary, An96 represent a novel ASR bioengineered fIX variant than can be utilized to increase the potency of fIX gene therapy completely independent of, or more beneficially in conjunction with, Padua R338L.

590. Genome Editing Strategy for the Treatment of Hemophilia A Patients with Inhibitors

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The CRISPR-Cas9 technology is revolutionizing modern medicine. With distinct genome-editing capabilities, CRISPR-Cas9 has diverse therapeutic implications for several genetic diseases including hemophilia A. With no cure yet on the market, treatment for hemophilia A routinely involves prophylactic administration of recombinant Factor VIII (FVIII). However, 30% of patients receiving FVIII replacement therapy develop inhibitors, rendering the current treatment options ineffective. In addition, most hemophilia gene therapy protocols propose to deliver FVIII to the liver with no consideration of limiting presentation to the immune system. In large, hemophilia patients with inhibitor formation are excluded from clinical studies. Therefore, we hope to design a treatment plan specifically for these individuals. Our innovative targeting strategy utilizes the CRISPR-Cas9 biotechnology to target the transcriptionally active von Willebrand Factor (vWF) locus to express FVIII. In order to not disrupt the expression of vWF, the donor DNA template was designed to eliminate the vWF stop codon as well as contain a T2A element, which facilitates ribosomal skipping, allowing for bicistronic expression, yielding the synthesis of multiple proteins from a single messenger RNA. Further to this aim, we also designed six guide RNAs (gRNAs) for the purpose of directing the Cas9 protein to the vWF stop codon. TIDE analysis revealed cutting efficiencies ranging from 2 to 35%, indicating successful levels of gRNA-directed cleavage. Utilizing our most effective gRNA, preliminary modification of the myeloid-erythroid K562 cell line

confirmed the viability and efficacy of this therapeutic strategy. As a proof-of-principle, an exogenous GFP transgene was incorporated in the donor DNA template downstream of the T2A element. Targeting of the vWF locus by homologous recombination was confirmed by DNA sequencing. GFP expression was assessed by fluorescent microscopy and flow cytometry. Stable expression of the exogenous transgene was confirmed in that GFP expression was found to persist for a period of three weeks after nucleofection. In addition, expression of vWF was found to be unaltered. These experiments set the stage for targeting a clinically relevant FVIII transgene to the vWF locus of hematopoietic stem and progenitor CD34+ cells, an attractive option for gene therapy applications. While unconventional, our treatment approach ultimately has the potential to provide a cure to thousands currently without any approved treatment option.

591. Deletion of B Domain of F8 to Restore the FVIII Function in Patient-iPSCs and ECs

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Given that the cDNA of *F8* is too large to be packaged into AAV capsids, gene transfer of some versions of B domain-deleted *F8* (BDD-*F8*) for hemophilia A (HA) treatment has been attempted with promising results. And up to now 775 mutations in the B domain *F8* that cause HA have been reported in the LOVD, approximately 85% of which result in premature termination. Here we described a targeted deletion of B domain of *F8* with CRISPR/Cas9 to restore the function of FVIII with a B domain frameshift mutation in HA-iPSCs. The expression and activity of FVIII was restored in corrected HA-iPSC-derived endothelial progenitor cells (C-iEPCs) *in vitro* and *in vivo*.The bleeding phenotype was rescued in HA mice after C-iEPC infusion. Our results demonstrate an efficient approach for targeted gene correction via introduction of B domain deletion using CRISPR/Cas9 to restore the *F8* transcript and FVIII function in HA-iPSC-derived EPCs with potential clinical impact in HA gene therapy. For the first time, we demonstrated *in vitro* and *in vivo* the FVIII function that is encoded by the endogenous B domain-deleted *F8*.

592. coreHEM, a Multistakeholder Core Outcome Set Project for Gene Therapy in Hemophilia

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Introduction: Gene therapy trials have demonstrated the potential to "cure" hemophilia by introduction of a functioning gene that enables production of the missing clotting factor (factor VIII, hemophilia; factor IX, hemophilia B). As pivotal trials were set to be begin, we recognized the need to define outcomes that would distinguish and differentiate gene therapy from the existing standard of care of prophylactic factor replacement. Achieving this goal required both a standardized set of outcomes that could be used for comparisons across trials and to existing therapies, and consideration of novel outcomes that capture the transformative potential of gene therapy. coreHEM was an international multistakeholder consensus project that aimed to develop a clearly-defined core outcome set (COS), a minimum set of outcomes that should be measured and reported in all clinical trials of a specific condition. **Methods:** The COS was developed using a modified Delphi consensus process of online surveys plus an in-person meeting. A literature review and participant/patient interviews were used to create a list of candidate outcomes. Participants (patients, healthcare providers, payers, HTA agencies, regulators and industry) condensed and prioritized the list by rating each outcome on a scale of 1-9 (not important to include in a core set to essential). In methods adapted from the COMET Initiative, outcomes were eliminated from consideration if <70% rated the outcome from 7-9 ("high consensus"), unless the patient stakeholder group average score was ≥7 (categorized as "patient-important" and discussed at the meeting). The "patient-important" criterion was dropped for the final vote; outcomes were retained if they met the criteria for "high consensus. **Results**: After two voting rounds, three outcomes reached "high consensus": frequency of bleeds, factor activity level and duration of expression. In the final round, 3 more outcomes met the criteria and were included in the COS: chronic pain, utilization of the healthcare system (direct costs), and mental health condition. 49 voters participated. **Conclusions**: For the first time in hemophilia, a COS has been developed, with the involvement of representatives of all relevant stakeholder groups. Three of the outcomes represent the novelty of gene therapy treatment. Only frequency of bleeds is a "legacy" outcome, consistently used in past hemophilia trials; its inclusion in the COS will enable comparing efficacy and effectiveness with existing treatment and also calculating derived measures such as impact on target joints, a surrogate endpoint for long-term joint function deterioration. Achieved factor activity level (i.e. restoration of lacking clotting capacity) is directly and linearly correlated to the risk of bleeding. Factor activity level is recommended as a measurement to reflect that patients who receive gene therapy will be freed from the regular peaks and troughs of factor level with consistent protection from bleeds. Duration of expression relates to the endurance of the transfected gene and the potential long-term value. Chronic pain and impact on mental health are proposed to capture the entire spectrum of the impact of gene therapy as a definitive cure (as such, with a huge potential for transforming the patient experience) for the functional and social impact of hemophilia. These outcomes will inform a value-based approach, along with measurement of direct healthcare resource use.

593. Combination of CRISPR/CAS9-Triggered Re-Activation 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 closely correlates with the level of gamma-globin expression after HSC gene therapy. 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 targe 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/B6 recipient mice exhibited stable, high-level gamma-globin expression. 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. We are currently testing the combined system in CD34+ humanized mice and in a mouse model for sickle cell disease.

594. Nanoparticle-Mediated Correction of the Sickle Cell Mutation

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Monogenic blood disorders, such as sickle cell disease (SCD) and β-thalassemia, can be potentially cured or treated through genome editing of hematopoietic stem/progenitor cells (HSPCs). While many gene editing technologies have been developed to date, few, if any can be effectively administered *in vivo*. This is particularly true of nuclease-mediated methods, which require *ex vivo* manipulation to efficiently deliver these agents to cells. As an alternative, we have shown that triplex-forming peptide nucleic acids (PNAs) can be used to initiate targeted recombination of "donor DNA" fragments into genomic DNA, resulting in site-specific correction of genetic mutations. These materials can be delivered efficiently and safely to cells *in vitro* and *in vivo* using polymeric nanoparticles (NPs) composed of a poly(lactic-co-glycolic) acid (PLGA), a biocompatible and biodegradable polymer, which has been approved by the U.S. Food and Drug Administration (FDA) for various clinical uses in humans[3]. Using polymeric NPs to encapsulate and deliver PNAs and donor DNA fragments, we have now been able to correct genetic mutations underlying cystic fibrosis as well as β-thalassemia postnatally and in utero. Here we demonstrate for the first time that PNAs can be designed and delivered via PLGA NPs for the correction of the SCD mutation. We also demonstrate that novel poly(lactic acid)-hyperbranched polyglycerols (PLA-HPG) can efficiently deliver PNA and donor DNA combinations, demonstrating superior loading and gene editing as compared to PLGA. We designed and screened 9 PNAs targeting regions near the SCD mutation site to induce recombination and repair with a correcting 'donor' DNA. Encapsulation of these molecules into PLGA resulted in NPs that were negatively charged and 300 nm in diameter. *Ex vivo* screening in primary bone marrow cells derived from the Townes mouse model of SCD resulted in editing as high as 6% in bulk bone marrow and 9% in HSPCs. Using our best PNA, PLA-HPG NPs were subsequently synthesized, resulting in negatively charged NPs with a diameter of ~150 nm. Though significantly smaller, PLA-HPG NPs loaded PNA and donor DNA more efficiently, resulting in elevated levels of gene editing with frequencies up to 11% following a single treatment. To further validate the clinical translatability of our approach, human CD34+ cells from the peripheral blood of patients with SCD were treated *ex vivo*with our optimal PNA/DNA combination. Preliminary results using PLGA have shown editing frequencies of up to 3%. *Ex vivo* treatment of PNA/DNA-loaded PLA-HPG NPs is expected to result in elevated gene editing. Additional studies are currently underway to compare these delivery systems *in vivo.*

595. HbA and HbF Simultaneous Induction by Double-Pronged Lentiviral Vector ATM1.1 Outperforms Beta-Globin-Based Gene Addition and miRNA-Based Gamma-Globin Reactivation in SCD-Derived Erythroblasts

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Gene therapy is a promising therapeutic approach in many genetic disorders including b-thalassemia (b-thal) and sickle cell disease (SCD) with several ongoing clinical trials spanning a wide range of strategies such as gene replacement and gene editing. However, no lentiviral vector (LV)-based therapy has shown to be effective in the most severe phenotypes of both SCD and b-thal with VCN levels of 1-2 copies per genome. Here we present a new therapeutic approach that combines two of the strategies presently in clinical trial. We aimed to potentiate the beta-globin-based vector ALS10-T87Q with the expression of a miRNA for the purpose of increasing HbF levels. The shRNA^{miR} sequences targeting BCL11A (Guda et. al. Molecular Therapy 2015) were flanked by an optimized backbone termed "miR-E" (Fellmann et. al. Cell Reports 2013) and incorporated in the non-coding regions of ALS10-T87Q. We screened several positions and secondary structures for the miR-E-BCL11A in ALS10-T87Q and looked for the optimal intronic location to express the beta-globin mRNA and the shRNA^{miR} sequence. Screening in HUDEP-2 M#9 cells identified the vector ATM1.1 as the most potent vector, showing the highest concurrent induction of HbF and transgenic HbA. To evaluate the potential use of this vector in gene therapy for SCD, we transduced CD34^{+} hematopoietic progenitor cells isolated from peripheral blood of three SCD patients. Moreover, we compared the total production of HbF+HbA of ATM1.1 with its counterparts ALS10-T87Q and SEV1.1 (a LV expressing the miR-E-BCL11A driven by the core regulatory elements of ALS10-T87Q). HPLC protein analysis showed a dose-dependent induction of HbF and HbA for ALS10-T87Q, ATM1.1 and SEV1.1. Moreover, our results showed that ATM1.1 induces the highest levels of curative hemoglobins (HbF+HbA), outperforming the single vectors ALS10-T87Q and SEV1.1. Western blot analyses confirmed the induction of g-globin chains in both ATM1.1 and SEV1.1 treated cells, and showed an incomplete suppression of BCL11A in cells treated with ATM1.1 but not in cells treated with SEV1.1. The partial knockdown of BCL11A observed in cells treated with ATM1.1 could be advantageous in light of recent data suggesting that disruption of BCL11A leads to an erythroid differentiation defect or survival disadvantage. Additionally, we introduced silent mutations in the coding sequence of the b-globin gene, allowing for detection of splicing variants and specific amplification of the transgenic mRNA. Cells treated with ALS10-T87Q and ATM1.1 showed identical results by RT-PCR and qPCR indicating that the presence of the miR-E-BCL11A in ATM1.1 does not affect the splicing nor lessen the production of the b-globin mRNA. Furthermore, when exposed to low oxygen tension, cells transduced with ATM1.1 were less prone to sickle than control cells. ATM1.1 treated cells showed the lowest percentage of sickle-like morphology (58.1%) when compared to cells transduced with ALS10-T87Q (81.9%) and SEV1.1 (65.1%). Additional studies are in progress to validate the *in vitro* studies using BERK-SCD mouse model as well as SCD-patient BM cells transduced and transplanted into NBSGW mice. Our results are the proof-of-principal that combined strategies are feasible and outperform the current single-pronged methods used in clinical trials. With this approach we expect to generate more powerful and versatile vectors, which can be successful at treating the most severe genotypes with minimal integrations per genome. Moreover, many more genetic disorders can benefit from the combination of gene transfer with RNA interference as one single therapeutic approach.

596. Fetal Hemoglobin De-Repression Following CRISPR/Cas9 Mediated Targeting of the γ-Globin Promoters as a Therapeutic Strategy for β-Hemoglobinopathies

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β-hemoglobinopathies (β-thalassemia and sickle cell disease, SCD), the most prevalent genetic disorders worldwide, are anemias caused by mutations affecting quantitatively or qualitatively the production of the adult hemoglobin (Hb) β-globin chain. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of genetic mutations causing a sustained fetal γ-globin chain production at adult age, a condition termed hereditary persistence of fetal hemoglobin (HPFH). Naturally occurring HPFH mutations cluster at several loci in the promoters of the two γ-globin genes, *HBG1* and *HBG2*, and generate *de novo* DNA motifs recognized by transcription activators or, conversely, disrupt binding sites for transcriptional repressors (e.g., BCL11A and LRF). Genome editing technologies could be employed to mimic HPFH mutations in *HBG* promoters by generating insertion and deletions (InDels) leading to disruption of DNA motifs recognized by transcriptional repressors. This strategy relies on the occurrence of InDels in *HBG* promoters of hematopoietic stem cells (HSCs) that are known to be preferentially edited via non-homologous end joining (NHEJ) mechanism. On the contrary, microhomology-mediated end joining (MMEJ) repair pathwaythat typically generates ≥2-bp deletions, might be less active in HSCs since it shares with HDR the same DNA resection machinery. Here, we have compared the extent of fetal hemoglobin (HbF) de-repression following CRISPR/Cas9-mediated targeting of binding sites for known and putative HbF repressors in the *HBG1* and *HBG2* promoters in an adult erythroid cell line (HUDEP-2) and erythroid cells derived from SCD patient hematopoietic stem/progenitor cells (HSPCs). Efficient genome editing (up to ~90%) was obtained in both HUDEP-2 cell line and primary

patient HSPCs. Upon targeting of LRF and BCL11A binding sites, we achieved a potent and pancellular γ-globin de-repression and amelioration of the SCD cell phenotype. In particular, targeting of the LRF binding site resulted in the highest HbF reactivation. Targeted deep sequencing of the *HBG* promoters, showed gRNA- specific InDel profiles characterized by the co-occurrence of NHEJ- and MMEJ-mediated events. Although the MMEJ repair mechanism generated larger deletions that more efficiently disrupt the binding site for LRF γ-globin repressor, shorter NHEJ-mediated InDels were still able to induce a potent γ-globin de-repression that is sufficient to improve the SCD cell phenotype. Preliminary experiments evaluating genome editing efficiency in different HSPC subsets (sorted according to increasing stemness features) showed a similar InDel profile in all the populations, suggesting that these binding sites are targetable in *bona fide* HSCs. Overall, this study identified the binding site for the LRF HbF repressor as a novel and potent target for HSC-based gene therapy of β-hemoglobinopathies.

597. Novel Lentiviral Vectors to Induce High Levels of HbF in Adult Human Haematopietic Stem Cell Derived Cultured Erythroid Cells

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Induction of fetal hemoglobin (HbF; α2γ2) in sickle cell disease (SCD) and β-thalassemia is a promising approach to ameliorate the disease phenotype. B-cell lymphoma/leukemia 11A (BCL11A) is a transcription factor that represses γ globin gene (HBG) expression in adults. Down regulation of BCL11A induces HbF levels. BCL11A is therefore a prime candidate for targeted therapy aimed at induction of HbF in the patients with β-globin disorders. As depletion of BCL11A in hematopoietic stem cells can result in impaired B-cell growth and render aging like changes in HSCs, it is important that the knock out or knock down of BCL11A be erythroid specific. We generated two novel lentiviral shRNA vectors (CSCREUv1 and CSCREUv5) for the knock down of BCL11A in human erythroid cells. The CSCREUv1 has three DNA hypersensitivity sites, HS2, HS3 and HS4 and a 180 bp HBB promoter. The CSCREUv5 lacks HS4, and has a 266 bp β - globin (HBB) promoter. Both carry a polycistronic GFP-IRES-puromycin-shBCL11a cassette cloned downstream of the HBB promoter. For efficient RNAi, the shRNA was designed using the Sherwood algorithm and UltramiR miR30 scaffold for more effective knock down of the target gene. We measured the efficiency of these lentiviral vectors to downregulate BCL11A and upregulate HbF in an erythroid progenitor cell line, HUDEP2. We observed that >90% knock down of BCL11A by western blot and real time PCR analysis in the transduced GFP⁺HUDEP cells. HbF analysis by flow cytometry in the HUDEP cells differentiated to the later stages of erythropoiesis showed that HbF expressing cells was 50%, while the cells transduced with scrambled shRNA showed only in 12% erythroid cells, suggesting that knockdown of BCL11A caused an increase in HbF expressing cells by nearly 40% for both CSCREU v1 and v5. This experiment was then repeated in CD34+ hematopoietic stem and progenitor cells (HSPCs), which, after transduction, were differentiated to erythroid cells. The transduction efficiency was again noted to be 30-40%. The GFP+ HSPCs were flow sorted and differentiated to erythroid cells using an erythroid culture system supplemented with cytokines, SCF, EPO and IL3. BCL11A expression was analysed by real time PCR and western blot analysis on day 10 after obtaining 97% of CD71+CD235a+ erythroid cells, which showed >80% downregulation of BCL11A. Similar to the erythroid cell line data, these vectors increased the HbF expressing cells by 50% in the primary erythroid cells. We also observed significant upregulation of HBG transcripts with both CSCREUv1 and v5 respectively. Interestingly, consistent with the recent reports, the CSCREUv5 vector lacking HS4 appears to be produced at higher titers. Higher titers resulted in increased mean fluorescence intensity of GFP for the v5 vector. Thus, we were able to generate a lentiviral vector that provided efficient knockdown of our targeted BCL11A protein in erythroid lineages. Therefore, these vectors can provide an opportunity to enhance HbF expression in clinical applications for SCD and thalassemia.

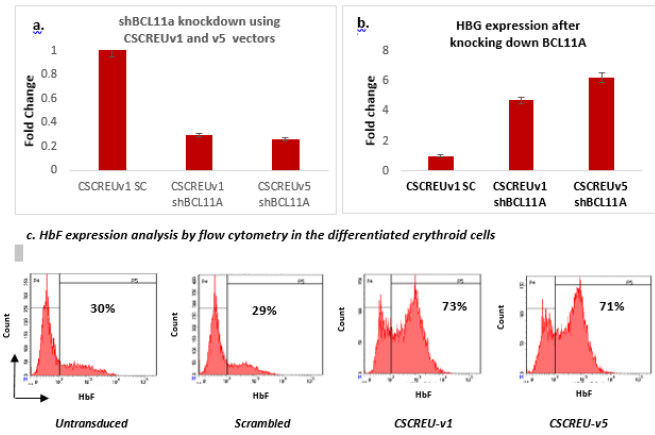

Figure. Induction of γ - globin and HBF expression in the cultured erythroid cells after knocking down the expression of BCL11A using CSCREUV1 and V5 vectors. (A) Real time PCR analysis of knock down efficiency of shBCL11A in the V1 and V5 vectors (B) Induction of γ – globin expression measured by real time PCR analysis and (C) Flow cytometry analysis of HbF expression. The real time PCR results were normalized with the data obtained from the scrambled shRNA (SC).

598. Genome Editing at the Globin Locus in a Sickle Cell Disease Model

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Sickle cell disease (SCD) is caused by a mutation of the β-globin gene (*HBB*), resulting in abnormal hemoglobin molecules that polymerize when deoxygenated, forming "sickle" shaped red blood cells (RBCs). Sickle RBCs lead to anemia, multi-organ damage and pain crises, beginning the first year of life. The onset of symptoms coincides with the developmental switch from fetal stage γ-globin to adult stage β-globin resulting in a shift from fetal hemoglobin (HbF, α2γ2) to adult hemoglobin (HbA, α2β2). Some individuals harbor mutations and deletions throughout the extended γ-globin-β-globin gene cluster, resulting in constitutively elevated postnatal HbF, a benign condition known as hereditary persistence of fetal hemoglobin (HPFH) which,

when co-inherited with SCD, alleviates symptoms of the latter. Previously, we showed that CRISPR-Cas9-mediated genome editing can recreate a naturally-occurring HPFH mutation in the distal CCAAT box region of the γ-globin (*HBG1* and *HBG2*) promoters *in vitro.* Editing at this region led to induction of HbF in an erythroid cell line (HUDEP-2) and in human CD34$^+$ cell-derived RBCs, most likely by disrupting a TGACC binding motif for the γ-globin gene repressor BCL11A. In order to study this potential therapy using an *in vivo* model, we disrupted the BCL11A binding site in the Townes SCD mouse strain, in which homologous recombination was used to replace all endogenous mouse globin genes with human *HBB^s*, *HBG1* (including 1400 nt of the proximal promoter), and *HBA1*. This model has been shown to partially recapitulate human γ-to-β globin switching and exhibit overlapping pathophysiology with human SCD. In this strain, disruption of the endogenous BCL11A de-repressed the human *HBG1* gene, causing persistent postnatal HbF expression. We isolated lineage negative (Lin$^-$) hematopoietic stem and progenitor cells (HSPCs) from Townes SCD mice, electroporated them with Cas9/guide (g) RNA ribonucleoprotein (RNP) complex targeting the distal CCAAT box/BCL11A site of the *HBG1* promoter and cultured them in erythroid cytokines. We achieved robust editing, >85% insertion-deletion (indel) mutations at or near the target site, resulting in HbF protein induction up to 9.0 ± 0.3% compared to 0.28% HbF in control cells. We transplanted the Townes SCD mice *HBG1*-gene-edited HSPCs into lethally irradiated wild-type C57.Bl6 host mice. At 14 weeks post-transplantation, donor engraftment was 93 ± 1% with 39 ± 3% indels in peripheral blood nucleated cells of *HBG1*-gene-edited mice (n=3 mice). HbF protein in circulating RBCs rose from undetectable in controls (unedited donor cells, n=4) to 2.7 ± 0.4% in edited donor-derived RBCs, as measured by ion-exchange HPLC. Notably, this magnitude of HbF induction is approximately 10-fold less than that achieved using the same editing strategy in human HSPCs followed by xenotransplantation into NBSGW mice. At similar levels of human HSPC editing (40-45%), we typically observe approximately 15-23% HbF in donor-derived human RBCs isolated from recipient bone marrow. Thus, our preliminary studies indicate that the functions of cis elements regulating γ-globin gene expression are not faithfully recapitulated in the Townes SCD strain. This may be due the altered configuration of the endogenous human globin gene cluster in the SCD mice and/or altered activities of mouse trans-acting factors on the human genes.

599. Re-Activation of Gamma-Globin Expression in a Mouse Model of Sickle Cell Disease after CRISPR/CAS9-Mediated HSC Genome Editing

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Sickle cell disease (SSD) is an inherited, monogenic blood disorder. It is caused by a single base mutation on the first exon of β-globin gene (β^s allele), resulting in the formation of defective hemoglobin tetramers, which polymerize upon low oxygen concentrations, leading to destruction of erythrocytes. Hereditary persistence of fetal hemoglobin (HPFH) ameliorates the clinical symptoms of SSD. Based on this, we developed an approach of CRISPR/Cas9-mediated gene editing to disrupt the binding sites of the gamma globin suppressor BCL11A within the HBG1 and HBG2 promoters in order to reactivate the expression of γ-globin in a sickle cell anemia mouse model. For the delivery of CRISPR/Cas9 system we used a non-integrating helper-dependent adenovirus HDAd5/35++ vector with high affinity to hCD46 receptor, which is expressed uniformly on hematopoietic stem and progenitor cells (HSPCs). Due to the fact our vector targets hCD46$^+$ cells, we had to generate a mouse model which a) express hCD46 receptor in its HSPCs in similar levels as human and b) has a phenotype of severe sickle cell anemia, and c) contains the human HBG1/2 genes together with their promoters. We therefore bred the humanized SSD Townes model hα/hα::β^A/β^S , hα/hα::-383 γ-β^A/-1400 γ-β^S with CD46 transgenic mice. The F3 generation of these mice had a severe SSD phenotype and a greatly disturbed hematopoiesis, reflected by the presence of ~40% reticulocytes in the peripheral blood. We hypothesized that HBG1/2 promoter-edited HSPCs from CD46$^{+/+}$ Townes mice would have a survival advantage resulting in an amelioration of the SSD phenotype. To test this, we transduced lineage-depleted cells from their bone marrow with an HDAd-HGB1/2-CRISPR vector in vitro and transplanted them into lethally irradiated C57Bl/6 mice. In ongoing studies, we are evaluating the efficacy of HBG1/2 site editing, γ-globin expression in peripheral blood erythrocytes, as well as hematological parameters to assess phenotypic correction in comparison to C57Bl/6 mice transplanted with control HDAd5/35++-vector transduced Townes HSPCs.

600. Novel Lentiviral Vectors with Improved Beta Globin Expression for Hemoglobinopathies

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Several lentivirus-based gene addition therapies have been developed aimed at rescuing both Sickle Cell Disease (SCD) and beta-thalassemia (BT). Results from recent trials indicate that the vectors used are safe; however, their efficacy inversely correlates with the severity of patients' hemoglobinopathy and mutations. In fact, a relatively low number of integrations (1-2 copies/genome) or vector copy number (VCN) is sufficient to cure patients whose mutations are categorized as non-beta0 and express relative high levels of endogenous hemoglobins. In contrast, the same level of VCN alleviates transfusion regimen but does not cure patients with beta0 mutations. This study supplies a platform for rapid screening of lentiviral vectors expressing curative hemoglobin and compares new lentiviral vectors to those currently used in clinical trials side by side, with the goal to select the most robust vector, based on Hb increase. Using CRISPR-Cas9 we modified the erythroid HUDEP-2 cells (Kurita, 2013) to generate a clonal cell line, HUDEP#M13, which, upon differentiation, produces a hemoglobin variant (HbMut) that can be discriminated from that produced by the

lentiviruses (HbA). In parallel, we immortalized erythroid progenitor cells isolated from a SCD donor (SCD#13), using the HPV16-E6/E7 expression system. We compared the correlation between gene transfer and production of HbA in HUDEP #M13 treated with 5 lentiviral vectors (ALS16-20). Our constructs include the Ankyrin insulator in the 3' LTR (Breda et al 2012), the full beta-globin gene and its introns, the full 3' enhancer region, combinations of different portions of the beta-globin promoter, along with modifications and inclusion of novel genomic elements from the locus control region (LCR). ALS16-20 were compared to contructs currently utilized in clinical trials, which were reproduced based on the literature (Negre, 2015; Miccio, 2008; and Boulad, 2014) and indicated as CV-1 (similar to the bluebird bio-BB305 vector), CV-2, and CV-3, respectively. In Hudep #M13, analysis of the ratio of HbA to vector copy number (VCN) for each treatment, indicates that ALS17 and ALS20 yield roughly 40, 157 and 84% more HbA per copy than CV-1, CV-2 and CV-3, respectively. Similar increment in HbA% were confirmed on primary and SCD#13 SCD erythroblasts derived from $CD34^+$ cells isolated from patients' blood. In these specimens, ALS20 maintained a 40% HbA increase compared to CV-1, when exploring a range of VCN from 0 to 3 with a linear mixed effects model. Based on most recently reported data (Thompson et al, 2018), 1 copy of the vector we reproduced as CV-1, makes on average 6.8g/dL of HbA. Hence, 1 copy of our best vector has the potential to make up to 9.5g/dL HbA. This could lead to a much greater clinical impact especially for those patients who require higher Hb production to become transfusion independent, like those with the beta0 genotype. We are assessing the ability of our two most powerful vectors or CV1 to increase hemoglobin content in murine BT hematopoietic stem cells transplanted in BT recipients. Preliminary data obtained in mice that received BM treated with ALS17 indicate that this vector induces therapeutic effects at VCN as low as 1.38, with values of Hb, HCT, RBC and reticulocytes comparable to those of recipients of healthy BM. Currently, we are completing experiments with ALS20 and CVI. In vivo toxicology studies are ongoing and in vitro data show no potential of our vectors to generate clonal transformation (Ikawa's Abstract). These studies are intended to provide preclinical evidence to support initiation of a new clinical trial with our best vector.

601. Genotypic and Phenotypic Characterization of CD46-Transgenic Mouse Models of Sickle Cell Disease for In Vivo HSC Gene Therapy with HDAd5/35++ Vectors

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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 expressing the human gamma globin gene under the control of a micro beta-globin LCR, we demonstrated the safety and efficacy of our approach in a mouse model of thalassemia intermedia. Next, we plan to test our approach in a more severe model of a hemoglobinopathy, the Townes model for sickle cell disease: hα/hα::β^A/β^S , hα/hα::-383 γ-β^A/-1400 γ-β^S. To make this model suitable for HDAd5/35++ mediated HSC gene therapy, we bred Townes mice with human CD46 transgenic mice and analyzed various combinations of transgenes in F1, F2, and F3 generations. Mice homozygous for the human alpha and beta locus displayed sickle-like erythrocytes, severe anemia, ~40% reticulocytes in the peripheral blood as well as leukocytosis. The latter indicates that the disturbance of hematopoiesis extend beyond the erythroid lineage. Various combinations of the human alpha and beta transgenes resulted in different degrees of anemia, leukocytosis, reticulocytosis, and deformation of erythrocytes. Considering the spectrum of SSD phenotypes in humans, these intermediated models could be relevant for testing new gene therapy approaches.

602. Comparison of DNA Break-Inducing and Base-Editing CRISPR/CAS9 Approaches for Fetal Globin Re-Activation after HDAd5/35++ Transduction of Human and Monkey CD34+ Cells

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Current SIN-lentiviral-based approaches for gene therapy of hemoglobinopathies are challenged by *i)* the relatively low transduction efficiency, *ii)* an insert capacity of less than 8kb, and *iii)* the semi-random integration of LVs with preference for active genes, generating a non-negligible risk for transformation, especially in the context of vectors carrying very powerful enhancers as the globin vectors. Recently we generated helper-dependent HDAd5/35++ adenovirus vectors with an insert capacity of 30kb to express CRISPR/Cas9 for potential hematopoietic stem cells (HSCs) gene therapy of β-thalassemia and sickle cell disease through re-activation of fetal γ-globin expression. Transduction of human $CD34^+$ cells with CRISPR/Cas9 expressing vectors exerted toxicity to primitive HSCs, i.e. multi-lineage progenitor colony formation cells and NSG mice-engrafting cells. Controlling the duration of Cas9 expression could only partially abrogate toxicity. We therefore hypothesized that CRISPR/Cas9-mediated DNA double-strand breaks trigger intracellular signaling that negatively affects the survival of primitive HSCs. Furthermore, we postulated that base editing (without DNA breaks) using the CRISPR/Cas9-i platform would exert less toxicity. To test these hypotheses, we generated HDAd-CRISPR/Cas9 and HDAd-cytidine-editor vectors to disrupt the erythroid BCL11a enhancer. We transduced human and macaque $CD34^+$ cells *in vitro* at different MOIs and analyzed *i)* the efficacy of genome editing by T7E1 assay, *ii)* intracellular signaling

using proteome arrays for stress-induced and apoptosis pathways, *iii)* genome-wide RNA-Seq, *iv)* progenitor colony formation and erythroid differentiation, *v)* engraftment in NSG mice, and *vi)* HBF reactivation after erythroid differentiation *in vitro* and *in vivo*. Results will be reported.

603. Comparison of Genome Editing Outcomes In Vivo Using rAAV6 vs. ssODN Donor Template Delivery to Drive Homology-Directed Repair at the HBB Gene

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Sickle cell disease (SCD) is caused by a single nucleotide transversion in exon 1 of the *HBB* gene, resulting in a glutamic acid to valine substitution at the 6th amino acid (E6V). This change increases the hydrophobicity of the adult globin (β^A) and renders it susceptible to polymerization resulting in the characteristic sickling pattern of erythrocytes. Sickle patients have increased morbidity and a reduced life-span. HLA-matched allogeneic transplant from healthy donors can be curative, but is complicated due to limited donor availability, possibility of graft-versus-host disease (GvHD) and possible adverse effects following higher intensity myelo-ablative conditioning. Gene editing in autologous hematopoietic stem cells (HSCs) has the potential to circumvent some of these limitations. Designer nuclease cleavage in the presence of a donor template (e.g. recombinant adeno-associated virus (rAAV) or single-stranded donor oligonucleotides (ssODN)) leverages the cellular homology-directed repair (HDR) machinery to correct genetic mutations. In the absence of a donor template, a second cellular pathway that resolves double-strand breaks via error-prone non-homologous end joining (NHEJ) will be favored. For optimal clinical benefit in SCD, gene editing should yield a high ratio of precise homology-directed repair (HDR) events relative to on-target nuclease-driven gene disruption via NHEJ. In this study, we directly compared the efficiency of rAAV6 vs. ssODN donor templates to generate high HDR: NHEJ ratios when co-delivered with a Crispr/Cas9 ribonucleoprotein (RNP) targeting exon 1 of the *HBB* gene. Both the rAAV6 and ssODN donor templates were designed to introduce either a sickle mutation (GAG > GTC or GTG change, both encoding the E6V amino acid change) or a silent nucleotide change that does not alter the final protein sequence (GAA; encoding E6optE) in adult $CD34^+$ human mobilized peripheral blood cells (hPBSCs). We found that rAAV6 donor template delivery drives proportionately more HDR than NHEJ outcomes (37.5% HDR and 12.7% NHEJ) and ssODN donor template delivery introduces more NHEJ than HDR outcomes (11.9% HDR and 17.4% NHEJ) measured in erythroid cells at 14 days post-editing. Whether these alternative modes of template delivery differentially impacted the efficiency of editing engraftable HSC was then tested *in vivo*. The persistence and engraftment potential of edited cells introducing a GTC change (E6V) were measured longitudinally in the bone marrow of NBSGW mice at 3 weeks and 12-14 weeks. Interestingly, despite having a lower HDR: NHEJ ratio, mice that received ssODN-edited cells had a higher proportion of correctly edited cells (4.1%) in the bone marrow at 12-14 weeks, compared to cells edited with rAAV6 (0.66%). In parallel, as a functional assessment, sickle globin expression (β^S) was detected in the bone marrow of NBSGW recipient mice engrafted with ssODN edited cells and directly tracked with our molecular assessment of gene editing efficiencies. We conclude that, in this scenario where the nucleic acid change is small, ssODN donor delivery outperformed rAAV6 delivery by improving the engraftment potential of edited progenitor cells *in vivo* and through increasing the persistence of gene corrected stem cells in the bone marrow.

604. A Highly Efficient and GMP-Compliant Protocol to Manufacture CCR5-Edited Cells to Treat HIV Infection

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Targeted genome editing in blood and immune cells enable new therapeutic applications, especially for infectious diseases. We present a GMP-compliant protocol to manufacture *CCR5*-edited CD34+ hematopoietic stem and precursor cells (HSPCs) with the goal to cure patients suffering from chronic infection with human immunodeficiency virus type 1 (HIV1). We hypothesize that genetic disruption of the *CCR5* gene, which encodes the major HIV1 co-receptor, in HSPCs will give rise to an HIV-resistant immune system after transplantation. We have developed engineered nucleases based on transcription activator-like effector nucleases (TALENs) targeting *CCR5*. Electroporation of CD4+ T-cells and CD34+ HSPCs with mRNAs encoding TALENs revealed disruption of up to 80% of *CCR5* alleles in CD4+ T-cells and over 90% of alleles in HSPCs. The high gene editing frequencies in T-cells and HSPCs were confirmed by deep sequencing, and no cleavage activity above background levels were detected at the top 20 predicted off-target sites. *CCR5*-edited CD4+ cells preserved their proliferation capacity and their biological function. Importantly, these cells showed significantly reduced CCR5 expression and became resistant to infection with the R5-tropic HIV-$1_{JR\text{-}FL}$ virus. The *CCR5*-edited HSPCs maintained their proliferation potential and their capacity to differentiate into the various blood lineages *in vitro* and *in vivo*, and clonal analysis revealed bi-allelic *CCR5* disruption in more than 75% of cells. In summary, our developed protocol enables

highly efficient and GMP-compliant knockout of the *CCR5* locus in clinically relevant cells, so forming the foundation for a planned phase I/II clinical study.

605. Preferential Long-Term Engraftment of Hematopoietic Stem and Progenitor Cells with Low Numbers of Lentiviral Vector Integrations in the NSG Xenograft Model

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Many ex vivo gene therapies rely on the efficient genetic modification of hematopoietic stem and progenitor cells (HSPCs) and their subsequent ability to engraft and repopulate the hematopoietic system. The degree of gene modification in the cell product can be determined by vector copy number (VCN) analysis. However, while high VCNs ensure sufficient levels of transgene expression for therapeutic impact, it is widely assumed that increasing the average number of integration events per cell also increases the theoretical risk of insertional oncogenesis. This conclusion assumes that the ultra-high VCN clones within the cell product are capable of supporting long-term engraftment. To ask if VCN distributes independently of the cell phenotype, we stratified transduced HSPCs into low to high VCN cell populations by sorting. Briefly, we transduced mobilized peripheral blood-derived CD34+ cells with a lentiviral vector (LVV) encoding a GFP reporter and sorted cells based on GFP expression (GFP-negative, GFP-low, GFP-mid, and GFP-high). First, 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. Interestingly, we saw that colonies were increasingly erythroid biased with increasing GFP expression, a finding supported by CyTOF immunophenotyping of the sorted cell populations. Next, to determine if the degree of transduction was independent of stem cell phenotype, the sorted cell populations were transplanted into NSG mice at equivalent cell doses to evaluate the long-term engraftment potential of the different cell populations differing only in initial transduction level (GFP expression and VCN). Interestingly, VCNs were maintained from the sorted cell product to bone marrow VCNs after 4 months, confirming the near-clonal homogeneity of the populations attained post-sort. However, there were statistically significant differences between the engraftment of human cells from the GFP-negative groups (6.43 %hCD45) as compared to the GFP-mid (1.11 %hCD45) and GFP-high (0.02 %hCD45); the GFP-low group had reduced engraftment (2.47%hCD45) that was not statistically significant compared to the GFP-negative group. These results suggest that either (i) the subset of the HSPC population that is permissive to ultra-high levels of transduction is mutually exclusive with cells possessing long-term engraftment potential in an NSG mouse model; or (ii) that cells that have undergone the process of multiple productive vector integrations are rendered incapable of supporting long-term engraftment. Regardless of mechanism, the data suggest that clones with the highest VCNs are likely more lineage-committed cells that do not support long-term engraftment in the NSG mouse model.

606. Efficient Transgene Marking in Peripheral Blood Cells and Targeted Integration by HDAd5/35++ Vector-Mediated In Vivo HSC Transduction in AAVS1- Transgenic Mice

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A major task in the HSC gene therapy field is targeting transgene integration to pre-selected sites, so-called "Safe Harbor Loci" through homology-dependent repair (HDR) mechanisms. The adeno-associated virus integration site 1 (AAVS1) locus is one of these target sites. We evaluated helper-dependent HDAd5/35++ adenovirus vectors for targeted integration in AAVS1 transgenic mice (from Jackson Labs) after in vivo HSC transduction. HDAD5/35++ vectors use CD46, a receptor that is expressed on primitive HSCs. The first HDAd5/35++ vector expressed an AAVS1-specific CRISPR/Cas9 (HDAd-CRISPR). The HDAd5/35++-based donor vector contained either a GFP/mgmt or a γ-globin/mgmt transgene cassette flanked by AAVS1 homology regions and AAVS1-CRISPR cleavage sites (HDAd-donor). Target site-specific CRISPR/Cas9 cleavage and efficient donor cassette release were demonstrated after HDAd-CRISPR + HDAd-donor co-transduction of bone marrow lineage-negative cells from AAVS1/hCD46-transgenic mice. For in vivo HSC transduction of AAVS1/CD46-transgenic mice, HSCs were mobilized from the bone marrow into the peripheral blood stream by subcutaneous injections of G-CSF/AMD3100 and transduced in vivo by intravenously delivered HDAd-CRISPR+HDAd-donor vectors. After in vivo selection with three cycles of low-dose O^6BG/BCNU, on average 35% of PBMCs expressed GFP. We then tested another donor vector expressing human γ-globin, which can be used as a therapeutic gene for hemoglobinopathies. The γ-globin/mgmt cassette was flanked by longer AAVS1 homology regions than in the GFP donor vector. After in vivo HSC transduction/selection, we observed, on average, 70% γ-globin-positive peripheral red blood cells. Inverse PCR analysis on total bone marrow cells and progenitor colonies revealed predominant integration of the released donor cassette into the AAVS1 site through HDR. Low frequency non-HDR mediated integration was also seen in two of the AAVS1 CRISPR off-target sites. By targeted locus amplification and PCR, we found that AAVS1 transgenic mice have multiple repeats of the 8.2 kb AAVS1 locus inserted in tandem, with one repeat lacking a complete target homology region. This probably contributed to the observed off-target integration. The HDAd5/35++ vector system is a new tool for achieving targeted transgene integration in HSCs. Notably, HDAd5/35++ vectors have an insert capacity of 30kb which allows delivering long therapeutic genes and incorporating larger stretches of homology, which, as our data indicate, can mediate more efficient targeted integration. Additionally, our system may be further improved by controlling the duration of Cas9 expression using naturally existing anti-Cas9 peptides or self-inactivating mechanisms. We are currently testing these approaches.

607. High Levels of Transduction in CD34 Positive Cells and Toxicology Studies Using New Lentiviral Vectors for the Cure of Hemoglobinopathies

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Introduction: Lentiviral vectors (LV) have been demonstrated as a safety gene transfer tool and a variety of clinical trials are ongoing to cure patients affected by hemoglobinopathies. However, these studies suggest that current vectors require high number of integrations (~4) in a pancellular fashion. This could increase the risk of genome toxicity, limiting the application of these vectors and preventing their use in a reduced myeloablative regimen. While we address this issue elsewhere (ref Breda's abstract), aiming to indentify a vector that can express higher levels of beta globin chain, here we focus on perfecting transduction protocol and performing the toxicology studies to validate our approach. **Methods:** We prepared LV constructs carrying the GFPreporter gene or the β-globin gene under the control of phosphoglycerate kinase (PGK) promoter (LV-GFP) or internal β-globin promoter (ALS17), respectively. Frozen human hematopoietic stem and progenitor cells (HSPC) derived from bone marrow (BM) were used as target cells to study the transduction efficiency.The cells were exposed to LVs at a multiplicity of infection (MOI) of 10 and 100, respectively. For identification the best transduction protocol, HSPC were transduced with eight different enhancers (polybrene, PGE2, protamine sulfate, LentiBoost, Vectofusin-1, SR1, UM171 and CsH). The exposed LV was psuedotyped with different envelopes (VSVg, Cocal and Baboon). Transduction efficiency was calculated by GFP positivity using flow cytometry (7 days post-transduction) and vector copy number (VCN) using droplet digital polymerase chain reaction (ddPCR; 14 days post-transduction).For toxicology studies, we performed an in vitro immortalization assay (IVIM), an analysis of the clonal dynamics in NSG-mice transplanted BM cells, and vector integration sites analysis. **Results:** LentiBoost promoted highest LV-GFP transduction, up to 3.5-fold compared to the transduction without enhancers. Higher doses of LentiBoost further increased transduction, but were toxic to the cells. Cocal-pseudotyped LV-GFP transduced at highest efficiencies (1.3-fold) compared to VSVg-pseudotyped. Interestingly, Baboon-pseudotyped LV-GFP did not respond to LentiBoost. Long-length VSVg-pseudotyped ALS17 vector (total-length; 14.1 kb) reached VCN up to 4.2 copies/cell. Of note, these levels were close to those achieved with a vector similar to BB305 (total-length; 11.9 kb), which is currently used in the bluebird bio clinical trials (NCT01745120 and NCT02151526). To date, IVIM assay resulted in no immortalized line. An analysis of the clonal dynamics in NSG-mice transplanted BM cells and vector integration sites analysis is in progress. **Conclusions:** Our preliminary results confirm that this protocol allows efficient gene transfer of ALS17 into HSPC derived from bone marrow. This vector already showed promising results in achieving curative levels of β-globin production at 1-2 copies per cell (or even less) in HSPC derived from peripheral blood of patients affected by hemoglobinopathies. It is therefore fundamental to move forward with these studies to minimize the number of genomic integrations and reduce the chances of genome toxicity.

Cell Therapies II

608. In Vitro Treatments Reported to Improve the Engraftment of Muscle Precursor Cells in Mice Did Not Make a Difference in Non-Human Primates: Limitations of Cell Therapy Studies in Mice to Define Protocols for Humans

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Introduction. Cell therapy is a major strategy for the potential future treatment of disease of the skeletal muscle. Indeed, the protocol of cell therapy that we developed in non-human primates (NHPs) based on myoblast transplantation allowed dystrophin restoration with better efficacy than previously in patients with Duchenne muscular dystrophy, but would benefit of improvements to obtain a broader engraftment and dystrophin restoration. Studies in mice reported methods that substantially increased cell engraftment, based on *in vitro* treatments and co-injection of the cells with growth factors or other molecules. Since these methods would be easily applicable in the clinic, we wanted to verify them in NHPs, given that this animal model is crucial in preclinical research in several areas of medicine before translating protocols to humans. **Methods.** We allotransplanted muscle precursor cells (MPCs) labeled with ß-galactosidase (ß-Gal) in muscle regions of 1 cm^3 in cynomolgus monkeys. Tacrolimus was administered daily to control acute rejection. The strategies to improve the MPC engraftment were: in vitro treatment with or without co-injection of the cells with the following molecules: insulin growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), IGF-1 + bFGF + plasmin, concanavalin A and SB203580 (a p38 MAP kinase inhibitor that should conserve muscle cells in a condition similar to satellite cells). One of two cell-grafted regions was treated by electroporation to induce broad myofiber regeneration concomitant to the graft. Cell grafted regions were sampled one month after transplantation, snap frozen in liquid nitrogen and sectioned in a cryostat. Muscle cross-sections were stained with hematoxylin and eosin and for histochemical demonstration of ß-Gal. ß-Gal+ myofibers were quantified as an indication of engraftment success. **Results.** The only factor that substantially increased the number of ß-Gal+ myofibers was the extensive muscle regeneration induced by electroporation. Otherwise, for the same cell graft conditions (injection with or without electroporation) none of the cell treatments increased the amount of ß-Gal+ myofibers and therefore the success of the cell graft in terms of genetic complementation. **Conclusion.** *In vitro* cell treatments reported to increase the MPC engraftment in mice had no incidence in the cell engraftment in NHPs. The only strategy capable of increasing the cell engraftment in NHPs remained the induction of extensive muscle regeneration concomitant with the cell graft. As in other areas of preclinical research, this study also questions the clinical validity of results reported only in mice.

609. Dissolvable Alginate Particles Functionalized with αNKp46/αCD2 Successfully Activate and Expand NK Cells in G-Rex Plates

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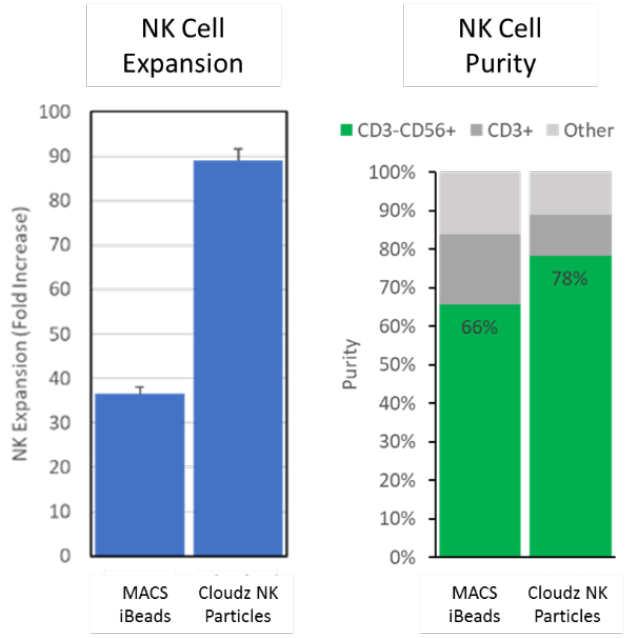
Introduction: Generating clinical scale doses of natural killer (NK) cells is currently a challenge for manufacturing CAR-NK cell therapies. Current expansion methods are restrictive due to high costs, scale-up challenges, and licensing restrictions for clinical and commercial applications. Cell activation technologies, cytokines, and easy-to-use bioreactor platforms are key parameters for successful activation and expansion of NK cells. We hypothesized that using a dissolvable phase-change hydrogel that is functionalized to promote NK cell activation may provide a more efficient and defined alternative for NK manufacturing processes. Additionally, we investigated cytokine combinations that optimize the expansion of NK cells from PBMCs in G-Rex® plates. **Methods:** Hydrogel microspheres (9 µm median diameter) - referred to as Cloudz™ particles - were functionalized with αNKp46 and αCD2 antibodies using standard bioconjugation techniques. In this study, human peripheral blood mononuclear cells (PBMCs) were cultured for 7 days in G-Rex® 24-well plates containing NK cell-functionalized Cloudz™ particles along with various cytokine combinations. Prior to expansion, PBMCs were analyzed for $CD3^-/CD56^+$ NK cells and $6x10^5$ NK cells were seeded into each well with a 2:1 ratio of Cloudz™ particles:NK cells. Miltenyi MACS iBeads were used as a control and were prepared according to the manufacturer instructions. During days 0-3, the cells were cultured with IL-2 (40 U/mL) along with combinations of IL-12, IL-18, and IL-21 (10 ng/mL each). After 3 days, the IL-2 concentration was increased to 200 U/mL, while the other cytokine concentrations remained constant. The cytokines and media were refreshed every 3 days with ½ media changes. After 7 days in culture the cells were analyzed by flow cytometry for $CD3^-/CD56^+$ NK cell expansion and purity. NK cell purity was defined as the percentage of $CD3^-CD56^+$ cells relative to the total population. **Results:** Cultures using NK cell-functionalized Cloudz™ particles combined with IL-2 resulted in a 69% NK cell purity and 29.9-fold expansion. In comparison, cultures using MACS iBeads combined with IL-2 resulted in 49% NK cell purity and a 6.8-fold expansion, suggesting that Cloudz™-based methods provide better expansion. When IL-12 was added to cultures with Cloudz™ particles, both the purity and expansion decreased (45% and 7.2-fold, respectively). When IL-12 and IL-18 were added, the expansion increased to 33.9-fold and the purity increased to 60%. The best combination was IL-2/IL-12/IL-18/IL-21 which had an 88.9-fold expansion with a 78% purity. IL-12 and IL-18 mimic the activating signals released by dendritic cells and IL-2 and IL-21 mimic the activating signals released by activated T cells. **Conclusion:** In summary, these data demonstrate that Cloudz™ particles functionalized with αNKp46 and αCD2 can efficiently expand NK cells with high purity in G-Rex® cell culture plates in specific cytokine combinations. In future studies, we will determine if NK cells can be expanded in larger G-Rex culture vessels to manufacture NK cells to reach desired cell therapy levels. Additionally, we will also further explore the possibilities of this novel system for rapid expansion and recovery of specific NK cell subtypes that exhibit different cancer cell killing abilities.



610. Utilising Non-Viral Episomal S/MAR DNA Vectors for the Persistent Genetic Modification of hiPSCs

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The use of patient-derived induced pluripotent stem cells (iPSCs) is a promising approach for a variety of cell therapies. They combine the advantages of both, a decreased immune reaction due to the derivation from the individual patient, and the characteristics of stem cells with their self-renewing capacity and competence to differentiate into all kinds of cell types. When aiming to apply these cells for cell therapy, there is often the need to genetically alter or repair these cells before they can be reintroduced to the patient. Modification of cells in this context is mainly done using integrating vectors. While being highly efficient at modifying cells, they harbour the risk of integration-mediated genotoxicity, which requires clinical monitoring of the patient for decades after the treatment.Our lab has developed an alternative DNA vector system based on a Scaffold Matrix Attachment Region (S/MAR) and a minimally sized-antibiotic free Nanoplasmid™ as a novel technology to persistently genetically modify human cells without causing any molecular or genetic damage. With this novel vector platform, we now have a powerful tool which offers the functionality of viral vectors while providing advantages such as easy and economical production, no limitation in transgene size and minimal impact on the host cell. We have previously shown that S/MAR vectors can provide sustained transgene expression while remaining episomal and have utilised them to genetically modify a variety of cell types. We also demonstrated that we can genetically modify fibroblasts, reprogram

them to hiPSCs and randomly differentiate them while maintaining transgene expression throughout reprogramming and differentiation. However, when working with transgenes which are involved in these processes, it will almost certainly be necessary to modify the cells directly at the stem cell stage. Human induced pluripotent stem cells (hiPSCs) are typically refractory to transfection due to the stringent control mechanisms that their nature as progenitor cells entails. Here, we show for the first time that we can efficiently deliver our S/MAR DNA nanovectors to hiPSCs and generate stable cell lines without altering their stem cell properties. These stable genetically modified hiPSCs maintain transgene expression throughout expansion, passaging and freeze-thawing cycles. An area of research our group has an interest in is the development of a gene or cell therapy for inherited kidney cancer. In place of fibroblasts for generating iPSCs, we are also utilising a novel source of cells by isolating exfoliated renal epithelial cells from urine samples. Based on the reported epigenetic memory of iPSCs, which leads to favoured respective origin cell lineage redifferentiation, iPSCs established from renal epithelial cells might be more suitable to direct along the mesoderm lineage into kidney progenitor cells than iPSCs derived from fibroblasts. Furthermore, urine samples provide a continuous, non-invasive source of cells. We show that, as with fibroblasts, we are now able to isolate and culture cells derived from urine, effectively reprogram them into hiPSCs, maintain them in a feeder dependent or independent culture system and establish stable modified iPSC lines using S/MAR DNA vectors. Finally, we have differentiated these modified hiPSCs from different sources into kidney progenitors and demonstrate the sustained transgene expression throughout and without hindering this process. Together, this work shows for the first time that our improved S/MAR DNA vector platform can be applied to modify hiPSCs from different sources without altering their characteristics persistently and widens the opportunities for a range of cell therapy approaches.

611. Fat Derived Stromal Cells Can Act as Useful Biomarker to Monitor Metabolism and Cell Differentiation, Post Exercise

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Diabetes will very soon become a global epidemic. Aerobic exercise is known to show positive effects on cardiovascular health in diabetes and pre-diabetes subjects. We investigated if fat derived MSC can act as useful biomarkers in exercise. We also invested if endothelial conditioned media can affect MSC differentiation in adipogenic environment. **Methods:** For the in-vivo study, five prediabetic subjects, were enrolled in a 12-week exercise program. Stromal cells were obtained from subcutaneous abdominal fat pre and post exercise and cultured for 2-3 weeks. Stromal cells were then analyzed for gene expression. For the in vitro study, commercially obtained human fat-derived stromal cells were exposed to adipogenic media with endothelial conditioned media (obtained from mature endothelial cells, HUVECs). MSC differentiation was examined by RT-PCR. **Results:** mRNA expression of patient's fat-derived stromal cells showed an upregulation of glucose transporter GLUT1, GLUT4 (3.6, 1.14 folds). Gene expression of most of the antioxidants such as SOD2, SOD3, GPX1, GPX3 and CAT (1.5, 1.7. 1.16, 1.6 and 1.6 fold respectively) increased post exercise with a statistically significant upregulation of SOD1 (p= 0.04, 1.8 fold). An increase in bone formation marker osteocalcin, BMP6, SMAD3 and TGF-β (1.3, 2.6, 2, 3.1 fold respectively) were noted, post exercise.. Exercise increased METs significantly (p=0.04, 12%) while reduced body weight, systolic, diastolic blood pressure, HbA1c, cholesterol by 4.2%,9%, 9%, 10%, 9.8 % respectively (p=0.04, 0.04, 0.05, 0.001, 0.0003 respectively) indicating this exercise regimen in our study was effective in improving both biophysical and biochemical parameters When we analyzed in-vitro data, we noted a reduced expression of markers for adipogenic differentiation such as PL1N, CEBPA, and PPARG (40.3, 14.8, 5.2 fold, p= 0.002, 0.00003, 0.00001, 0.00002 respectively). We also observed increased expression of bone formation markers RUNX2 (1.9, fold, p=0.05), and angiogenesis markers NOS3, VEGF and KDR (1.6 and 1.4, 2.8 fold respectively). **Conclusion:** Addition of endothelium-derived factors in vitro and start of exercise in vivo, both appear to augment bone formation markers in fat derived MSCs indicating a cross-talk between endothelium, fat differentiation, and bone formation, post-exercise. We conclude that fat derived MSC (subcutaneous fat biopsy) can act as important biomarkers to study metabolic changes and changes in mesenchymal stromal cell differentiation, post exercise.

612. Abstract Withdrawn

613. Expansion of Human Cardiac Progenitor Cells in Physiological Oxygen Promotes Cell Proliferation and an Endothelial Cell Phenotype

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Human cardiac progenitor cells (CPCs) are actively being used in the clinic to treat patients affected by cardiac disease. Despite current controversy and ambiguity regarding the role of CPCs in the heart, preclinical and clinical trials that have tested efficacy of exogenously delivered CPCs in a variety of models have produced encouraging results. Numerous intrinsic and environmental stresses diminish cellular therapeutic potential. Specifically, oxygen concentration has been identified as a modulator of CPC proliferation and migration, where physiological oxygen concentration has been identified to be optimal for culture expansion. Here, we tested the hypothesis that CPC product potency would be enhanced when manufactured under physiological oxygen. Neonatal cardiac biopsies were obtained from 2 donors undergoing routine heart surgery at the University of Miami Holtz clinic and transferred to Interdisciplinary Stem Cell Institute for processing under an approved IRB protocol. The biopsies were digested and plated for expansion under 21% or 5% O2. After 1 passage, expanded cells were subjected to magnetic selection by anti-CD117 beads. Positively selected cells were further expanded and analyzed for passage 2 and 3. Cell characterization was completed by flow cytometry analysis, PCR gene expression, and matrigel angiogenesis assay. Cell growth analysis re-confirmed the finding that physiological oxygen is optimal for the expansion of CPCs. Doubling time was found to be reduced in both products (34.7 hours in 5% O2 vs 42.0 hours in 21% O2). Importantly, PCR analysis of CD117 confirmed the enrichment

of CD117 expressing cells, as KIT RNA expression was found to be increased immediately post sorting (average of 20.1-Fold in 5% O2 and 7.29-Fold in 21% O2). Interestingly, KIT expression decreased after expansion in 5% O2 (-11.70-Fold) compared to 21% (1.00-Fold). Surface protein analysis by flow cytometry revealed greater expression of endothelial lineage markers CD31 CD106 in 5% than 21% O2 after 2 and 3 passages. Furthermore, preliminary results of the angiogenic potency of final product revealed an increased angiogenesis potential of sorted cells versus unsorted cells, however no difference between 21% O2 and 5% O2 cultured cells. Together, these preliminary results suggest that although expansion of CPCs in 5% O2 is optimal for cell proliferation, the decrease in KIT RNA an increase in endothelial lineage markers is indicative of a push towards expansion of an endothelial phenotype. Continuing studies will dive deeper into the therapeutic potency of these two populations to determine which condition is optimal for regenerative repair.

614. CRISPR-Based Activation of Adipose Stem Cell Endogenous Gene Expression for Peripheral Nerve Regeneration

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Peripheral nerve regeneration is a complex and delicate process that requires coordinated functions of neurotrophic factors and neuronal cells. CRISPR activation (CRISPRa) has been adapted as a powerful tool with the potential for multiplexed endogenous gene expression. Here we developed a hybrid baculovirus (BV) vector to express a CRISPRa system that enables simultaneous activation of multiple genes. We demonstrated that the expression of CRISPRa system in rat adipose-derived stem cells (ASC) enabled robust activation of neurogenesis-related genes (e.g. BDNF, GDNF and NGF). The CRISPRa-mediated enhanced expression of neurotrophic factors stimulated the migration of Schwann cell and neurite extension in vitro. Importantly, implantation of the hybrid BV-engineered ASCs into sciatic nerve transection site in rats significantly improved the nerve regeneration as judged from the enhanced functional recovery, integrity of nerve conduction, electrophysiological functionality, axon regeneration and remyelination. These data demonstrate that the baculovirus-delivered CRISPRa system can activate endogenous gene expression and enable functionalization of rat ASC for peripheral nerve regeneration.

615. Moving towards a Closed CAR-T Cell Manufacturing Process

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The field of cancer immunotherapy has seen a surge of activity in recent years stemming from the success of checkpoint inhibitors and recent marketing approvals of CAR-T therapies for the treatment of hematologic malignancies. Despite the excitement and clinical success of these CAR-T treatments, challenges remain with regards to manufacturing, including lengthy production times (11-21 days), product variability associated with open handling steps, and high manufacturing costs. We addressed these issues by evaluating individual CAR-T cell unit operations, commercial reagents, and equipment with process closure potential to develop an improved workflow and increase product consistency. Here, we present our workflow for CAR-T cell manufacturing: Thawed apheresis units were processed using the Sepax™ C-Pro and T-cells were isolated with EasySep™ Release CD3 Positive Selection Kit. Isolated T cells were seeded in shake flasks, activated with ImmunoCult CD3/CD28/CD2 T cell activators, and transduced with an eGFP-encoding lentiviral vector 24 hours post-activation using the Sepax™ C-Pro. Transduced T cells were expanded in the Xuri™ Cell Expansion System W25, where a perfusion feed strategy allowed for greater than 1.0E10 expanded T cells with upwards of 80% eGFP transduction efficiency across an 8-day manufacturing process. The Sefia™ Cell Processing System and VIA Freeze™ platforms were then used for downstream processing and cryopreservation, respectively. Our innovative use of shake flasks for initial T-cell expansion can be performed in a closed manner and reduces manual manipulation times by greater than 50%. Collectively, this semi-automated CAR-T cell manufacturing process offers a readily adoptable platform for manufacturing of autologous immunotherapies to meet the growing demand for patient treatment.

616. Development and Preclinical Characterization of a Solid Oral Formulation of a Synthetic Biotic Medicine for the Treatment of PKU

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Phenylketonuria (PKU) is an inherited metabolic disorder resulting from the inability of the liver to break down Phenylalanine (Phe) leading to the disregulation of metabolites in the brain and neurotoxicity. To develop new alternative approaches to a strict low-protein diet we have designed a genetically engineered strain ofthe probiotic/non-pathogenic bacterial strain *Escherichia coli* Nissle that can metabolize Phe in the mammalian gut. The engineered stain, SYNB1618, has been designed to covert Phe into trans-cinnamate (TCA) via the phenylalanine ammonia lysase (PAL) enzyme or to phenylpyruvate (PP) via the L-amino acid deaminase (LAAD) enzyme. Previously we have demonstrated that oral dosing of SYNB1618 can mediate dose dependent decreases in circulating Phe levels and increases in TCA (or its downstream metabolite, Hippurate, HA) in animal models (Isabella et al. Nature Biotechnology 2018), and in healthy human volunteers. Initial studies used a liquid cell suspension of SYNB1618, however, a solid dosage form is desirable to improve stability, storage, and ease of use for patients. To that end, we have developed a process

for lyophilization of SYNB1618 cells which does not impact biologic activity or cell morphology when viewed by electron microscopy. Lyophilized cells retain the ability to consume Phe and produce TCA or PP *in vitro* and produce TCA in an *in vitro*, intestinal simulation model. Furthermore, an equivalent live cell dose of lyophilized SYNB1618 demonstrated similar urinary levels of HA relative to the liquid cell suspension in both a mouse model of PKU and in healthy non-human primates. We conclude that lyophilization of SYNB1618 does not result in significant loss of activity and there is a clear path forward for a solid oral formulation in the development of SYNB1618 for the potential treatment of PKU.

617. Cellular Reprogramming of Human T Cells Using Non-Animal Origin Conditions Followed by 3D Expansion of T Cell-Derived iPSCs

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Induced pluripotent stem cells (iPSCs) are derived from somatic cells that have been reprogrammed into an embryonic-like pluripotent state. These iPSCs can ultimately be differentiated into any cell type in the human body, thereby providing an unlimited source of differentiated cells which have the potential to be used in disease modeling, pharmacological drug screening, regenerative medicine and cell-based therapy. iPSCs can be utilized clinically for treating cancers and viral infections by using them as a source for producing T lymphocytes (T cells) and natural killer (NK) cells. Recent studies have reported that antigen-specific iPSCs can be generated by reprogramming CD8+T cells (T-iPSCs) isolated from cancer patients. These antigen-specific T-iPSCs can be differentiated and further matured into functional cytotoxic T lymphocytes (CTLs) which are highly proliferative. These ″rejuvenated″ T cells retain the same T cell receptors (TCRs) pattern as the original T cell population from where the T-iPSC clone was derived and thus kill the cells expressing those particular antigens (antigenic memory). T-iPSCs can also be genetically engineered to express chimeric antigen receptors (CARs) and then differentiated into T cells that express both the CARs and an endogenous TCRs (CAR-Ts) thus making them more potent in specifically targeting and killing cancer and/or virally infected cells. Antigen-specific T cells and CAR-Ts thus have a therapeutic potential to treat a variety of disease conditions. Recently, several Immunotherapy-based approaches to treat cancers have been approved by FDA and many are in the approval process indicating the potential of T cell-based therapy. Here, in this report, we demonstrated that human T cells can be reprogrammed into iPSCs (T-iPSCs) using non-viral and non-animal origin (NAO) components. We further characterized these T-iPSCs and show that T-iPSCs are positive for alkaline phosphatase staining and pluripotency markers (Oct4, Nanog, SSEA4,Tra1-81 and Tra-1-60) by immunofluorescence (IF) staining and flow cytometry.We further validated that T-iPSCs are vector free and maintain normal Karyotype. STR analysis verified that T-iPSCs are indeed derived from the parental T cells. Furthermore, T-iPSCs have the potential to differentiate into cells of the three germ layers. Additionally, we were able to successfully expand the T-iPSCs in 3D suspension culture using NAO cell culture system. These 3D expanded T-iPSCs could be potentially differentiated into CTLs, NK cells etc. in 3D suspension culture providing end-to-end solution for large volume T cell expansion and be further used for either autologous or allogeneic clinical applications To summarize, human T cells were successfully reprogrammed into iPSCs using non-viral vectors, and utilizing NAO cell culture system. iPSC lines were further characterized and were shown to present hESC characteristrics of self-renewal and pluripotency, along with normal karyotype. Finally, we demonstrated high fold expansion of T-iPSCs in a suspension culture to produce large quantities of high quality of T-iPSCs needed in clinical therapies.

618. Activation of DOR on Human Mesenchymal Stem Cells Promotes Cardiomyocyte Differentiation, Angiogenesis and Anti-Inflammation via the DOR/NOTCH1/HES1 Signaling Cascade

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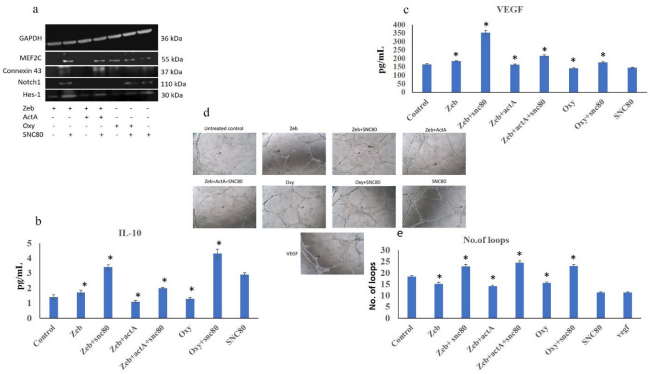
Aim: Ischemic myocardium or myocardial infraction is the leading cause of mortality worldwide. Utilization of mesenchymal stem cells (MSCs) in cardiac tissue regeneration is hindered due to their inefficient invivo differentiation, lack of angiogenic trigger and presence of excessive inflammatory damage. In the present study we demonstrated the enhanced differentiation, angiogenesis and anti-inflammatory effect of the delta (δ)-opioid receptor (DOR) agonist SNC80 on human MSCs, in combination with known cardiomyocyte inducers like zebularine (Zeb), activin-A (Act A) and oxytocin (Oxy). **Main methods:** Human wharton jelly derived MSCs (Hu-MSCs) were either treated with Zeb (10nM), Act-A (10nM) and Oxy (100nM) alone or with combination of SNC-80 (1μM) for 21 days. Cardiomyocyte specific markers and pathways were studied using immunoblot and PCR. The spent media collected from the above-mentioned conditions were used to study its angiogenic (invitro human endothelial tube formation) and anti-inflammatory effect (ELISA). **Key findings:** SNC-80 treatment (DOR activator) in combination with known cardiomyocyte differentiation agents showed enhanced expression of cardiomyocyte markers (MEF2c, Connexin 43) expression (Figure 1a). The expression of pro-angiogenic protein (VEGF) was also significantly increased upon DOR activation with SNC80 as follows: Zeb vs Zeb+SNC80 (183.5pg/mL vs 354pg/mL), Zeb+ActA vs Zeb+ActA+SNC80 (164 pg/mL vs 216.6 pg/mL), Oxy vs Oxy+SNC80 (142.6 vs 177.3 pg/mL) as confirmed by ELISA (Figure 1c). Also, DOR activated conditioned medium increased the invitro endothelial tube formation in terms of number of loops (Figure 1d and 1e). In addition, SNC-80 treated Hu-MSCs significantly increased the release of anti-inflammatory cytokine (IL10) as follows: Zeb vs Zeb+SNC80 (1.7 pg/mL vs 3.4 pg/mL), Zeb+ActA vs Zeb+ActA+SNC80 (1.1 pg/mL vs 2 pg/mL), Oxy vs Oxy+SNC80 (1.3 pg/mL vs 4.3 pg/mL) as measured by ELISA (Figure 1b). Interestingly NOTCH1 and its downstream effector molecule Hes1 were found to be upregulated with the activation of DOR in combination with zebularine, oxytocin (Figure 1a). **Significance:** Activation of DOR in combination with already reported cardiomyocyte inducers could enhance the differentiation of Hu-MSCs, its pro-angiogenic property and production of anti-inflammatory cytokines. Warranting further

investigation, the DOR signaling pathway could have potential therapeutic benefit for ischemic myocardial tissue regeneration.
Figure 1: a. Immunoblot analysis for cardiomyocyte markers- MEF2C, Connexin 43 and molecular signaling markers-Notch1, Hes1 in the different treatment conditions. b. Anti-inflammatory cytokine IL10 as measured by ELISA from spent media of the different treatment conditions (*p < 0.05 compared to the respective controls). c. Pro-angiogenic VEGF cytokine levels as measured by ELISA from spent media of the different treatment conditions (*p < 0.05 compared to the respective controls). d. Representative images of invitro tube formation on Matrigel® in respective treatment conditions. e. Quantification of number of loops formed from d. (*p < 0.05 compared to the respective controls). All experiments were done in triplicates.



619. Inhibition of Interferon Signaling Does Not Enhance Lentiviral Vector Transduction of Activated Primary Human T Cells

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Lentiviral vector (LV) is commonly used to manufacture T cell based therapeutic products such as chimeric antigen receptor (CAR) T cells. Since LV transduction of resting human T cells is inefficient, T cells are activated prior to transduction. T cell activation (TCA) results into expression of many genes required for T cell proliferation, and effector function including those with broad antiviral activity such as type-I and type-II interferons (IFNs). Although transduction efficiency is higher in activated T cells compared to resting T cells, a high multiplicity of infection (MOI) is required to achieve adequate transgene expression. We hypothesized that expression of IFNs and subsequent IFN-signaling during TCA reduces the efficiency of LV transduction of activated T cells. Ruxolitinib, an FDA-approved inhibitor of JAK1/2 tyrosine kinases was used to inhibit type-I and type-II IFN signaling and GFP encoding LV was used to assess the efficiency of transduction. We found that Ruxolitinib at low concentrations [1-250nM] did not affect T cell receptor (TCR) mediated TCA and did not enhance LV transduction; however, at high concentrations [500-1000nM] inhibited TCA and reduced LV transduction. Ruxolitinib at high concentrations did not affect PMA/Ionomycin induced TCA and did not enhance LV transduction. Furthermore, exogenous addition of IFN-alpha (10^3 units/ml) did not significantly reduce LV transduction of TCR-activated T cells. Together, these data suggest that inhibition of IFN-signaling during TCA does not enhance LV transduction of activated human T cells. Since exogenous addition of IFN-alpha did not significantly reduce LV transduction, we conclude that IFN-signaling does not play a major role in limiting LV transduction of activated primary human T cells.

620. Evidence of Improved Transduction Efficiency and Preservation of Relevant T Cell Subpopulations through Novel Media Formulations

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Adoptive immunotherapy involves a process in which patient T cells are expanded in optimal conditions for the purpose of re-infusing their progeny as therapy. Replacing components of animal origin within the cell culture medium with human or chemically defined components that conserve the bioactivity and efficacy of T cells has been challenging. One of the main roadblocks with this effort is that several key parameters, such as proliferation and functionality, are often diminished possibly due to the lack of physiologically relevant protein sources in concentrations suitable for biological activity. Therefore, there is an important need for the generation and characterization of novel media supplements and formulations to boost the progress of the cell therapy market. Physiologix™ XF Human Growth Factor Concentrate (hGFC) is a cGMP, xeno-free media supplement made for stem cells and T cells that replaces standard serum supplements such as fetal bovine serum or human serum. In this study, conventional T cell manufacturing basal media such as OpTmizer and X-VIVO 15 were supplemented with either 5% human serum or 2% Physiologix™ XF. In addition, RPMI 1640 with 10% FBS was compared due to its prevalent use in non-clinical settings. Bulk T cells from three healthy donors were activated with CD3/CD28 Dynabeads before being cultured for up to 14 days in varying media formulations. Proliferative capacity was assessed using bead-based flow cytometry. We show that Physiologix™ XF demonstrates increased potency relative to human serum when added to X-VIVO 15 or OpTmizer media. Differentiation status was assessed using well established markers to distinguish naïve-like, central memory and effector T cells within the CD4 and CD8 T cell compartment. We detected an increase in naïve and central memory populations when cells were cultured in 2% Physiologix™ XF compared to human serum across all tested basal media. These populations are highly relevant for the outcome of patients undergoing CAR-T cell therapy. In order to further define the potential advantage of our supplement in generating these populations, we compared the transduction efficiency of a lentiviral-GFP in various media (Optimizer, X-VIVO 15 or RPMI) supplemented with 2% Physiologix™ XF compared to 5% human serum at different multiplicities of infection (MOIs). The higher transduction efficiency that was observed when using 2% Physiologix™ XF at a relatively high MOI of 4 was conserved across all tested ratios including a MOI of less than one. These differences were observed with both OpTmizer and X-VIVO 15. These data indicate that Physiologix™ XF preserves less

differentiated T cell subpopulations, even when used at a lower final percentage. Physiologix™ XF also maximizes transduction efficiency even at a low MOI, thus increasing the efficiency of vector usage. This increased efficiency can translate into a higher number of transfusable CAR+ cells with relevant phenotypes which would allow for reduced overall costs and better clinical outcomes. Future work will examine the ability of anti-CD19 CAR-T cells which were expanded in medium supplemented with Physiologix™ XF to control tumor burden in an aggressive leukemia xenograft model.

621. Expansion of Tregs from CD4⁺ Cells Using Dissolvable Hydrogel Particles Functionalized with CD3/CD28 Antibodies

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Introduction: Cost of goods concerns are driving increased focus on improving the efficiency of manufacturing unit operations for regulatory T (Treg) cell therapies. Treg cell manufacturing workflows frequently employ feeder cells or antibody-coated magnetic particles, which must be removed from the final cell product. This removal adds additional workflow steps and increases manufacturing costs. In this study, we used a dissolvable phase-change hydrogel to develop a technology that potently activates and expands Treg cells and eliminates magnetic particles from the manufacturing process. **Methods:** Ionotropic hydrogel microspheres of 9 µm median diameter were prepared. In the presence of a chelating agent these microspheres rapidly depolymerize and enter liquid phase. To provide co-stimulatory activation signals, the hydrogel microspheres were functionalized using two different ratios of humanized anti-CD3/CD28 antibodies to generate Cloudz™ Treg Expander 1 and Cloudz™ Treg Expander 2. Dynabeads™ Treg Expander was used for comparison, according to the manufacturer's instructions. Human CD4⁺ T cells were seeded (Day 0) at 0.5x10⁶ cells/well in 24 well plates (n = 3) in complete RPMI supplemented with 10% Fetal Bovine serum and Recombinant Human IL-2. Cloudz™ Treg Expander 1 or Cloudz™ Treg Expander 2 was added (25 µL per 0.5x10⁶ cells) in a single stimulation. Media addition was performed every 2-3 days. Following expansion, flow cytometry was used to assess expression levels of CD4, CD25, CD127 and FoxP3. **Results:** Addition of Cloudz™ Treg Expander 1 induced expansion of the CD4⁺ cells similar to the Dynabeads Treg Expander (Population Doublings: 6.8 ± 0.3 and 6.9 ± 0.3, respectively). Cloudz™ Treg Expander 2 had a slower growth than the other two conditions (Figure 1). After 9 days, it was hypothesized that Treg expanders would increase the cell population expressing the phenotypic Treg markers, CD4⁺/CD25⁺/CD127⁻. Compared to the starting population, all three expanders induced expansion of CD4⁺/CD25⁺/CD127⁻. However, both Cloudz™ Treg Expanders had a higher percentage of CD4⁺/CD25⁺/CD127⁻ cells (94% and 97%, respectively) compared to the Dynabeads Treg Expander (76%) (Figure 2). The percentage of CD4⁺/CD25⁺/FoxP3⁺ cells in the expanded cells also showed similar trends. **Conclusions:** This study demonstrates the utility of Cloudz™ Treg Expanders (particles functionalized with CD3/CD28 antibodies) to potently activate and expand Treg cells from CD4⁺ cells. No magnetic beads are present, thus simplifying activation, expansion, and harvesting workflows within cell therapy manufacturing operations. In the future, we will improve methods to increase yields of specific Treg phenotypes, assess the functionality of expanded Tregs using suppression assays., and develop GMP-grade manufacturing of the Cloudz™ Treg Expander for use in clinical cell therapy manufacturing.

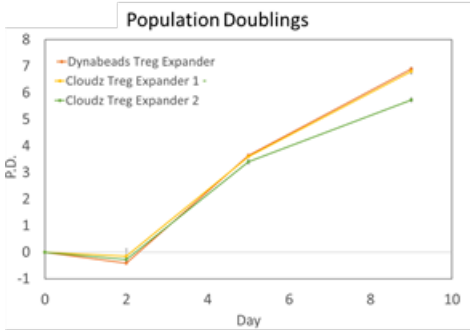

Figure 1: CD4⁺ T cells were cultured for 9 days in 24-well plates using a variety of Treg Expanders. The growth was monitored, and population doublings were calculated for each sample.

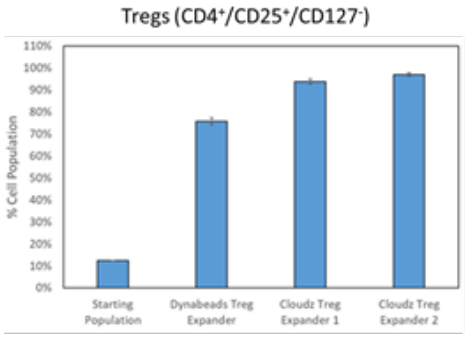

Figure 2: The percentage of CD4⁺/CD25⁺/CD 127⁻ Tregs was calculated after 9 days of expansion and compared to the starting population.

622. Increase Inflammatory Cytokines Expression of Mesenchymal Stem Cells Expanded in Platelet Lysate Pre- and Post-Cryopreservation

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Traditional production of MSCs involves the addition of fetal bovine serum (FBS) into cell culture media as a growth stimulant. Although proven to be safe, investigators have begun to use human derived platelet lysate (hPLT), an effective alternative to FBS, as a nutrient source to avoid the use of animal products. The main limiting factors of using FBS are the lot variation, cost, and availability. FBS is commonly used as a growth stimulant for Mesenchymal stem cell (MSC) expansion in culture. Although FBS or calf serum from USDA approved animals with well-monitor health status has been proven to be safe as a growth stimulant, but the use of xenogeneic serum always retain the risk of contamination with non-human pathogens and inducing an unwanted immune response. A xeno-free growth medium that has similar growth stimulation is always desired. We hypothesize that hPLT would induce MSC growth but would not change any phenotype comparing to the MSCs using FBS. Our preliminary results show that hPLT significantly reduced the cell doubling time over 3 passages, while maintaining high viability and MSC cell surface protein identifiers (CD105⁺, CD90⁺, CD45⁻). The inflammatory cytokine profiles of conditioned media collected

from pre and post- cryopreserved MSCs grown in FBS or hPLT had significant differences. In thawed, post-cryopreserved hPLT MSCs, expression of IL-6, IL-8, RANTES, MCP-3, and angiogenin were significantly elevated. The pre- and post- cryopreservation comparison is an important factor to consider as clinical products are commonly freshly thawed cells. The influence of elevated pro-inflammatory cytokines in the freshly-thawed, post cryopreserved hPLT products on anti-fibrotic and anti-inflammatory properties of MSCs is unknown. Although, cytokine changes were found to be most dramatic in the post-cryopreserved cells, elevation of RANTES and MCP-1 in pre-cryopreserved cells was evident. MSC therapeutic response is dependent upon micro-environmental stimuli and cues. A more proliferative MSCs may not have the same cytokine profile as a less proliferative MSCs because the relationship between MSC proliferation and cytokine secretion profile has not been studied. Thus, the cytokine profile immediately after thaw should be examined more in detail.

623. Development and Validation of a GMP Process to Generate Retinal Pigment Epithelial Cells (RPE) Derived from Autologous Induced Pluripotent Stem Cells (iPSCs)

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Age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly and no cure exists for the "dry" or atrophic form to date. We have developed a GMP compliant manufacturing process to differentiate iPSC into RPE cells to be used in a phase I clinical trial for patients with AMD. In our process, iPSC derived from patient CD34 cells are used as starting material. iPSC are carefully differentiated using a defined cocktail of cytokines and reagents and are assessed for the loss of iPSC-specific markers and the gain of RPE-specific markers outlined here. After RPE differentiation and maturation, cells are seeded onto a PLGA scaffold-snapwell plate and cultured for an additional month. The scaffold containing RPE cells is transplanted into retinal surface of the patients' eye. The validated manufacturing process outlined herein utilizes an open manufacturing system with quality measurements and criteria built in at multiple stages. We predict that this phase I clinical trial will be open and enrolling patients in first quarter of 2019.

624. Large-Scale Expansion and Characterization of Human CD3+ T Cells in an Automated Functionally-Closed Hollow Fiber Bioreactor System

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Background: The rapid evolution of cell-based immunotherapies such as chimeric antigen receptor (CAR) T-cells for treatment of hematological cancers has prompted the need for a platform to expand these cells ex vivo in a safe, efficient, and reproducible manner. In the Quantum® Cell Expansion System (Quantum system), we evaluated a method to expand T-cells from healthy donors in a functionally-closed environment, thus reducing time and resources needed to produce a therapeutic dose. The Quantum system's hollow-fiber bioreactor dual loop fluidics design, consisting of an intracapillary (IC) loop and an extracapillary (EC) loop, together with a semi-permeable membrane, creates an environment that permits continuous perfusion of new medium and cytokines, while simultaneously removing waste metabolites and replenishing dissolved oxygen in a highly efficient manner. **Methods:** Mononuclear cells (PBMCs) from leukapheresis products from 5 healthy donors were activated with anti-CD3/CD28 Dynabeads®, loaded into the Quantum system IC loop and expanded for 8-9 days using serum-free, defined medium and IL-2. Harvested cells were phenotyped by flow cytometry and evaluated for cytokine secretion by multiplex assays. **Results:** From starting products of 30 or 85×10^6 PBMCs, $CD3^+$ T-cell populations expanded up to 1800-fold following stimulation and provided yields up to 28×10^9 cells within 8 days, reaching a maximum cell concentration during culture of 225×10^6 cells/mL. T-cell expansion yields from all donors were similar with respect to starting product (30 or 85×10^6 PBMCs), viability (mean 93%, ~2% CV) and doubling times (mean 20 h, ~4% CV). Functionality (as measured by secretion of IFN-γ, IL-2 and TNF-α) was retained in harvested T-cells upon restimulation in vitro and T-cells displayed predominantly less-differentiated phenotypes of naïve ($CD45RA^+$, $CCR7^+$) and central memory ($CD45RO^+$, $CCR7^+$) T-cells, with low expression of exhaustion markers LAG-3 and PD-1. In addition, the Quantum system's dual loop design for nutrient supply reduced the amount of IL-2 required. Although a total of 20 L medium (3.6 L IC medium, 16.4 L EC medium) was used to culture an average of 25×10^9 cells, IL-2 was added only to the IC medium, thus reducing the amount of IL-2 required for culturing by 82%. **Discussion:** The Quantum system has been successfully used to produce functional T-cells at clinical dosing scale and within a short timeframe. T-cells generated were of less-differentiated phenotypes, which have been shown to be important for durable therapeutic effect. Savings in growth factor use were also demonstrated and may be of importance particularly for cells requiring very high amounts of growth factors, e.g. expansion of tumor infiltrating lymphocytes requires 3000 U/mL IL-2. Expansion occurred from input cell numbers as low as 7×10^6 $CD3^+$ T-cells from a total of 30×10^6 PBMCs. This is an important consideration for expansion of T-cells from patients, particularly pediatric patients, who may have low numbers of circulating $CD3^+$ T-cells and for whom apheresis blood collection may be restricted or challenging. Though the T-cell yields reported here for the Quantum system are higher than current autologous CAR T-cell dose ranges, the Quantum system potentially reduces the culture time required for preparation of autologous doses. The cell expansion rate the system supports would allow expedient treatment of patients with rapidly-advancing disease. Future directions for this platform include evaluation for expansion of other types of suspension cells, in particular for gene-modified T-cells.

625. High-Dimensional Proteomic Characterization of Immune Cell Phenotypes with Data-Independent Acquisition Mass Spectrometry

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Background Recent success with cell-based therapies has demonstrated their utility for control of multiple cancers. These successes have also spurred interest in characterizing immune cell subpopulations to understand mechanisms of activation and suppression, and ultimately their relationship to therapeutic response. Currently, antibody-based approaches are commonly used to characterize immune cells, however these methods are limited to 30-40 markers and are driven by previous hypotheses, limiting new discovery. Here, we demonstrate how data-independent acquisition mass spectrometry can be used for high-dimensional characterization of immune cells, even with very limited cell numbers. These studies open the way for driving further understanding of cellular sub-populations contained within those generated from sorting with classical cell markers. **Methods** Primary human CD8+ T cells, CD4+ T cells, CD14+ monocytes and natural killer (NK) cells, isolated from peripheral blood mononuclear cells, were prepared for mass spectrometry using standard sample preparation workflows. 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. DIA data was extracted using Spectronaut X (Biognosys) with both peptide assay resource libraries and directDIA data searching. **Results** Cytotoxic CD8+ T cells were evaluated using 100,000 cells of input material, which resulted in >3500 proteins quantified in the primary cells. When combined with resource spectral libraries >4,500 proteins were quantified. In the current experimental setup, 30k cells represents the lower limit of detection of CD8A and CD8B. Among other previously characterized proteins associated with CD8+ T cells, KLRG1, CCL5, TBX21, GZMH, PRF1, GNLY, CST7 were all detected at 30k cell input, except KLRG1 which was detected at 50k cell input. Detected proteins are mapped into relevant processes including: antigen presentation, T-cell activation, target cell recognition, proliferation and metabolism. Data will be presented for the additional cell types (CD4+ T cells, CD14+ monocytes, and NK cells) to further map the immune cell phenotypic landscapes. **Conclusions** DIA-MS enables deep proteomic phenotyping of sorted immune cell samples, even with limited numbers of cells. These new data sets make available broad and un-constrained biomarker investigation for deconvolution of the processes driving immune cell activation and suppression.

626. Modification of Urine-Derived Stem Cells with piggyBac Transposons

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Cell and gene therapy approaches for kidney disease are lacking. This is unfortunate as chronic kidney disease affects up 14% of the US population carrying with it higher mortality. Urine is a practical and completely painless source of cells for gene and cell therapy applications. Urine-derived stem cells are adult kidney cells that have been isolated from a urine sample and propagate well in tissue culture on gelatin-coated plates. There are approximately one million nephrons per kidney that process approximately one hundred liters of urinary filtrate each day. Not surprisingly, several thousand cells become detached and can be collected in the urine. Among these, dozens of cells have the potential to grow into clones of urine-derived stem cells. We have isolated, expanded, and optimized transfection of these cells in order to develop regenerative therapies based on *piggyBac* transposon-modified urine-derived stem cells. Urine-derived stem cells from a healthy 23 year old male donor were isolated according to established protocols. Within two months, ten clones had been expanded, analyzed, and frozen. Analysis of individual clones by FACS characterization revealed that they expressed the characteristic marker panel of CD44 (97%), CD73 (98%), CD90 (99%), and CD146 (98%) positive and CD31 (0%), CD34 (0%), and CD45 (0%) negative (the percentage is the average percent positive within the live cell population of N=10 clones). The isolated urine-derived stem cells have been successfully differentiated along the osteogenic and adipogenic lineages, suggesting multipotent differentiation capacity. Transfection by several commercially available lipophilic reagents was optimized to achieve 61% transfection of live cells following transfection with a strongly expressing GFP plasmid. Transfection of luciferase-expressing plasmids were detectable by IVIS imaging. Promoter choice of transgene was compared among five *piggyBac* transposons with the CMV promoter producing the highest luciferase signal, followed by EF1-alpha. In conclusion, urine-derived stem cells represent an easily isolated, clinically relevant cell type that can be manipulated with non-viral genetic tools. Future studies will be aimed at using these cells for regenerative therapy approaches for kidney injury.

627. Tumor-Specific T Cell Engineering for Enhanced Effector Function via Microfluidic Delivery of Bioactive Molecules

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Background: Tumor-specific T cells possess unique potential for cancer therapy but are limited by T cell exhaustion and anergy induced in the tumor microenvironment. *Ex vivo* manipulation of these T cells to maintain their full function is critical to their success

clinically. Yet, limitations of existing *ex vivo* delivery approaches, such as electroporation, dramatically restrict their function and thus limit their therapeutic use. **Methods:** Genome-wide profiling was used to identify the impact of optimized electroporation treatment and the SQZ cell therapy platform on gene expression in human T cells. The profiling was paired with a 42 analyte T cell cytokine-multiplex panel to assess perturbation of cytokine secretion. We then compared the *in vivo* functionality of immune checkpoint deleted antigen-specific T cells, modified by either electroporation- or SQZ-mediated delivery of CRISPR/Cas9, and adoptively transferred into tumor-bearing mice. Finally, genomic editing of tumor infiltrating lymphocyte (TIL)-derived T cells was compared using either electroporation or SQZ and subsequent effector response upon re-exposure to tumor cells. **Results:** Impactful disruptions in transcript expression after treatment with electroporation (17% of genes mis regulated, FDR q <0.1) were identified, whereas cells treated with SQZ had similar expression profiles to untreated control cells (0% of genes mis-regulated, FDR q <0.1). These genetic disruptions result in concomitant perturbation of cytokine secretion and effector response. Ultimately, the effects at the transcript and protein level resulted in functional deficiencies *in vitro* and *in vivo* with electroporated antigen-specific and TIL-derived T cells failing to demonstrate sustained antigen-specific effector responses and tumor control with or without immune checkpoint editing. **Conclusions:** This work demonstrates that maintaining the desire phenotype and functionality of modified T cells is essential to their activity when re-exposed to their specific antigen and that the delivery mechanism used for modification is critical to this process. The significant differences in outcomes between SQZ and EP underscores the importance of understanding the impact of intracellular delivery methods on cell function for research and clinical applications. For both research and therapeutic applications with primary T cells, the functional consequences of the selected intracellular delivery technique and its impact on cell phenotype should be carefully evaluated.

Non-Nuclease Mediated Genome Editing

628. 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 targeting rate. Targeting rate may be influenced on a site-specific basis by factors thought to affect accessibility of the target site such as nucleosome occupancy, position-effect variegation, transcription across the target site, or the collision of processive nucleoprotein complexes leading to genomic instability. Our preliminary studies in mammalian cells have suggested up to a three-fold increase in HR rates at a single locus upon transcriptional induction. Yet, it is unclear if transcription itself or other factors that secondarily influence transcription, such as chromatin state, are directly linked to AAV-HR. We therefore set out to establish how transcriptional rate and/or chromosomal position effects influence this type of HR. To do this, 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. To this end, we used lentiviral vectors to generate a pooled population of HAP1 cells each harboring a single-copy, doxycycline-inducible genomic site expressing eGFP, which are mapped using ligation-mediated PCR and next-generation sequencing. The population is subsequently infected with a library of AAV serotype DJ vectors designed to integrate mScarlet and a unique barcode that allows for sorting of properly targeted cells based on gain of mScarlet and loss of eGFP expression. The genetic barcode is detectable in both genomic DNA and RNA transcripts originating from the doxycycline-inducible promoter; barcode heterogeneity is a measure of the targeting rate at a given genomic site. To date, we have targeted a cell population comprising greater than 1000 unique target sites with the barcoded rAAV library, with and without transcriptional induction at the time of rAAV vector administration. We used inverse PCR to directly map integrated barcodes to the target genomic sites to identify preferred target sites in the pooled cell population. Finally, we amplified the integrated barcodes from mRNA to quantify transcriptional activity from the doxycycline-inducible promoter at each targeted genomic site. Over half of the targeted genomic sites in either population overlap, and barcode heterogeneity is significantly higher in the population as a whole when under transcriptional induction compared to without, with on average approximately three more targeting events occurring per site. We see a moderate positive correlation between the number of targeting events at a site and barcode expression at that site, and we will discuss our statistical analyses to determine whether the transcriptional level at distinct genomic sites is independently predictive of targeting rate. Moreover, we are examining other feature sets at the targeted genomic sites to determine if ATAC-Seq, ChIP-Seq or Repli-Seq profiles contain features that improve the prediction of targeting rates.

629. Novel CRISPR Cytosine Base Editors with Minimized Off-Target Effects and Improved Editing Properties

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The combination of nucleobase deaminases with CRISPR-based DNA targeting has led to the development of base editors (BEs) that can induce targeted alteration of single DNA bases without introducing double strand breaks (DSBs). BEs have been rapidly adopted and widely applied in many different organisms and cell types and are being explored for use as therapeutics for human genetic diseases. Cytosine base editors (CBEs) allow for the programmed conversion of C·G to T·A base pairs and the predominantly used CBE harbors and uses the APOBEC1 cytosine deaminase to induce cytosine deamination. Here we show that CBEs with APOBEC1 can induce a novel type of off-target alteration in human cells and describe engineered versions of these editors that reduce these unwanted changes. In addition, we show that these CBE variants also show improved precision with more narrowed editing windows and increased sequence requirements for the base preceding the target cytosine. These results have important implications for the research and therapeutic uses of CBEs, illustrate the feasibility of engineering improved CBE variants with reduced off-target activities and increased precision, and suggest the need to more fully define and characterize the off-target profiles of other base editor platforms.

630. Targeted Transcriptional Modulation with Type I CRISPR-Cas Systems in Human Cells

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The development of CRISPR-Cas systems for targeting DNA and RNA in diverse organisms has transformed biotechnology and biological research. Moreover, the CRISPR revolution has highlighted bacterial adaptive immune systems as a rich and largely unexplored frontier for discovery of new genome engineering technologies. In particular, the class 2 CRISPR-Cas systems, which use single RNA-guided DNA-targeting nucleases such as Cas9, have been widely applied for targeting DNA sequences in eukaryotic genomes. Here, we report DNA-targeting and transcriptional control with class 1 CRISPR-Cas systems. Specifically, we repurposed the effector complex from type I variants of class 1 CRISPR-Cas systems, the most prevalent CRISPR loci in nature, that target DNA via a multi-component RNA-guided complex termed Cascade. We designed codon-optimized mammalian expression cassettes for each component of the Cascade complex from the *E. coli* strain K12 (termed EcoCascade). Following transfection of human cells, we validated expression of each component of the Cascade complex by Western blot as well as proper nuclear localization by immunofluorescence staining. We also confirmed crRNA-dependent EcoCascade complex formation in human cells by co-immunoprecipitation. We demonstrated programmable CRISPR RNA (crRNA)-mediated targeting of specific loci in the human genome via ChIP-seq analysis. By tethering transactivation domains to Cascade, we modulated the expression of targeted chromosomal genes in human cells. Specifically, we demonstrated robust endogenous *IL1RN* activation with multiple crRNAs, including >3,000-fold *IL1RN* activation comparable to activation with dCas9-based effectors. Transcriptome-wide analysis by RNA-seq showed highly specific crRNA-dependent activation of the targeted gene. To repurpose other Cascade complexes, we applied our understanding of the model type I-E EcoCascade to the type I-B CRISPR-Cas system of *L. monocytogenes* Finland_1998 (termed LmoCascade). Following tethering of transactivation domains to LmoCascade, we show endogenous *IL1RN* activation in human cells with almost all tested crRNAs. Additionally, we tethered the KRAB effector to LmoCascade for targeted transcriptional repression. In human cells with stable LmoCascade-KRAB expression, we demonstrated robust endogenous *HBE* repression following lentiviral transduction of crRNAs. This study expands the toolbox for engineering mammalian genomes and establishes Cascade as a novel CRISPR-based technology for targeted mammalian gene regulation.

631. Mechanisms of Non-Enzymatic PNA-Mediated Gene Editing

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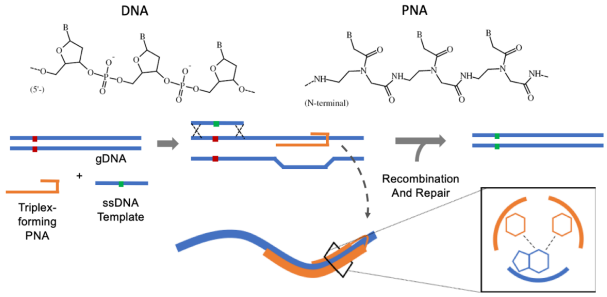
Nuclease-based reagents have dominated modern approaches to site-specific gene editing and rely on the induction of DNA double-strand breaks (DSBs) to elicit endogenous repair machinery. This strategy, however, often results in insertions, deletions, and translocations at target sites rather than template-mediated editing. In contrast, our recent work on non-nuclease, triplex-forming peptide nucleic acid (PNA)-mediated approaches has demonstrated efficient and safe site-specific editing without mutagenic outcomes. Animals treated via intravenous or inhalation administration of polymeric, biodegradable nanoparticles loaded with PNA molecules and single-stranded donor DNA demonstrate *in vivo* corrective editing and disease-amelioration in relevant models. Here we explore the repair mechanisms by which PNA technology achieves targeted gene correction without the introduction of an exogenous nuclease. We investigate the roles of select repair factors using targeted siRNA knockdown and quantitative assays for PNA editing frequency *in vitro*. In contrast to nuclease-based approaches, our data suggest nucleotide excision repair and single-strand annealing pathways are implicated in PNA-mediated editing.

We conclude with a detailed proposed model of PNA editing featuring triplex-forming PNA targeting of a single strand of genomic DNA and non-mutagenic template-mediated repair. We also suggest future experiments, including high-throughput screens, to further elucidate how triplex-forming PNA molecules elicit faithful corrective repair.



632. A Novel and Highly Sensitive In Vitro Platform for Detection of Gene Editing Nuclease and Base Editor Off-Target Sites

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Gene editing nucleases, such as Cas9, provide the ability to induce targeted mutations or transgene insertions with high efficiency at specified loci in the human genome. The recent addition of base editors to the genome editing toolbox has further enabled the introduction of precise mutations with single nucleotide resolution. In addition to editing their intended target sites, CRISPR nucleases and base editors can also induce unintended mutations at off-target genomic loci. Identification of these off-target mutations is particularly important for therapeutic translation of gene-editing nucleases and base editors. Many screening methods to identify potential off target sites experimentally have been developed. In vitro methods, such as Digenome-seq and the CIRCLE-seq method recently described by our lab, offer the advantage of high sensitivity and have been shown to be effective at identifying *bona fide* off-target mutations both in cells and *in vivo*. However, despite these advantages, Digenome-seq is not as sensitive as CIRCLE-seq and requires access to whole genome sequencing capabilities, making it less accessible to most labs. CIRCLE-seq requires large amounts of input DNA, may suffer from potential biases resulting from the need to circularize genomic DNA, and is experimentally complex and challenging for non-specialist labs to implement. Here we describe a novel in vitro method, which provides a generalizable, highly sensitive, and user friendly screening platform for identifying off-target sites of gene editing nucleases and base editors. The method is more robust than CIRCLE-seq, can be performed in one to two days, and only requires the sequencing capacity of a benchtop sequencer. We demonstrate higher sensitivity than previously described off-target detection methods both for nucleases and base editors. Because the method is generalizable, the platform could also be used to identify off-target sites of other nucleases including non-Cas9 CRISPR-Cas nucleases, zinc finger nucleases, and transcription activator-like effector nucleases (TALENs). The system could also be adapted to identify off-target sites of action for DNA-binding domains used in synthetic gene regulatory proteins. Given its higher sensitivity, ease of use, and broad applicability, we envision that the new method can be broadly adopted to characterize the specificity of a wide variety of gene- and epigenetic-editing technologies.

633. Cell-Specific CRISPR-Cas9 Activation by MicroRNA-Dependent Expression of Anti-CRISPR Proteins

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The emergence of CRISPR-Cas technologies revolutionized genetic studies and brought a personalized and targeted treatment of genetic disorders into closer reach. With respect to *in vivo* applications, strategies to confine CRISPR-Cas9 activity to selected cells and tissues are highly desired, both, to avoid artefacts that would result from unspecific genetic perturbations in animal genetic studies and to obviate side effects in gene therapy applications in humans. Until today, however, virtually any mode of efficient *in vivo* delivery of the CRISPR-Cas components is likely to affect many cell types and tissues beyond the one of actual interest. To address this limitation, we here generated a cell-specific Cas9-ON switch, which renders the activity of CRISPR-Cas9 dependent on the presence of cell-specific miRNAs, i.e. miRNAs that are abundant solely within the target cell type. To translate the abundance of a miRNA, which typically is a negative stimulus (causing gene expression knockdown), into a positive output (CRISPR activation), we established a negative feedback based on anti-CRISPR proteins, a recently discovered class of phage-derived CRISPR-Cas inhibitors. To this end, we inserted target sites for miR-122 or miR-1, which are abundant specifically in liver and muscle cells, respectively, into the 3'UTRs of Acr transgenes. Co-expressing these alongside Cas9 and sgRNAs via Adeno-associated virus (AAV) vectors resulted in Acr knockdown and concurrently release of Cas9 activity solely in hepatocytes or myocytes, while Cas9 was efficiently inhibited in off-target cells. First, we demonstrate control of genome editing and gene activation using a miR-dependent AcrIIA4 in combination with different *Streptococcus pyogenes (Spy)*Cas9 variants (full-length Cas9, split-Cas9, dCas9-VP64). Importantly, by varying the dose of the supplied Acr vector or the strength of the Acr-driving promoter, we could easily fine-tune the system towards low background activity in off-target cells as well as a high dynamic range (up to 100-fold). Finally, to showcase its modularity, we adapted our Cas9-ON system

to the smaller and more target-specific *Neisseria meningitidis* (*Nme*) Cas9 orthologue and its cognate inhibitors AcrIIC1 and AcrIIC3. Our novel Cas9-ON switch (Hoffmann et al., 2018, BioRxiv) complements existing strategies for cell-specific CRISPR-Cas activation, e.g. via tissue-specific promoters or targeted vectors, thereby promoting informative *in vivo* genetic studies and safe gene therapies.

634. Engineered Anti-CRISPR Proteins for Precision Control of CRISPR-Cas9

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The CRISPR-Cas9 technology transformed our ability to manipulate genomes and study molecular networks in living cells. To enhance the accuracy of CRISPR-Cas9 genome perturbations and to decrease adverse side effects, universal strategies enabling spatiotemporally confined Cas9 activation are highly desired (see complementary abstract by MD Hoffmann *et al.*). Anti-CRISPR (Acr) proteins, a diverse class of small, potent Cas9 inhibitors derived from bacteriophages, hold great potential to become the next generation tools for Cas9 regulation. However, Cas9 inhibition by Acrs is difficult to confine in time and space and not always sufficiently tight, thereby limiting the utility of this approach. Our lab has recently engineered a set of Acr proteins, which can be switched on or off with high spatiotemporal precision using blue light (Bubeck et al., Nat. Methods, 2018). More specifically, we created hybrids between AcrIIA4, a potent inhibitor of the *Streptococcus pyogenes* CRISPR-Cas9, and the light-oxygen-voltage 2 (LOV2) domain, a small blue light sensor from *Avena sativa*. When co-expressed together with Cas9 and a g(uide)RNA in mammalian cells, the AcrIIA4-LOV2 hybrids block Cas9 DNA binding in the dark, while photoexcitation releases Cas9 activity. Our technology named CASANOVA (for **C**RISPR-Cas9 **a**ctivity **s**witching via **a** **n**ovel **o**ptogenetic **v**ariant of **A**crIIA4) solely requires co-expression of the AcrIIA4-LOV2 hybrid to confer optogenetic regulation on in principle any *Spy*Cas9-based tool and can thus be used, e.g. to control genome editing, gene activation and CRISPR labeling. Here, we extend our CASANOVA method to the smaller and more target-specific *Neisseria meningitidis* (*Nme*)Cas9 and its cognate inhibitors AcrIIC1 and AcrIIC3. We find that despite their profoundly different inhibitory mechanisms, i.e. impairing Cas9 catalytic function, but not DNA binding (AcrIIC1) and inducing Cas9 dimerization, thereby blocking DNA targeting (AcrIIC3), both inhibitors are amenable to engineering by domain insertion. Similar to CASANOVA, AcrIIC3-LOV2 hybrids enable efficient light control of *Nme*Cas9 activity. In contrast, LOV2 insertion into AcrIIC1 did thus far not yield a light-dependent hybrid, but - very unexpectedly - improved inhibition potency as compared to wild-type AcrIIC1. This effect was preserved even when replacing the LOV2 domain by an unrelated domain (mCherry). While the underlying molecular mechanism remains elusive at this point, we speculate that the inserted domain strengthens *Nme*Cas9 inhibition by interfering with DNA binding. Taken together, our work facilitates precision control of CRISPR-Cas9 activity and paves the way towards engineering of anti-CRISPR proteins with novel, desired properties.

635. ProxyBE: Improving Efficiency of Single Base Editors by Proximal Cas9 Targeting

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Genome editing with CRISPR-Cas9 typically relies on introducing double-stranded breaks (DSBs), which when repaired through non-homologous end joining (NHEJ) create stochastic mutations and undesirable outcomes such as chromosomal translocations. While increased precision can be achieved by stimulating DNA repair by homologous recombination, homology directed repair pathways are inefficient and active only in dividing cells. These limitations can be overcome by using base editors (BEs), a genetic engineering system consisting of cytidine or adenine deaminase domains fused to Cas9 nickases that enable C>T (cytidine base editors, CBEs) or A>G (adenine base editors, ABEs) without the need for introducing DSBs. BEs have been applied to a broad range of genetic engineering applications including correction of point mutations, gene knockout, and exon skipping. Despite their potential widespread applications in gene therapy, implementation of BEs remains limited due to inconsistent editing activity across targets. While it is not possible to predict the activity of BEs at specific targets, it has been established that separation of the strands of DNA to form single stranded DNA as well as the identity of the bases surrounding the target cytidine/adenine are important parameters to consider for successful base editing. In order to broaden the editing capabilities of BE, we hypothesized that two sgRNAs targeting adjacent DNA sequences will separate the DNA strands more effectively and enable more efficient base editing. To test this hypothesis, we created a novel base editing method, which we named ProxyBE, for improving cytidine base editing activity by targeting a proximal protospacer with a second Cas9. First, we integrated an artificial sequence into the genome of a mammalian cell line to study base editing in a controlled setting. This experiment allowed us to position the ProxyBE at regular intervals from the target sequence of the base editor. This system also allowed us to use different Cas9 scaffolds for the BE and proximal Cas9, which prevented confounding effects. In these experiments we tested ProxyBE at distances ranging from 5 to 100 bases from the target site with

sgRNAs binding different strands. These studies not only revealed that ProxyBE enhances base editing more efficiently when it binds closer to the BE target -- presumably because ProxyBE facilitates DNA strand separation -- but also identified patterns for efficient use of ProxyBE. Finally, we tested ProxyBE at multiple native target sequences in different loci within the human genome using combinations SaKKH-BE3 and Sp-dCas9 base editors and proxies and our results demonstrate improvements in base editing efficiency that ranged from 2 to over 30-fold over control samples in which the base editors were used without proxies. Based on these data, we anticipate that ProxyBE will enable base editing of a broader range of DNA sequences than current approaches and will ultimately facilitate the development of novel gene therapies for correction of genetic diseases with improved efficiency.

Immune Response Mechanisms in Gene and Cell Therapy

636. Reducing AAV-Mediated Immune Responses and Pathology in a Subretinal Pig Model by Engineering the Vector Genome

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Studies have shown that the DNA genome of AAV vectors triggers Toll-like receptor 9 (TLR9), a pattern recognition receptor that activates inflammatory and innate immune defenses after sensing foreign DNA. We have developed a strategy termed "coupled immunomodulation", where we engineer AAV vectors to be intrinsically less immunogenic by incorporating directly into the vector genome short DNA oligonucleotides that antagonize TLR9 activation. We have demonstrated the utility of this strategy in liver and muscle-directed AAV therapy in mice *in vivo*. We now extend our studies to large animals *in vivo* and primary human immune cells *in vitro*. Intraocular inflammation and loss of visual acuity have been reported in subretinal AAV clinical trials in a dose-dependent manner, in spite of prophylactic immunosuppression, and large animal studies recapitulate the observed intraocular inflammation. Thus, we utilized a pig model to study immune responses to subretinal AAV administration and pathology in the retina. We injected AAV8.GFP (parental vector) or AAV8.GFP.io2 (engineered vector, carrying TLR9-inhibitory sequences) at an intermediate dose of 4 x 10^{11} vg per eye, based on published work showing inflammation in patients at 10^{11} and 10^{12} vg per eye. We performed in-life clinical examinations and optical coherence tomography (OCT) to detect intraocular inflammation and examine the health of the retina, as well as histological analyses to study photoreceptor pathology and immune cell activation in the retina. Our findings showed that, unlike the parental vector, the engineered vector markedly reduced eliciting photoreceptor pathology as well as innate and adaptive immune cell responses in the retina. To evaluate the effect of our approach on human innate immune responses, we tested peripheral blood mononuclear cells (PBMCs) from eleven donors and found that an engineered vector (AAV2.GFP.WPRE.io2) elicited lower IL-1beta cytokine responses than the parental vector (AAV2.GFP.WPRE) in different dendritic cell populations, indicating reduced innate immune responses. Our data indicate that this "coupled immunomodulation" strategy may be broadly applicable for reducing AAV-induced immunogenicity.

637. Immune Analysis Following Intrathecal Gene Transfer: 3-Year Data from Clinical Intrathecal Gene Transfer Trial for Patients with Giant Axonal Neuropathy

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OBJECTIVE: Giant axonal neuropathy (GAN) is a rare childhood onset autosomal recessive neurodegenerative disorder affecting the nervous system. Mutations in the *GAN* gene cause loss of function of gigaxonin, a cytoskeletal regulatory protein, clinically leading to progressive sensorimotor neuropathy, reduced coordination, slurred speech, seizures, and progressive respiratory failure resulting in death by the 2nd to 3rd decade of life. We are conducting an ongoing first-in-human intrathecal (IT) AAV9 mediated gene transfer trial for GAN (NCT02362438). The immunologic consequence of an IT gene transfer approach is not fully characterized, especially to further timepoints to follow up post gene transfer. Here we present a review of updated pre-and post- gene transfer immunologic data, including neutralizing antibody (NAb) titers in serum and cerebrospinal fluid (CSF), ELISpot data, CSF cell counts, oligoclonal band formation, IgG index, and overall assessments of safety. We are including biallellic null mutation or "CRIM (-)" patients who are on added immune modulation to further characterize the impact of targeted T cell immune modulation on immune profiles following gene transfer. METHODS: Eleven GAN patients [8 CRIM (+) (with at least one missense mutation in the GAN gene) and 3 CRIM (-)] have been dosed at one of three dose levels (ranging from 3.5x10e13 vg to 1.8x1014 vg) with scAAV9-JeT-GAN under an IRB approved protocol at NIH/ NINDS. Patients receive pre-treatment with IV methylprednisolone within several hours prior to gene transfer, and then are maintained on moderate dose oral prednisone for 4 months followed by a 1 month taper. CRIM (-) patients receive tacrolimus and rapamycin in addition. Clinical safety is assessed by neurologic exam, neuroimaging, serum counts/ chemistries, EKG/ Echocardiogram, pulmonary function testing, and CSF studies.

Preexisting AAV9 NAb titers are measured both in serum and CSF and are followed after gene transfer. In addition, interferon-γ ELISpot analysis to GAN and AAV9 epitopes pre-and post- gene transfer is performed. RESULTS: 11 patients have received IT gene transfer and follow up ranges from 6 months to 3 years. 3 of 11 patients had positive preexisting AAV9 NAb titers in serum at trial entry, but none had positive NAb titers in CSF at baseline. There is a rise in both CSF and serum NAb titer levels following IT gene transfer, but is diminished in patients on T cell directed immune modulation combined with steroids. Most patients show a delayed CSF lymphocyte predominant pleocytosis, that is otherwise clinically asymptomatic and self resolving at 6 to 12 months post gene transfer. This CSF pleocytosis appears to be diminished by the use of corticosteroids with T cell targeted immune modulation. ELISpot analysis demonstrates T cellIFN-γresponse to AAV9 epitopes in CRIM (+) patients, but not in the CRIM (-) patients who are on T-cell directed immune modulation. Thus far, no patient, including with CRIM (-) status has shown evidence of an anti-transgene T cell response by ELISpot or any clinical signs of inflammation. CONCLUSIONS: The phase I first-in-human intrathecal gene transfer study for GAN represents the longest systematic monitoring of immune profiles following IT gene transfer. Safety and immunological analysis in the trial is ongoing but can already inform safety monitoring and immune modulation protocol design for this and future gene transfer trials targeting the nervous system with an IT approach.

638. Adaptive Immune Responses Directed Against Residual VSVG on Transduced Hematopoietic Stem Cells

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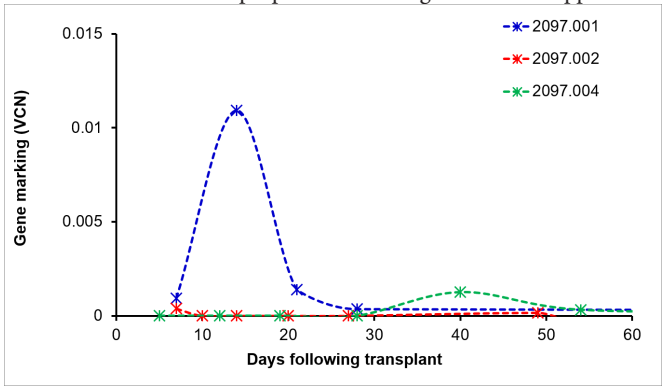
Fanconi anemia (FA) is a rare genetic order in which the FA core complex, consisting of DNA damage repair genes are mutated, resulting in abnormal cell death, uncontrolled cellular proliferation, and bone marrow (BM) failure. The median age of survival in patients with FA-associated genetic mutations is 25 years of age. Of the approximately 20 possible genetic mutations associated with FA, 90% of patients have mutations in *FANCA*, *FANCC*, or *FANCG*. The only cure currently available for FA patients is BM transplantation from an allogeneic donor. Because of the dysregulated state of the lymphoid and myeloid compartments in FA patients, conditioning is not available to them, making graft versus host disease a potential complication. To circumvent this, autologous transplantation of gene-modified hematopoietic stem cells (HPSCs) that have received a functional copy of the mutated FA gene could serve as an alternative therapy without the requirement for conditioning. In a recent clinical trial, recruited FA patients (n=3) received a functional replacement for the *FANCA* gene delivered by a VSVG-pseudotyped lentiviral vector. Shortly after transplantation, evidence of gene marking failed to be observed in 2 of 3 patients, with short-lived gene marking observed in the third patient. Failure of engraftment was presumed to be due to an immune response against the delivered transgene or viral vector components. Vesicular stomatitis virus glycoprotein (VSVG) is commonly used to pseudotype lentiviral vectors to improve tropism and transduction efficiency. Prior research has shown that VSVG pseudotyped lentiviral and retroviral vectors produced in human cells can be inactivated by human serum. Due to a lack of clarity in the mechanistic aspects of the neutralization of VSVG-pseudotyped vectors *in vivo*, as well as the failure of HPSC transplantation in unconditioned transplant patients receiving VSVG-pseudotyped gene delivery, we set out to determine if antigen-specific humoral or cellular immunity to VSVG in transplanted patients was driving rejection of transduced HPSCs. Here we show a serum component capable of blocking *ex vivo* transduction of cells with the VSVG pseudotyped FANCA clinical vector. We also show that transduced hematopoietic stem cells (HPSCs) in nonhuman primates retain VSVG from the vector on the cell surface. This VSVG can be detected weeks after transplant in the peripheral blood. Developing immune responses can be detected in the transplanted animals when compared to baseline, including increased VSVG-specific T cell responses, anti-VSVG IgG, and cytotoxic responses that can specifically kill VSVG-expressing target cell lines. Finally, we show that some humans have preexisting VSVG-specific CD4+ and CD8+ T cell responses, as well as VSVG-specific IgG (n=10). Taken altogether, these data show that VSVG-pseudotyped vectors are immunogenic in a nonhuman primate model, and that pre-existing immune responses to VSVG in human patients may prevent engraftment of lentivirally-transduced cells without proper conditioning or immunosuppression.



639. Immunoprophylaxis of Influenza Using AAV Vector Delivery of Recombinant Cross-Neutralizing Nanobodies

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Influenza continues to pose a significant threat to public health and there is an urgent need for alternative counter-measures that can provide broad protection especially in high-risk patient groups. Amongst these, cross-neutralizing human monoclonal antibodies (Mabs) which bind the highly conserved hemagglutinin (HA) stem either using passive or gene therapy vectored delivery are showing great promise. Several of the human Mabs currently in clinical development use only their heavy chain for antigen recognition suggesting that a light chain is superfluous to requirements in binding to the HA stem. This has led to our isolation of naturally occurring 'heavy chain only' antibodies, also known as nanobodies, which are emerging as an important next generation of biotherapeutics with several advantages over conventional Mabs. These include their small size, cleft binding properties, easy engineering and high stability. We have previously described a highly potent nanobody, R1a-B6, which binds to the HA stem and is capable of neutralizing *in vitro* key group 1 influenza viruses (A(H1N1)pdm09, highly pathogenic avian influenza H5N1, H2N2 and H9N2). This study investigates the potential for immunoprophylaxis of R1a-B6 using gene delivery in a mouse influenza challenge model. In order to extend the serum half-life and engage effector functions, we appended antibody Fc regions to R1a-B6. We have designed four constructs in a recombinant adeno-associated virus (rAAV 2/8) vector, (i) R1a-B6 mouse Fc IgG1, (ii) R1a-B6 mouse Fc IgG2a, (iii) monovalent R1a-B6 and (iv) negative control nanobody Fc fusion. A single intramuscular (IM) injection of 1 x 10^{11} viral genomes of rAAV into BALB/c mice gave robust and stable expression of nanobody Fc fusions at levels in excess of 800 μg/mL for a duration of up to 6 months. Monovalent R1a-B6 showed barely detectable serum levels demonstrating the importance of the Fc in prolonging the serum half-life. Sera from mice that received AAV expressing R1a-B6-mIgG1 and R1a-B6-mIgG2a neutralized both A/California/07/2009(H1N1)pdm09 and A/Vietnam/1194/2004(H5N1) pseudotyped virus *in vitro* and potency was seen to directly correlate with the serum concentration of the nanobody Fc fusions. Following lethal challenge with A/California/07/2009(H1N1)pdm09, mice expressing R1a-B6-mIgG1 and R1a-B6-mIgG2a showed no signs of illness or weight loss for the duration of the study. Whereas all mice expressing the negative control nanobody Fc fusion showed dramatic weight loss and symptoms of influenza, exiting the study by day 6. Mice expressing monovalent R1a-B6 without Fc function showed limited protection and the onset of disease was delayed by 3 days indicating that an extended serum half-life is required to achieve the protective level of R1a-B6. Our studies have demonstrated the prophylactic potential of gene delivery of broadly reactive nanobodies against influenza. This paves the way for the development of a "universal influenza vaccine" capable of protecting at risk patient groups such as the elderly, children or immunocompromised independent of vaccination and prior knowledge or availability of the influenza strain.

640. Single Cell Transcriptome Analysis of Responses of Mouse Liver Immune Microenvironment Following Intravenous Administration of AAV Vectors

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Intravenous (IV) administration of adeno-associated virus (AAV) vectors is widely used as a strategy to genetically modify the liver to express therapeutic genes. While extensive attention has been focused on systemic immune responses to the AAV capsid and transgene, little is known about the response of the inherent liver immune microenvironment in response to IV AAV vector administration. In this study, we have leveraged the power of single-cell RNA-sequencing to characterize the immune microenvironment in the mouse liver following intravenous administration of AAVrh.10 vectors. C57Bl/6 mice were randomly divided into 3 groups (each n=3) and intravenously injected with PBS, AAVrh.10Null or AAVrh.10mCherry (10^{11} genome copies/mouse). Two wk after vector administration, livers were harvested, dissociated into single cells, and Drop-seq single cell sequencing performed to obtain transcriptomic profiles of single liver cells. The datasets were combined using canonical-correlation analysis followed by dynamic time warping, and unsupervised clustering of the cells was performed using the t-SNE method. Of the total 46,500 cells sequenced, besides hepatocytes, endothelial cells and cholangiocytes, there were 318 Kupffer cells, 204 B cells, 151 double-negative αβT cells and 92 NKT cells identified. Assessment of the distribution of mCherry expression demonstrated expression in hepatocytes, endothelial cells and cholangiocytes, but also expression in all classes of liver immune cells, including Kupffer, T, B and NKT cells, with highest expression in Kupffer cells. Strikingly, AAV capsid interaction with the liver significantly altered the transcriptional patterns of the liver immune cells. Compared to the PBS control, the AAVrh.10Null vector induced immune-related genes in Kupffer cell (e.g., Csf1r, Ccl6, Maf), B cells (e.g., Jak1, Ifi27, Ifi203), T cells (e.g., Itgb2, Tagln2, Il2rb) and NKT (e.g., Aim2, Cd81, Sdc4). As expected from expression of a foreign transgene, AAVrh.10mCherry also induced genes in Kupffer cells (e.g., Mbl2, Atg3, Fgl1), B cells (e.g., Clec7a, Lyz2, Marcks), T cells (e.g., Cxcr6, Cd2, Cdo1) and NKT cells (e.g., Fli1, Elf1, Gzmb). Analysis of pathways in liver immune cells modulated by AAVrh.10Null included complement and coagulation cascades, drug metabolism (non-cytochrome P450 enzymes), protein processing in endoplasmic reticulum and B cell receptor signaling. These observations bring a new dimension to assessing the host defenses responding to AAV vectors. In addition to systemic immune responses, the liver has its own immune microenvironment that responds to intravenous administration of AAV vectors, a response that should be considered in evaluating the safety and efficacy of AAV-mediated gene therapy.

641. Assessment of Anti-Cocaine Vaccine dAdGNE in Clinical Cohort 1 of Cocaine Addicts

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Cocaine addiction, a chronic relapsing illness characterized by cycles of drug use and abstinence, is a major problem for which there is no effective therapy. This report summarizes our experience with a killed adenovirus-based anti-cocaine vaccine designed to evoke high titer, high affinity anti-cocaine antibodies designed to prevent administered cocaine from reaching the central nervous system. The challenge in developing an anti-cocaine vaccine is that cocaine is a small molecule, invisible to the immune system. We overcame this barrier by linking a cocaine analog (GNE) to highly immunogenic $E1^{-}E3^{-}$ serotype 5 adenovirus capsid proteins creating the vaccine dAd5GNE. In mice, rats and nonhuman primates dAd5GNE elicited high-affinity, high-titer antibodies against cocaine, sufficient to sequester systemically administered drug from access to the brain, with consequent reduction in cocaine-induced behavior. Following IND-enabling preclinical efficacy and safety testing, and an approved IND, we initiated a clinical trial in cocaine addicts with clinical-grade dAd5GNE formulated with Adjuplex® adjuvant. The phase I placebo controlled, double blind, dose-ranging clinical study is designed with 3 dose cohorts (100, 316 and 1,000 μg) administered 1x/month for 6 months. In each cohort of n=10, n=7 receive vaccine and n=3 placebo. In addition to safety, subjects are assessed for serum anti-cocaine titers, urine cocaine and a scale for cocaine craving. All subjects fulfilled DSM-V criteria (9.3±0.5). Adverse events included pain/ tenderness/ soreness at injection site (39%), fever (7%), erythema at injection site (6%), nausea (6%), and chills (4%). There were no serious adverse events related to the vaccine. The n=3 receiving placebo had background anti-cocaine titers ($p<10^3$). In contrast, the monthly vaccine administration resulted in stable average anti-cocaine titers over 6 months of $2.4x10^5$, just below the threshold of $4x10^5$ that, based on our prior nonhuman primate studies (Moaz A et al, Neuropsychopharmacology 2013; 38:2170), should be necessary to protect the brain from systemically administered cocaine. As expected from the low dose cohort in which titers were less than the $4x10^5$ protective threshold, there were no differences in % cocaine positive urines, nor in the cocaine craving scale. In summary, at the 100 μg monthly dose, the dAdGNE vaccine can be safely administered to cocaine addicts, and elicits sustained average anti-cocaine antibody titers of $2.4x10^5$, just below the predicted "efficacy" threshold. Studies in cohort 2 (316 μg monthly) have been initiated.

642. Engineering AAV Vectors to Reduce CD8+ T Cell Responses Following Intramuscular Injection in Mice

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Nucleic acid systems are used in therapeutic approaches to treat genetic diseases. However, the introduction of foreign nucleic acids can elicit an immune response that may decrease the safety and efficacy of the therapy. It has been demonstrated that adeno-associated virus (AAV) vectors set off an immune response when their DNA genomes are detected by Toll-like receptor 9 (TLR9), a pattern recognition receptor found in the endosomes of immune cells. Upon detection of CpG motifs in vector DNA, TLR9 initiates an inflammatory response and primes T cell responses, potentially leading to destruction of transduced cells. To engineer an intrinsically less immunogenic AAV vector, we have directly incorporated TLR9 inhibitory oligonucleotides into an untranslated region of the vector genome. Previous clinical trials have suggested that intramuscular delivery of AAV vectors can lead to CD8+ T cell infiltration in muscle biopsies. To test whether our designed inhibitory oligonucleotide (termed "inflammation-inhibiting oligonucleotide 2", io2) reduces the level of CD8+ T cell infiltration *in vivo*, we used a combination of intramuscular delivery and the AAV capsid AAVrh32.33 to model immunogenic conditions in mice. These conditions have been previously shown to elicit robust CD8+ T cell responses against AAVrh32.33 capsid and local infiltration of cytotoxic T cells into the muscle. C57BL/6 mice were injected in the quadricep muscle with PBS (5) or 1×10^{10} viral genomes (vg) of either AAVrh32.33.GFP (8) or AAVrh32.33.GFP.io2 (7). Twenty-one days post injection, the animals were sacrificed and their quadriceps harvested for histological analysis. Tissues were embedded in paraffin and sectioned before being stained for CD8 and Granzyme B (GZMB), a serine protease that serves as a cytotoxic T cell activation marker. A DAPI counterstain was also included to verify that cells had a nucleus and thus were not autofluorescing red blood cells. CD8+ T cells were scored by viewing four random fields of the muscle section and manually counting the amount of CD8+ and CD8+/GZMB+ T cells present in the field. These were summed for each section, and the percentage of overall CD8+/GZMB+ T cells was calculated. We did not observe CD8+ T cells infiltrating PBS injected quadricep tissues. Analyses of AAVrh32.33.GFP injected quadricep tissues showed robust CD8+ T cell infiltration as expected, and we observed that one-third to one-half of were Granzyme B+. In contrast, the AAVrh32.33.GFP.io2 injected quadriceps showed a significant reduction in the number of infiltrating CD8+/GZMB+ T cells. This work demonstrates that our approach can not only reduce the induction of inflammatory responses, but also works to dampen the infiltration of CD8+ T cells into tissues following intramuscularly injected gene therapy. Our approach may be beneficial for enhancing safety and efficacy in gene therapies.

643. Monitoring T Cell Responses Toward the Streptococcus Pyogenes Cas9 Nuclease

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Previous studies showed that there is a pre-existing adaptive immunity toward CRISPR-associated nucleases (Cas) in humans. Clinical studies of viral gene therapy suggest that immune responses toward vectors can decrease the efficacy and safety of these treatments. Thus, clinical translation of CRISPR-Cas-based therapies will require careful immune monitoring as well as strategies to suppress unwanted immune responses in patients. We characterized the T cell responses toward the whole protein of the CRISPR-associated nuclease derived from *Streptococcus pyogenes* (SpCas9) in the peripheral blood of healthy human donors. SpCas9-reactive T cells were found in 95% of all the human donors comprising both effector and regulatory subsets with distinct functional properties. SpCas9-reactive regulatory T cells suppressed the proliferation and cytokine production of their effector counterpart *in vitro*. Through depletion of regulatory T cells, we established SpCas9-reactive effector T cell lines that lysed SpCas9-expressing target cells in a dose-dependent manner. Further, we established an efficient and rapid method to identify the entire SpCas9-reactive T-cell repertoire using a customized peptide library covering the complete amino-acid sequence of SpCas9. Our method allows immune monitoring for risk assessment before and during clinical trials employing Cas-derived therapeutic approaches. Moreover, it provides the basis for an unsupervised characterization of the antigenic structures that mount the pre-existing Cas-reactive T-cell response. In addition, SpCas9-reactive effector T cell lines could be used to evaluate the immunogenicity of autologous Cas-modified cell products before infusion into the patient. Regulatory T cells with specificity for Cas epitopes may be an attractive option for a targeted immunosuppression strategy during gene therapy.

Oligonucleotide Therapeutics II

644. Amino-Acylated LeuCAG3′tsRNA Mediates Translational Elongation of Ribosomal Protein S28 mRNA and is a Key Regulatory Step in Ribosome Biogenesis

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Transfer-RNA-derived small RNAs (tsRNAs) have been implicated in regulating cellular processes in health and disease including cancer. From the more than 400 tRNA genes in the human genome, there are potentially 161 unique 3′tsRNAs that can be generated in the human genome. We recently found that a 22 nucleotide (nt) 3′ end of the LeuCAG transfer-RNA-derived small RNA (LeuCAG3′tsRNA) binds to the human *RPS28*mRNA, unwinds the double-stranded secondary structure, which enhances *RPS28*mRNA translation. Small changes in RPS28 protein production was also shown to regulate rRNA processing and ultimately ribosome biogenesis. Inhibition of this specific tsRNA induced apoptosis in rapidly dividing cells in culture and suppressed the growth of human hepatocellular carcinomas *in vivo,* making it a *bona fide* target for cancer therapeutics (Kim et al., 2017 *Nature*). The mechanism involved in the generation of the 3′tsRNAs, the structure of the 3′terminus that contains the universal CCA terminal sequence, and the mechanism and species conservation of the step regulating translation has yet to be elucidated. In the current study, we used a battery of various enzymatic and chemical assays to establish that the LeuCAG3′tsRNA is fully amino-acylated under physiological conditions in mammalian cells. This strongly suggests the 3′tsRNAsare generated from the mature tRNA after it is charged. We also found that Rps28mRNA and ribosome biogenesis were similarly regulated by the same 3′tsRNA in the mouse. In addition, harringtonine-treated polysome analysis in both mouse and human cells showed that the 3′tsRNA regulates translation at the elongation step. We are currently developing algorithms to predict the *bona fide* binding sites of other tsRNAs and mRNAs, and our preliminary analyses suggest that the 3′tsRNAs may fine-tune production of various proteins involved in protein synthesis. Overall our results suggest a conserved functional role for fine regulation of various mRNAs in cells and that at least some of these non-coding RNAs may be valid therapeutic targets for treating cancer.

645. Splice-Switching Antisense Oligonucleotides for the Treatment of CLN3 Batten Disease

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CLN3 Batten disease, also known as juvenile neuronal ceroid lipofuscinosis (JNCL), is a fatal, pediatric lysosomal storage disease caused by mutations in a gene encoding the lysosomal protein CLN3. There is an urgent need to develop an effective treatment for this severe neurodegenerative disease, which shows its first symptoms in early childhood and typically causes death in early adulthood. The most common mutation associated with CLN3 Batten disease is a deletion encompassing *CLN3* exons 7 and 8 ($CLN3^{\Delta ex78}$), which disrupts the mRNA open reading frame by creating a premature termination codon that results in the production of a truncated, nonfunctional protein. We have devised a potential therapeutic approach to treating CLN3 Batten Disease using splice-switching antisense oligonucleotides (ASO), which base pair to *CLN3* pre-mRNA and alter splicing to re-frame the mutated transcript. We designed and tested ASOs in mouse and human cell models of the disease and effectively altered the splicing of *CLN3* mRNA to correct the open reading frame. Treatment of $CLN3^{\Delta ex78}$ neonatal mice by intracerebroventricular injection of the ASO resulted in the desired splicing effect throughout the central nervous system, improved motor deficits associated with the disease in mice and reduced histopathological features of the disease in the brain. Together our results suggest that ASO-mediated reading frame correction may be a promising therapeutic for CLN3 Batten disease.

646. Chemically Modified hCFTR mRNAs Recuperate Lung Function in a Mouse Model of Cystic Fibrosis

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Objective: Gene therapy has always been a promising therapeutic approach for Cystic Fibrosis (CF). However, numerous trials using DNA or viral vectors encoding the correct protein resulted in a general low efficacy. Consequently, lung function, as one of the main outcome parameters, probably having the most significant influence on life quality of CF patients, is rarely tested in preclinical models. In fact, actual effects of (modern) existing drugs on lung function, with forced expiratory volume in one second (FEV_1) as a key parameter, are quite low. Here, we tested chemically modified (cm)RNA h*CFTR* as a viable and potent therapeutic alternative. **Method:** Consequently, we first explored the expression, function and immunogenicity of human (h)CFTR encoded by cmRNA h*CFTR in vitro* and *ex vivo*, quantified the hCFTR expression by flow cytometry, determined its function using a YFP based assay and checked the immune response in human whole blood. Similarly, we examined the function of cmRNA h*CFTR in vivo* after intratracheal (i.t.) or intravenous (i.v.) injection of the assembled cmRNA hCFTR together with Chitosan-coated PLGA (poly-D, L-lactide-co-glycolide 75:25 (Resomer RG 752 H)) nanoparticles (NPs) by FlexiVent. The amount of expression of human hCFTR encoded by cmRNA h*CFTR* was quantified by h*CFTR* ELISA, and cmRNA h*CFTR* values were assessed by RT-qPCR. **Results:** In extensive immune assay tests *in* vivo, we could not detect any immunostimulatory effect arise from NPs or the cmRNA h*CFTR* (NPs); therefore, the loaded NPs could be administered safely to the lungs without any substantial increase in cytokines, or inflammatory-related cells such as macrophages or neutrophils. Systemic delivery did also show no impact on proinflammatory cytokine secretion. Intriguingly, the application of NP-cmRNA h*CFTR in vivo* efficiently restored lung function in a Cftr-deficient mouse model, close to levels of healthy control mice. In addition, our study compared - apart from two well-known mRNA modifications and pDNA hCFTR - also two different delivery routes (i.t. and i.v.), demonstrating that systemic (i.v.) administration of cmRNA targets lung cells more efficiently at lower dosages. **Conclusion:** This study provides the first proof of concept for a very promising, alternative treatment of patients suffering from CF. It utilizes cmRNA h*CFTR* transcript supplementation, which may be broadly applicable for most *CFTR* mutations, not only in adults but potentially already in the postnatal stage (i.v.!), thereby protecting the lungs from exacerbations from the very beginning of life.

647. Small Hairpin RNAs Compete with Heart MicroRNAs and Lead to Cardiomyopathy

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RNA interference (RNAi) is a promising gene therapy strategy for targeting dominant mutations. This therapy leverages small hairpin RNAs (shRNAs) to knock down gene expression; however, delivery of too much shRNA can disrupt the processing of endogenous microRNAs (miRNAs), and lead to toxicity. In this study, we sought to understand the effect that excessive shRNAs have on muscle miRNAs by transducing several constructs in mice and performing small RNA sequencing on their muscle and liver tissues. Specifically, we assessed tissues from ROSA26 mice that had previously been injected via tail vein with $2x10^{12}$ vector genomes of rAAV6 vectors expressing shRNAs. These shRNAs were driven by the U6 promoter and targeted beta-galactosidase mRNA, with either 19- or 21-nucleotide (nt) complementary sequences. We

then performed small RNA sequencing on liver, heart, gastrocnemius, tibialis anterior, quadriceps, and diaphragm samples from biological triplicates of mice at two, six, or 12 weeks after shRNA administration. We found that shRNA expression was highest in the heart, and that when shRNAs accumulated to about 27% of total small RNAs, mice experienced substantial cardiomyopathy. At this level in the heart, shRNAs in other muscle tissues were only ~1.8-7.6% of total miRNAs. Regardless of treatment, the four predominant heart microRNAs, which represent about half of all heart microRNAs, remained stable across samples. Instead, the lower-expressed microRNAs - most notably miR-451, one of the only microRNAs processed in a Dicer-independent manner - decreased significantly in direct correlation with shRNA level and toxicity. Our data suggest that certain muscle miRNAs compete with exogenous shRNAs, leading to the observed cardiomyopathy. Moreover, the mechanism of cardiotoxicity in these mice in response to shRNA treatment was in contrast to what has been previously shown in the liver, where loss of the most predominant microRNA, miR-122, is the cause of shRNA-induced hepatotoxicity. Quantifying microRNA profiles after excessive shRNA delivery illuminates the host response to rAAV-shRNA, allowing for safer and more robust therapeutic gene knockdown, and gives us insight into the basic functions of muscle microRNAs.

648. A Common Deep Intronic Mutation Causing Collagen VI-Related Muscular Dystrophy: Validation of Splice-Modulating Approaches In Vitro and Development of a 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 is no effective treatment. Our group has recently identified a new, and unexpectedly common, *de novo* deep-intronic mutation in the collagen 6 alpha 1 (*COL6A1*) gene (c.930+189C>T, referred to as the +189T mutation), associated with a severe COL6-RD phenotype. The +189T mutation activates the insertion of an in-frame pseudoexon, that acts in a dominant-negative fashion, and represents an ideal target for splice-modulating therapy. To test this strategy, we designed a series of phosphorodiamidate morpholino oligomers (PMOs) that were screened on splicing reporters (minigenes) and patient-derived primary fibroblast cells. We identified PMOs that promoted up to 88.4% pseudoexon skipping in patient-derived cells, which in turn abrogated the dominant-negative effect of the pseudoexon on collagen VI matrix assembly. Alternatively, we used a CRISPR/Cas9-meditated gene editing strategy to precisely delete an intronic sequence containing the pseudoexon, and this efficiently abolished its inclusion while preserving wildtype splicing in patient-derived cells. We created a humanized knock-in mouse to model the intronic mutation, and show that the pseudoexon is expressed an processed similarly as it is in human cells. Here, we successfully demonstrate that splice-modulating oligos and a CRISPR/Cas9-induced deletion can rescue the in-frame, dominantly acting pseudoexon insertion of this common COL6-RD mutation, and that it is possible to model this mutation in an animal model, paving the way for rapid clinical translation.

649. Antisense-Mediated Increase of SCN1A Expression Using TANGO Technology for the Treatment of Dravet Syndrome

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Background: Dravet syndrome is a severe pediatric developmental and epileptic encephalopathy characterized by frequent seizures, intellectual disability and risk of sudden unexpected death in epilepsy (SUDEP). The majority of Dravet syndrome patients carry *de novo* mutations in *SCN1A* leading to haploinsufficiency of the voltage-gated sodium channel α subunit $Na_V1.1$. Currently, there are no disease-modifying, targeted therapeutics for Dravet syndrome. In the current studies, we employ TANGO (Targeted Augmentation of Nuclear Gene Output) a novel therapeutic approach using antisense oligonucleotides (ASOs) to increase the endogenous expression of *SCN1A*. As TANGO leverages the *SCN1A* wild-type allele, it operates in a mutation-independent manner. TANGO exploits a naturally-occurring non-productive alternatively spliced exon in human and mouse *SCN1A* that leads to the incorporation of a premature termination codon and subsequent transcript degradation.

Methods: We designed and tested ASOs to prevent the inclusion of the non-productive, alternatively-spliced exon and increase levels of productive *SCN1A* mRNA in cultured human neural-progenitor cells. The lead ASO was delivered via intracerebroventricular (ICV) injection into neonate wild-type mice as well as an F1:129 x C57Bl/6 *Scn1a*$^{+/-}$ Dravet syndrome mouse model that recapitulates many patient phenotypes, including severe seizures, developmental delay, ataxia, sleep disorders, and SUDEP. Results: Identified ASOs significantly increased the expression of *SCN1A* in vitro. This increase in *SCN1A* expression resulted from a gene-specific reduction in non-productive mRNA and an increase in productive mRNA. ICV injection of the lead ASO in wild-type mice yielded a dose-dependent increase in *Scn1a* mRNA as well as $Na_V1.1$ protein with no effect on the expression of other voltage-gated sodium channel genes. No deleterious effects were observed from $Na_V1.1$ overexpression. In addition, a single ICV injection of the lead ASO at postnatal day (P) 2 in *Scn1a*$^{+/-}$ Dravet syndrome mice led to a sustained restoration (~90 days) of the levels of $Na_V1.1$ protein back to that of *Scn1a*$^{+/+}$ mice. The ASO treatment also prevented SUDEP in 99% (79 of 80) of *Scn1a*$^{+/-}$ mice tested, with 33 animals monitored through ~90 days. In contrast, over 50% of littermate *Scn1a*$^{+/-}$ mice treated with PBS seized and died before 35 days. There were no deleterious effects on ASO-treated *Scn1a*$^{+/+}$ littermate mice that overexpressed $Na_V1.1$ through ~90 days. Additional ongoing studies are investigating the impact of ASO treatment at P14, immediately prior to the onset of seizures in the *Scn1a*$^{+/-}$ Dravet syndrome mice. Conclusions: These results indicate that TANGO technology can be used to rescue a mouse model of *Scn1a*-linked Dravet syndrome. This ASO therapy may provide a gene-specific, disease-modifying approach to restore physiological $Na_V1.1$ levels and prevent seizures, SUDEP, and cognitive decline in patients with Dravet syndrome.

650. Artificial microRNA Silences C9ORF72 Variants In Vivo and Decreases the Most Abundant Toxic Dipeptides in BAC Transgenic Mouse

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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. In 2011, it was discovered within chromosome 9 open reading frame 72(C9ORF72) the presence of an expanded hexanucleotide G4C2 repeat. This expansion has been shown to be the major cause for not only familial and sporadic ALS but also frontotemporal dementia (FTD) cases. Patient brain samples carrying this expansion have reduced C9ORF72 mRNA levels and RNA foci aggregates shown to be complimentary to the expansion. In addition, it has been shown that this RNA is giving rise to di-peptide chains by repeat-associated non-ATG translation. These findings have led to multiple hypotheses on the pathogenesis of C9ORF72: 1) Haploinsufficiency, 2) RNA gain-of-function, 3) RAN Translation, and 4) Disrupted nucleocytoplasmic trafficking. Due to lack of treatments for this disease, we have pursued an AAV-RNAi dependent gene therapy approach, using an artificial microRNA packaged in a recombinant adeno-associated virus (rAAV) serotype 9. To further validate our *in vitro* results, we advanced to *in vivo* experiments using a transgenic mouse model expressing a full human C9ORF72 gene, with a ~100-1000 repeat hexanucleotide expansion. This mouse model recapitulates the major histopathological features seen in human ALS/FTD patients such as: lower levels of human C9ORF72 mRNA, RNA nuclear aggregates, and the Non- ATG translation products. Neonatal mice with this expanded human allele where injected via an intracranial ventricular (ICV) as well as a peripheral temporal route, additionally adult mice were injected with the rAAV-RNAi vectors via bilateral striatal injections. Our results suggest that AAV9-mediated microRNA silencing not only reduced the mRNA levels of C9ORF72 but also both C9ORF72 protein isoforms. More importantly it also leads to a decreased production of the expansion derived toxic dipeptides hypothesized arise from repeat-associated non-ATG translation. Although our artificial microRNA is targeting exon 3, the evident dipeptide decrease is likely due to the presence of mis-spliced (expanded intron containing) transcripts that are being targeted by the RNAi pathway. These encouraging results warrant the continued testing of this this treatment as a therapeutic option for C9ORF72 - ALS patients.

651. In Vivo MicroRNA Delivery by Innovative Inorganic Carrier

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BackgroundSuper carbonate apatite (sCA) nanoparticle is an efficient *in vivo* DDS for miRNA and siRNA (PlosOne 2015). We reported that sCA delivered efficiently siRNA for survivin and PTBP as well as miR-340 (Mol Cancer Ther 2015, 2015) to colon tumors implanted into mice. MiR-4689 and miR-29b-1-5p suppressed KRAS mutated colon cancer cells (Mol Ther *NA*2015, 2018) and miR-302 and miR-369 realized in vivo cancer reprograming (PlosOne 2015). In every case the RNA function was fully realized. Moreover, sCA exhibited the superb efficacy in augmentation of influenza vaccination with CpG nucleic acid (Frontiers in Immunol 2018) and in inhibiting inflammatory bowel disease with miR-29a and b (Mol Ther *NA*2018). These innovative findings are based on the unique ability of sCA in enhancement of EPR effect and reduction of tumor stromal pressure, which would help various substances in blood circulation to be accumulated into the tumor. In other words, sCA alone can collect the anti-tumor drugs to the tumors without incorporating them. We here show the distinct usage of sCA, which serves to collect the chemo-agent oxaliplatin, polymer conjugate chemo-agent (P-THP), liposome-microRNA complex to the tumors, and also provides application to minimally invasive treatment of photodynamic therapy (Mol Cancer Ther 2018).
Methods and Results Tumor IFP of colon cancer HT29 xenografts in mice was measured under anesthesia using pressure measurement systems (Transonic Science) with 1.6 Fr fluied-filled catheter. We found that the tumor IFP at 2.5 h - 4 h after the intravenous injection of sCA markedly decreased to 13.4 ± 1.8 mmHg, which was significantly lower than that of control group (30.9 ± 2.9 mmHg, $P < 0.0001$). To

further investigate whether a decrease in IFP in tumor tissues would really lead to enhanced tumor uptake of agentssupplied from the tumor vessels,sCA and indocyanine green(ICG) were simultaneously administered via the separate routes to the mice bearing HT29 tumor; sCA was administered i.v. (intravenously) and ICG was administered i.p. (intraperitoneal). We found by IVIS imaging system that sCA enhanced intratumoral uptake of ICG, whereas atelocollagen (Atelo) did not have such effects (Figure 1A). Time course studies showed that ICG levels in tumors with treatment of sCA plus ICG were significantly higher throughout the examined time points than those treated with ICG alone (P= 0.0142 for 4 h, P= 0.0139 for 8 h; Figure 1B). *Ex vivo* imaging of the tumors treated with sCA (i.v.) + ICG (i.p.) at 8 h post administration confirmed higher fluorescence intensity than the tumors treated with ICG alone (i.p.) (Figure 1C). **Conclusion**Our data suggest that sCA itself, while designed as an *in vivo*drug/siRNA delivery device, can reduce tumor interstitial fluid pressure and enhance the uptake of agents in the blood stream into tumors.

Presidential Symposium & Presentation of the Top Abstracts

652. ASPIRO Phase 1/2 Gene Therapy Trial In X-Linked Myotubular Myopathy (XLMTM): Update on Preliminary Safety and Efficacy Findings

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Background: XLMTM is a rare monogenic disease caused by mutations in the *MTM1* gene, which encodes myotubularin, a protein required for normal development and function of skeletal muscle. XLMTM is characterized by extreme muscle weakness, respiratory failure and early death. **Methods:** ASPIRO is an ongoing Phase 1/2 open-label, randomized, ascending dose study to evaluate the safety and efficacy of an investigational gene therapy product (AT132) in patients with XLMTM. AT132 (rAAV8-Des-hMTM1) is designed to deliver functional copies of the *MTM1* gene to skeletal muscle cells. ASPIRO was designed for XLMTM pts <=5 years, randomized onto drug or delayed control, and enrolled into ascending dose cohorts to receive a single AT132 infusion. **Results:** At the time of 26Sep18 data cut, safety and efficacy data from 4-48 weeks of follow-up were reported for 8 patients, including 7 patients in Cohort 1 ($1x10^{14}$ vector genomes per kilogram (vg/kg); 6 treated and 1 untreated control) and the sentinel patient in Cohort 2 ($3x10^{14}$ vg/kg), as well as Week 24 muscle biopsy data for the first 4 treated patients. All treated patients showed meaningful improvements in neuromuscular and respiratory function; with increased limb and trunk strength; improved velocity, coordination, and movement accuracy; and improved ability to communicate (increased loudness during vocalization and crying). In addition, there have been improvements in airway clearance, secretions management and swallowing capability, to the extent that patients are now beginning to tolerate oral food. Efficacy assessments have shown clinically meaningful improvements in both the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) scale and maximal inspiratory pressure (MIP) (Table). Notably, patients achieved scores close to the maximum CHOP INTEND score of 64, a level of functional performance typically achieved in a healthy child by 3-6 months of age. All patients had attained the ability to sit without support, one patient was crawling, and two patients were standing with support and making stepping movements. At 24 weeks, all patients assessed showed significant reductions in ventilator use, with three patients reaching ventilator independence. All of these improvements are clinically meaningful and inconsistent with the natural course of this disease. Muscle biopsy results were consistent with marked functional improvements, demonstrating robust tissue transduction and myotubularin expression, and considerably improved muscle fiber morphology. The safety profile of AT132 has been manageable: a total of seven serious AEs occurred, five in Patient 3 of which four were possibly or probably related to treatment. Subsequent to the data cutoff date, the remainder of Cohort 2 has been enrolled. Updated safety and efficacy data will be presented at the 2019 ASGCT Annual Congress.

ASPIRO Cohort 1 (1e14 vg/kg) & Cohort 2 (3e14 vg/kg): Baseline and Follow-up.

Patient Information		Neuromuscular Assessment		Respiratory Assessments		Biopsy	
Cohort (C) / Patient (Pt) #	Age at Baseline (years)	CHOP INTEND: Baseline / Last Report	Δ From Baseline	MIP (cm H_2O): Baseline / Last Report	Δ From Baseline	VCN per diploid genome	MTM1 expression vs. Normal
C1 / Pt1	0.8	29 / 56 (Wk 48)	+27 (93%)	33 / 89 (Wk 24)	+56 (170%)	6.2 (Wk 24)	~120%
C1 / Pt2	4.1	45 / 64 (Wk 48)	+19 (42%)	44 / 112 (Wk 48)	+68 (155%)	7.1 (Wk 24)	~250%
C1 / Pt3	2.6	34 / 34 (Wk 36)	0 (0%)	26 / 70 (Wk 24)	+44 (170%)	2.7 (Wk 24)	~80%
C1 / Pt4 (Control)	4.0	49 / 47 (Wk 36)	-2 (-4%)	58 / 46 (Wk 24)	-12 (-21%)	NA	NA
C1 / Pt5	1.0	36 / 53 (Wk 24)	+17 (47%)	14 / 78 (Wk 24)	+64 (457%)	2.2 (Wk 24)	~52%
C1 / Pt6	0.8	39 / 52 (Wk 16)	+13 (33%)	35 / 87 (Wk 12)	+52 (149%)	NA	NA
C1 / Pt7	0.9	43 / 53 (Wk 16)	+10 (23%)	29 / 68 (Wk 12)	+39 (134%)	NA	NA
C2 / Pt8	1.2	36 / 55 (Wk 4)	+19 (53%)	31 / 67 (Wk 4)	+36 (116%)	NA	NA

653. Liver-Targeted Lentiviral Gene Therapy Achieves 100% of Normal Circulating FVIII Levels in Non-Human Primates

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Lentiviral vectors (LV) are gaining prominence as a gene delivery vehicle due to their large cargo capacity, rare incidence of pre-existing anti-LV antibody in patient population, and ability to sustain transgene expression via integration. In addition, LVs have also been evaluated in numerous ex-vivo cell therapy clinical programs with promising efficacy and safety profiles. To assess the systemic gene delivery potential of LV, we have developed LV with hepatocyte specific expression cassette encoding human B-domain deleted Factor VIII (FVIII) and have evaluated these LV in Hemophilia A (HemA) mice and non-human primates (NHPs). To improve FVIII expression, FVIII cDNA was codon optimized (coFVIII), and a DNA fragment encoding a non-structured, 144-aa XTEN polypeptide, known to increase the half-life and expression level of the payload protein, was incorporated into the B-domain region of FVIII (coFVIIIXTEN). We have shown in NHPs, LV with high surface level of human CD47 (hCD47) mediates 3-fold higher transgene expression compared to LV with basal level of hCD47, thus, a HEK-293T cell line that over expresses hCD47 and devoid of MHC-I complex was used to produce corresponding third generation, VSVG pseudotyped, LV-coFVIII and LV-coFVIIIXTEN. LV-coFVIII or LV-coFVIIIXTEN was administered into neonatal HemA mice or adult NHPs via intravenous injection and circulating FVIII level post LV treatment was monitored by human FVIII specific activity and antigen assays. Persistent FVIII expression was observed post-LV treatment of 2 day old HemA mice throughout the 6-month study period despite a greater than 10-fold increase in body weight of the treated animals. In theory, this could extend the eligible patient population of LV treatment to include pediatrics, a clear advantage over non-integrating viral vectors. At the same LV dose level, compared to unmodified FVIII transgene, coFVIII and coFVIIIXTEN conferred 100-fold or 500-fold improvement on plasma FVIII levels, respectively, which translates into a more than 10-fold reduction on LV usage. This dose reduction could lower the potential toxicity associated with LV treatment. LV-coFVIII and LV-coFVIIIXTEN were further evaluated in a dose response study in NHPs. Administration of LV via peripheral vein was well tolerated with no significant changes in body temperature and physical activity of the animals. While ALT levels remained within normal range, dose dependent, transient, self-limiting leukopenia and AST elevation were observed. LV-FVIII has a linear dose response profile in NHPs, inclusion of XTEN further increases FVIII expression similar to what observed in mice. FVIII levels in the therapeutic range were achieved in all treated animals, with immune suppression. At 3E9 TU/kg dose, LV-coFVIIIXTEN treatment resulted in 60-100% of normal circulating FVIII, a FVIII activity range considered sufficient to achieve normal hemostasis. Gene integration is a unique property of LV which is lacking from most of the gene therapy programs that are currently under clinical investigation for hemophilia, and in conjunction with the rare incidence of pre-existing anti-VSVG-LV antibodies, our data support the further development of the LV system as a potential systemic gene delivery vehicle for hemophilia patients.

654. Gene Therapy for the Treatment of AADC Deficiency in Children using MRI-Guided Convection-Enhanced Delivery to the Midbrain

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Aromatic L-amino acid decarboxylase (AADC) deficiency is a rare autosomal recessive genetic disorder that causes deficient synthesis of dopamine and serotonin. The condition typically presents in infancy with hypokinesia, hypotonia, oculogyric crises (OGC), dystonia, autonomic symptoms, sleep disruption, and motor developmental delay. Most patients derive little or no benefit from currently available medical therapies.In this dose escalation safety and efficacy trial, adeno-associated virus serotype 2 (AAV2)-hAADC was delivered to the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). Our MR-guided technique permits vector delivery to the target with submillimeter accuracy, with the ability to monitor and adjust infusate delivery in real-time. Six children (3 female and 3 male; ages 4-9 years) were treated with a low (Cohort 1) and a high (Cohort 2) dose of vector (1.3×10^{11} and 4.2×10^{11} vector genomes). We previously reported the results for Cohort 1 at 6 months after surgery. At the time of this submission, follow-up duration after treatment is 12-18 months for subjects in Cohort 1, and 1-6 months for subjects in Cohort 2. We present results for the first 5 subjects, who have been followed for 6-18 months.Real-time MR data confirmed accurate targeting and 70-100% coverage of the SNc/VTA. There have been no adverse events related to the surgical intervention. All children developed mild to moderate involuntary movements (dyskinesia) that peaked in severity 1-2 months after surgery and then improved. OGCs completely resolved in 4/5 subjects, and the remaining subject experienced a significant reduction in the severity and duration of episodes. Sleep and mood markedly improved in all 5 subjects. All subjects had severe motor impairment at baseline (Gross Motor Function Measure (GMFM-88) scores: 0-15), and all have achieved recognizable gains in head control and voluntary movement at 6-18 months. Two subjects have attained the ability to sit independently, weight-bear fully while standing, take steps with support, and reach and grasp with both hands. One subject has attained the ability to speak single words and to eat by mouth. CSF homovanillic acid (HVA) increased in all subjects from <10% at baseline to 24-47% of the lower limit of normal 6-12 months post-gene transfer, consistent with increased brain dopamine synthesis. ^{18}FDOPA PET demonstrated increased uptake in the midbrain, caudate and putamen.Our findings demonstrate that AADC gene transfer to SNc/VTA is safe, and leads to improvement in dystonic symptoms, mood, sleep, and motor function in children with AADC deficiency. We hypothesize that selecting nuclei in the midbrain that normally innervate the striatum (nigrostriatal pathway) and nucleus accumbens (mesolimbic pathway), may result in more physiologic dopaminergic transmission than targeting (non-dopaminergic) medium spiny neurons in the striatum for gene delivery.

655. Combinational T Cell Genome Modifications through Vector Coupled and Uncoupled Chemical Deamination

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Genome editing of T cells has broadened the clinical scope of chimeric antigen receptor (CAR) treatments, including bypassing critical barriers when using allogeneic T cells from mismatched donors. Proof of concept studies are underway using transcription activator-like effector nuclease (TALEN) edited 'universal' T cells devoid of TCRab expression and depleted of CD52, the target antigen for the lymphodepleting antibody Alemtuzumab. Similar effects can be achieved using clustered regularly interspaced short palindromic repeat (CIRPSR) / Cas9 editing tools, and efficient multiplexed effects against multiple genomic targets can be achieved. However, these approaches rely on nuclease mediated cleavage and cell mediated repair of double stand DNA (dsDNA) breaks, which trigger apoptosis pathways and increase the risk of chromosomal translocations. Base editing through targeted chemical deamination circumvents the need for nuclease mediated dsDNA breaks for gene disruption, but instead offers the possibility of seamless, nucleotide conversion to create disrupted coding sequence. sgRNA sequences targeted candidate regions of TCRα and β genes, B_2M and CD52 genes to disrupt splice sites or create premature stop codons. These were initially assessed individually in a CRISPR-CAR coupled lentiviral configuration which incorporated guide expression cassettes into the viral 3'LTR. Transient exposure by electroporation of transduced cells to mRNA delivering codon optimized base editor 3 (coBE3) resulted in high levels of 'on-target' base conversion, with results comparable to those achieved by spCas9 mRNA for NHEJ mediated gene disruption. Coupled TCR disruption enabled magnetic bead mediated depletion of residual TCRab T cells, and provided highly enriched CAR+TCR- populations. Thereafter, combinational delivery of additional coupled and uncoupled sgRNAs during electroporation was investigated for multiplexed effects. Primary T cells (n=4) could be modified to be (83%-92%) triple knockout for TCR/CD52/B_2M in the CAR+ population which following TCR- enrichment would correspond to high levels of CAR expression (87%-95%). Whereas spCas9 treated samples exhibited low levels of translocations involving the targeted chromosomal arms, similar events were not confirmed in coBE3 modified T cells using PCR based screening. Additional characterization of CAR T cells with multiple deamination mediated edits is underway, alongside scalability testing as part of preparations for translational applications.

Gene Therapies Directed at the Immune System

656. Optimized CRISPR/Cas9 Editing Strategy for the Correction of CD40LG Gene in T Cells and Hematopoietic Stem Cells

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X linked hyper IgM syndrome (HIGM1) is caused by mutations of *CD40LG*, whose absence in CD4 T cells impairs their helper signaling necessary for B cell activation and immunoglobulin class switching. Since its unregulated expression leads to lymphoproliferations/ lymphomas, we aimed to correct *CD40LG* while preserving its physiologic regulation. Corrected autologous T cells could provide immediate therapeutic benefit to patients by resolving pre-existing infections and bridge them towards a definitive cure by hematopoietic stem/progenitor cell (HSPC) transplant. To confirm this, we infused different doses of wild type (WT) T cells into HIGM1 mice, pre-conditioned or not with different lymphodepleting regimens. These mice showed long term and stable T cell engraftment, with the highest rate obtained in mice pre-treated with chemotherapy regimen (cyclophosphamide), and partially rescued antigen specific IgG response and germinal center formation in splenic follicles after vaccination with a thymus dependent antigen (TNP-KLH). Thus, we optimized on human T cells a CRISPR/Cas9 gene editing strategy, based on ribonucleoprotein electroporation and AAV6 transduction, to insert a corrective *CD40LG* cDNA within the first intron of the endogenous gene and correct most of disease causing mutations with the same set of highly specific reagents. By exploiting a stimulation protocol that preserves long term surviving T stem memory cells, we tested different configurations of the corrective donor and reproducibly obtained ~35% of editing efficiency in both healthy donor and patients derived T cells. While all donor configurations restored regulated CD40L expression, only codon optimized *CD40LG* sequence allows reaching ~60% of the physiologic expression level. By performing T-B cells co-culture assays, we confirmed that this expression level is sufficient to restore CD4 T cells ability to provide normal contact dependent helper function to B cells, as assessed by measuring their *in vitro* proliferation, class switching, and IgG secretion. Since HIGM1 patients are susceptible to autoimmunity and there is limited space in the lymphoid niche for T cell engraftment, we aimed to increase the purity of edited cells before infusion by coupling the corrective cDNA with a clinically compatible selector gene. With this strategy, we recapitulated previous results of efficiency and, surprisingly, we found that this longer transcript allows higher CD40L expression that was now comparable to that of healthy controls. After selection, we confirmed that edited T cells are able to engraft and persist in NSG mice similarly to the bulk and untreated counterparts. To possibly

provide an even broader and prolonged therapeutic benefit to patients who are amenable to HSPC transplantation, we then adapted our gene correction strategy to target autologous HSPC. First, we evaluated the threshold of correction required for therapeutic efficacy by performing competitive transplants of WT and *Cd40lg-/-* HSPC in HIGM1 mice and found that as low as 10% of WT HSPCs are sufficient to partially restore serologic immunity against different antigens (TNP-KLH, OVA). By exploiting our recently optimized gene editing protocol on human HSPC, we attained this threshold in long term repopulating cells, obtaining up to 50% of *CD40LG* editing in bulk treated CD34+ cells and 30% after xenotransplantation in NSG mice. Overall, our work establishes the rationale and guiding principles for clinical translation of T cells and HSPC gene editing for treating HIGM1.

657. Gene Editing Models and Remedies Severe Congenital Neutropenia in Healthy Donor and Patient CD34+ HSPCs

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Severe congenital neutropenia (SCN) is a life-threatening disorder of insufficient granulocyte number, which despite G-CSF therapy carries substantial risk of MDS and AML. The most common etiology is dominant mutationof the *ELANE* gene, altering the sequence of the neutrophil elastase protein product causing impaired protein folding and/or trafficking, which results in excess cell death of neutrophil precursors at the promyelocyte stage. Most SCN *ELANE* mutations are missense, although frameshift mutations within exons 4 and 5 predicted to escape nonsense mediated decay (NMD) have also been associated with SCN. We hypothesized that introduction of premature termination codons (PTCs) by nuclease-mediated frameshift mutations within early exons of *ELANE* could constitute a universal, highly efficient, simple therapeutic approach for *ELANE*-associated SCN. We predicted that an early PTC would trigger NMD of the mutant transcript resulting in its loss of expression and thus bypassing neutrophil precursor cell death. The mild phenotype of combined neutrophil serine protease deficiency (Papillon-Lefevre syndrome) supports the safety of this strategy. We delivered 3xNLS-SpCas9 and *ELANE* targeting sgRNA as ribonucleoprotein (RNP) complex to healthy donor human CD34+ hematopoietic stem and progenitor cells (HSPCs) and conducted *in vitro* neutrophil maturation. We observed that targeting exon 5 efficiently introduced indel mutations but did not trigger *ELANE* NMD. Cells bearing *ELANE* exon 5 frameshift mutations were blocked at the promyelocyte stage of development, similar to clinical observations (Figure 1). In contrast, introducing frameshift indels at exon 2 of *ELANE* efficiently triggered NMD, with >75% loss of transcript expression. These edited cells were fully competent for neutrophil maturation. By sequential electroporation (thus avoiding interstitial deletion) we found that frameshift mutations at *ELANE* exon 2 could bypass maturation arrest caused by frameshifts at *ELANE* exon 5. Finally, using CD34+ HSPCs from three *ELANE* mutant SCN patient donors, we demonstrated that exon 2 targeting RNP achieved highly efficient editing (98-99% indel frequency), triggered *ELANE* transcript decay (77-95% reduction of expression), and overcame promyelocyte stage maturation arrest (with 2-4 fold increase of *in vitro* derived neutrophil output). These results demonstrate a first-of-kind SCN model in primary human neutrophil precursors with which to explore the molecular pathobiology and therapeutic strategies. This approach provides a potential pathway for the development of primary hematopoietic cell models for many genetic blood disorders for which access to patient material may be limiting. In addition these results support the development of *ELANE* early exon targeting as a highly efficient universal therapy for *ELANE* mutant SCN that is feasible with existing gene editing technology.

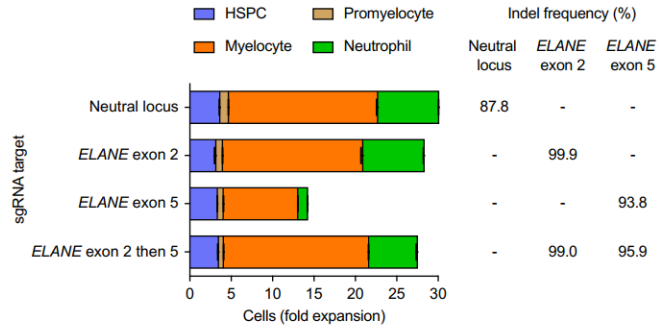

Figure 1. CD34+ HSPCs were subject to RNP electroporation (or sequential electroporation 48 hours apart) followed by liquid culture in G-CSF for 10 days. Cell distribution determined by flow cytometry CD34, CD117, and CD16 immunophenotype. Indel frequencies determined by Sanger sequence deconvolution.

658. A Potential CRISPR/Cas9-Based Gene Therapy for Severe Congenital Neutropenia

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Severe congenital neutropenia (SCN) patients lack matured neutrophils in their peripheral blood leading to recurrent infections. Autosomal dominant negative mutations of the ELANE gene account for more than 50% of SCN cases. CRISPR/Cas9-mediated gene correction in autologous human hematopoietic stem/progenitor cells (HSPCs) is a potential alternative for allogeneic bone marrow transplantation with its high risk of graft-versus-host disease. Here, we developed two independent approaches to rescue the disease phenotype of SCN patient-derived HSPCs carrying a novel mutation (ELANE-L172P) that blocks neutrophil differentiation at the promyelocyte stage. As the L172P mutation (CTC to CCC) generates a new PAM signal, we were able to design a guide RNA (sgRNA) which efficiently targeted and inactivated the mutant allele without affecting the wildtype one. On top of that, when providing a repair template via AAV6 infection, the mutant allele was corrected in up to 40% of the HSPCs. In addition, we established an efficient CRISPR/Cas9-based system

to repair mutational hotspots in exon 4 of the ELANE gene, using an sgRNA targeting both mutant and wildtype alleles, and AAV6 for repair template delivery. Both the mutant allele-deleted and mutant allele-corrected HSPCs differentiated into mature neutrophils *in vitro* as efficiently as normal HSPCs. Taken together, we describe a potential gene therapeutic approach for SCN patients.

659. In-Vivo Gene Therapy for Canine X1-SCID via Delivery of Cocal Enveloped Lentiviral Vector Expressing γ-Chain

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X-linked severe combined immunodeficiency (X1- SCID) is an inherited genetic life-threatening disease associated with mutations in the common cytokine receptor γ chain (γc) gene. X1- SCID is universally fatal within the first year of life unless restoration of the immune system via bone marrow transplantation (BMT). Hematopoietic stem and progenitor cells (HSPC) *ex-vivo* gene therapy utilizing viral vectors have been used in multiple clinical trials as a surrogate measure to circumvent the complications associated with BMT. Despite the undeniable therapeutic benefits offered by HSPC gene therapy treatment for X1- SCID, this approach poses several limitations which includes requirement of conditioning, lifelong administration of immunoglobulins, safety concerns due to vector toxicity (insertional mutagenesis) and requirement of GMP facility for production of the cell product. Considering these impediments, we previously developed a novel and accessible *in-vivo* gene therapy approach using Foamyviral vector (FV) without prior conditioning in a canine model of X1- SCID. In the current study, we further validated and broaden our *in-vivo* gene therapy approach for canine X1- SCID by investigating a third generation cocal envelope pseudotyped Lentivirus vector (cocal-LV) expressing human γc. We hypothesized that cocal-LV may help improve *in-vivo* targeting of HSPCs and multilineage gene correction since it results in higher transduction of human primary cells *in-vitro* as compared to FV or VSV-G enveloped LV. Similarly to FV, cocal-LV resist serum inactivation as compared to the commonly used VSV-G LV, which may prove beneficial in the context of *in-vivo* gene therapy. Clinical translation of this approach and large scale vector production will also be facilitated with cocal-LV since a producer cell line has previously been generated. We achieved rapid immune-reconstitution by intravenous delivery of a therapeutic cocal-LV (cocal LV-PGK- γC) vector into G-CSF and plerixafor mobilized X1- SCID neonatal canines. Within 6 weeks post-injection levels of CD3+T cells reached therapeutic levels with >90% gene correction in peripheral T-lymphocytes (Figure 1). The kinetics of immune reconstitution in the first 2 months is very much in corroboration with our FV mediated *in-vivo* gene therapy (FV-PGK- γC). Long-term follow-up studies will include validation of T-cell functionality, lentiviral integration site analysis, qualitative and quantitative measurement of antibody responses. Thus far our preliminary data suggest that *in-vivo* gene therapy with cocal LV-PGK- γC could be the preferred platform for the treatment of human X1- SCID.

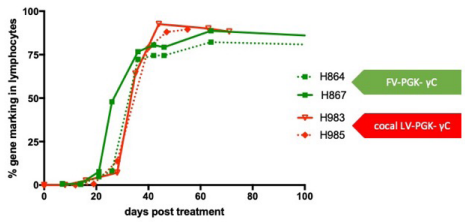

Figure1: Kinetics of gene marking based on fluorophore expression in circulating lymphocytes from dogs treated with Intravenous administration of Foamyviral vector (FV-PGK-γc) and Cocal pseudotyped Lentiviral vector (Cocal-LV-PGK- γc).

660. Gene Editing to Enforce FOXP3 Expression and a Rapamycin-Inducible IL-2 Signaling Complex in Human Primary T Cells Allows Selective Expansion of Immunosuppressive Treg-Like Cells

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Regulatory T cells are a distinct T cell lineage that suppress adaptive immune responses. Their importance in maintaining immune self-tolerance is evidenced in IPEX patients, who rapidly develop multiple severe autoimmune diseases caused by the lack of regulatory T cells due to *FOXP3* mutations. FOXP3 is a "master regulator" transcription factor that is required for the acquisition of regulatory T cell phenotypes. There is evidence that regulatory T cell defects may have roles in common autoimmune diseases such as Type I diabetes and Lupus. Numerous strategies are thus being developed to improve the numbers or functions of regulatory T cells, including *ex vivo* expansion and re-infusion of autologous regulatory T cells. This latter approach is limited by the biology of regulatory T cells: they comprise a low proportion of peripheral lymphocytes, are rapidly outgrown in normal culture conditions by other T cells, expand slowly in culture, and have unstable FOXP3 expression. We have previously shown a novel homology-directed repair-based gene editing strategy to generate regulatory-like T cells (edTreg) from bulk peripheral blood CD4 cells, using CRISPR/CAS9 and an AAV6 delivered donor template to stably express FOXP3. These edited cells express characteristic markers of regulatory T cells, down-regulate expression of inflammatory cytokines, and suppress expansion of activated effector T cells *in vitro* and *in vivo*. We have also previously demonstrated the development of a synthetic cell surface receptor we have termed a chemically-induced signaling complex (CISC), that delivers an intracellular IL-2 signal when dimerized by the drug rapamycin. In this study, we determined whether co-expression of FOXP3 with the IL2R-CISC could provide a selective advantage for edited vs. non-edited cells in the presence of rapamycin, improving *in vitro* manufacturing and/or *in vivo* expansion. Here we show a potentially manufacturing-scalable gene editing methodology for generating human CD4 T cells co-expressing FOXP3 and IL2R-CISC, resulting in a >90% pure population and ~20-fold expansion after two

weeks in rapamycin-containing cytokine-free media. When transferred into immunodeficient mice, these cells were selectively expanded in mice that were administered rapamycin. CISC edTreg were also able to suppress inflammatory T cell responses when co-administered with effector T cells, relative to mock-edited T cells. These results show the promise of this strategy for generating a regulatory T cell therapy. Further work is ongoing to determine the longevity and function of CISC edTreg *in vivo* in animal models, both with and without dimerizer therapy.

661. Immunoregulatory Cell Therapy with Lentiviral-Mediated FOXP3 Converted CD4+ T Cells into Treg Cells: Towards the Proof-of-Concept Application in IPEX Syndrome

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FOXP3 is an essential transcription factor for the regulatory T cell (Treg) function and therefore is a key regulator for the tolerance maintenance. Treg cell therapy, either with freshly isolated or expanded *in vitro*, has been proven to be safe but still remains challenging because of difficulties in isolating sufficient number of pure Treg cells, stability of the expanded preparations and Treg plasticity *in vivo* under pro-inflammatory conditions. The key role of FOXP3 and Treg cells in immune function is exemplified by immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a monogenic primary immune regulatory disease caused by mutations in *FOXP3*, that lead to impaired Treg cell function and early onset autoimmune manifestations.Our recent international retrospective study demonstrated suboptimal disease-free survival from current therapeutic options including immunosuppression and allogeneic hematopoietic stem cell (HSC) transplantation (Barzaghi et al, JACI, 2018) and therefore there is a great unmet medical need in patients with IPEX syndrome. We investigated lentiviral FOXP3 gene transfer (LV-FOXP3) in CD4+ T cells as an innovative approach to generate in large number stable Treg cells that could provide therapeutic benefit to IPEX patients lacking functional Treg. We have previously shown that LV-FOXP3 successfully converts IPEX patients-derived CD4+ effector T cells into Treg-like cells ($CD4^{LV\text{-}FOXP3}$ T cells) (Passerini et al, Sci Transl Med, 2013). To better investigate the safety and efficacy of this approach and to make it suitable for large scale GMP generation of $CD4^{LV\text{-}FOXP3}$ T cells, we optimized the original lentiviral vector and we further assessed the phenotype and function of $CD4^{LV\text{-}FOXP3}$ T cells both *in vitro* using transcriptome analysis and *in vivo* in different humanized mice models. $CD4^{LV\text{-}FOXP3}$ T cells express key molecules in common with freshly isolated Treg cells. $CD4^{LV\text{-}FOXP3}$ T cells can significantly extend the survival of *xeno* GVHD model mice, both in autologous and allogenic conditions ($p<0.01$) and they can also suppress *xeno* GVHD reaction caused by secondary infusion of responder cells ($p<0.05$). These *in vivo* data are clearly showing that $CD4^{LV\text{-}FOXP3}$ T cells have a sustained suppressive function in the various autologous *in vivo* mice models. In conclusion, we completed the preclinical requirement for the use of the $CD4^{LV\text{-}FOXP3}$ cell product in IPEX syndrome. The Proof-of-concept trial in a severe monogenic pediatric disease will open to future clinical applications of $CD4^{LV\text{-}FOXP3}$ cell therapy in other autoimmune and immunodysregulatory disorders of different origin.

662. Engineering B Cells as an Evolving Drug to Fight HIV

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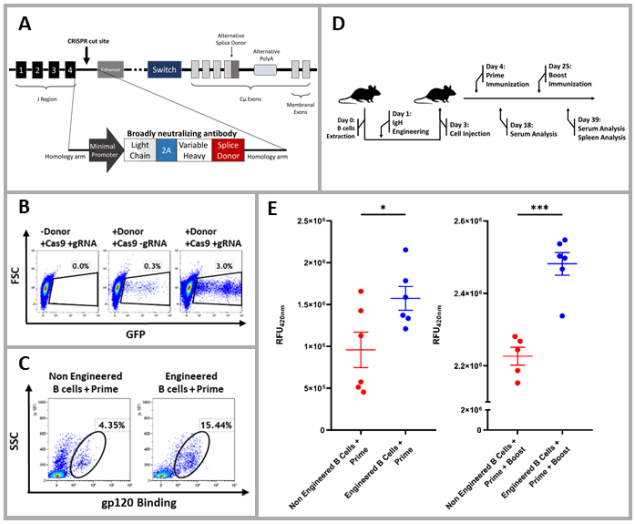
HIV viremia can be controlled by chronic antiretroviral therapy. However, treatment tolerability and adherence remain a challenge. Combination therapies of broadly neutralizing antibodies (bNAbs) can suppress viremia, but they may have to be chronically administered at a higher cost. bNAbs may be constitutively expressed from muscle, liver or mucosa following AAV transduction, but the antibody is of a single isotype, emergence of resistant strains is probable in lack of affinity maturation, and anti-drug antibodies (ADA) may develop due in part to improper glycosylation. In contrast, bNAbs integrated at the IgH locus in transgenic mice and human B cells were shown capable of undergoing class switch recombination (CSR) and somatic hyper mutation (SHM) and may be less prone to ADA. Here, we develop IgH engineering in B cells as a therapeutic approach for fighting HIV infections. We use CRISPR/Cas9 and AAV to introduce the anti-HIV bNAb 3BNC117 or GFP at the IgH locus of B cells. In particular, we target the intronic sequence downstream to the variable region and upstream to the IgM switch region (Figure A). Using TIDE and T7E1 assays we find cleavage efficiencies of up to 21% in a model human cell line and up to 33% and 15% in murine cell lines and activated murine splenic lymphocytes, respectively. The introduced cassette is flanked by appropriate homology arms and includes a derivative of a murine IgH promoter that is active only upon on-target integration in proximity to the endogenous enhancers. Indeed, in a model cell line, up to 10% of cells fluoresced when transduced with the GFP-encoding AAV, but only when co-electroporated with CAS9 and the correct gRNA. Up to 3% fluorescence was seen in primary splenic lymphocytes that were activated with LPS prior to the RNP electroporation and AAV transduction. Correct integration was verified by PCR and Sanger sequencing. Next, we used therapeutic cassettes coding for the anti-HIV bNAb 3BNC117. The light chain is coded in full, followed by a 2A peptide and the variable part only of the 3BNC117 heavy chain. The cassette then ends with a splice donor site, allowing splicing with endogenous Ig constant exons in an isotype-dependent manner. ELISA and RT-PCR demonstrated accurate splicing upon cassette integration in different cell lines as well as class switch recombination (CSR) in activated splenic lymphocytes. Flow cytometry showed bNAb expression as a B cell receptor (BCR) in up to 9% of cells in culture and up to 4% of activated splenic lymphocytes. Engineered cells could then further be activated by the HIV glycoprotein gp120, as evident from ERK phosphorylation. Importantly, adoptive transfer of the engineered cells into syngeneic mice allowed antigen-induced activation upon gp120 immunization. Post-immunization, germinal center B cells from mice injected with engineered cells had approximately twice as many gp120 binding B cells than mice injected with non-engineered cells. Importantly, mice with engineered cells generated a much higher serological response to gp120 than did control mice. Serological

response rose much further following boost immunization implying immunological memory and affinity maturation. Uniquely, our method enables antigen-induced bNAb secretion that may be further augmented by affinity maturation, class switch recombination, physiological glycosylation for diminished ADA, and the retention of immunological memory. B cells could thus be engineered as a living and evolving drug to counteract HIV escape.



A. Representative map of the targeting scheme B. CRISPR dependent targeting of a surrogate GFP cassette C. Accumulation of germinal center B cells binding gp120 in recipient mice D. Outline of the adoptive transfer and immunization experiment E. Serological response to Prime (left) and Boost (right) immunization

AAV Vector Biology II

663. Characterization of Assembly-Activating Protein (AAP) Variants among Natural AAV Isolates with High-Packaging Efficiencies

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The adeno-associated virus (AAV) genome encodes a unique 23 to 26 kDa protein called assembly activating protein (AAP) that is embedded within the ORF of capsid genes. AAP has been demonstrated to be the chaperone that mediates capsid stabilization, nucleolar transport, and assembly of the AAV capsid. AAP can be dissected into five functional domains: the hydrophobic N-terminal domain (HR), the conserved core (CC), the proline-rich region (PRR), the threonine/serine rich region (T/S), and the basic region (BR). The HR and CC domains are the determinants for viral protein recognition, and BR serves as the nuclear and nucleolar localization signal. Whether AAP can enhance genome packaging efficiency is currently understudied. We have recently identified a large library of natural AAV2 variant isolates from human surgical specimens. Approximately 27% of the AAV2 variants in our library exhibit greater genome packaging efficiencies than prototypical AAV2. Interestingly, analysis of this library revealed high variation within the AAP ORF, where the highest frequency of variation is within the HR and T/S regions. To determine whether the increase in packaging efficiency among this specific class of variants can be attributed to more robust AAP activities, we cloned AAPs from 12 capsids characterized with high packaging efficiencies, and co-expressed them in *trans* with a Rep2/Cap2 expression plasmid where AAP is knocked out. Surprisingly, we found that the AAP variants conferred similar amounts of packaged genomes compared to AAP from AAV2 (AAP2). We then selected a capsid that we have tentatively named AAVv229, which demonstrates 11-fold greater packaging efficacy than AAV2, and knocked out its ability to express AAP in *cis*. Co-transfection of this construct with AAP2 in *trans* resulted in a packaging efficiency that was very similar to that conferred by the fully intact AAVv229 construct. Taken together, we conclude that although AAP is required for capsid assembly, the variants identified in our library of natural isolates do not seem to improve genome packaging efficiencies during vector production. Importantly, this suggests that improving packaging efficiency of AAV vectors is independent of capsid assembly kinetics that is governed by AAP.

664. LY6A (SCA-1) Drives AAV-PHP.B Transport Across the Mouse Blood-Brain Barrier

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Efficient delivery of gene therapy vectors across the blood-brain barrier (BBB) is the holy grail of neurological disease therapies. A variant of the neurotropic vector adeno-associated virus (AAV) serotype 9, called AAV-PHP.B, was shown to efficiently deliver transgenes across the BBB in C57BL/6J mice. However, we recently observed that this phenotype is mouse strain dependent. Here, we used whole-exome sequencing-based genetics to map this phenotype to a specific haplotype of lymphocyte antigen 6 complex, locus A (*Ly6a)* (stem cell antigen-1, *Sca-1*). *Ly6a/Sca-1* encodes a glycosylphosphatidylinositol (GPI)-anchored protein whose function had been thought to be limited to the biology of hematopoiesis. Additional biochemical and genetic analyses definitively linked high BBB transport to the binding of AAV-PHP.B with LY6A (SCA-1). In conclusion, our study identifies LY6A as a receptor for AAV-PHP.B, and suggests a role for GPI-anchored proteins in BBB transport that could be hijacked by viruses in natural infections or by gene therapy vectors to treat neurological diseases.

665. Functional Consequences of Adeno-Associated Virus Capsid Deamidation

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Post-translational modifications (PTMs) can profoundly impact the efficacy and toxicity of protein-based therapeutics. Therefore, monitoring and controlling PTMs is critical for successfully developing and manufacturing many biologics. However, we currently know very little about the PTM landscape of adeno-associated viral vectors (AAVs). Here, we conducted a detailed investigation into recombinant AAVs of several serotypes using biochemical, structural, and mass-spectrometry approaches. We found that asparagine deamidation is common; at some sites, the extent of modification reaches nearly 100%. Primary-sequence and three-dimensional structural constraints explain the differing rates of deamidation. We observed that at sites with the sequence NG, deamidation occurred rapidly in all of the vectors we tested and was correlated with up to a 60% loss of *in vitro* transduction efficiency. We propose mutational strategies to stabilize AAV asparagine residues and discuss the impact of these mutations on manufacturing consistency and *in vivo* performance of the vectors.

666. Unbiased Proteomic Approach Identifies Novel Cellular Factors Involved in Aadeno-Associated Vector Production

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Producing sufficient vector quantity to meet patient need remains a practical yet highly relevant challenge for gene therapy. Augmenting cell lines and culture conditions to (1) maximize the number of cells producing vector and (2) maximize the quantity of vector produced per cell could exponentially increase vector yields from mammalian culture systems. Such an approach requires knowledge of the host cell factors that contribute either directly to assembly or indirectly to a permissive environment, or those that act as restriction factors. Host proteins with known roles in the AAV replication cycle are largely limited to entry factors, whereas the host cell machinery involved in assembly is almost completely unknown. This bias in knowledge is largely a function of experimental approaches employing purified vector preparations, which is the end product of assembly and thus unlikely to inform us on the production phases of the replication cycle. The AAV capsid is built from repeating structural subunits (VP proteins) and its assembly is dependent on the viral co-factor Assembly-Activating Protein (AAP). By expressing tagged VP1 and AAP in HEK293 cells, alone or in combination totaling eight different permutations, we examine co-precipitated cellular binding partners of monomers and assembly intermediates separately from fully assembled capsids. By Liquid Chromatography and Mass Spectrometry, we identify novel cellular interacting partners that bind only VP, those that bind only AAP, and those that bind preferentially to VP-AAP complexes. Expressing truncated versions of AAP increases the spatial resolution of some interactions. For example, Importin-5 and other proteins known to be involved in intracellular transport interact preferentially with the C terminal domain of AAP, which is in line with current literature describing NLS and NoLS sequences in this region. Initial functional validation also supports critical roles in production for Hsc/Hsp70 (and a specific subset of its cofactors), the ubiquitin carboxyl terminal hydrolase isoenzyme L1, and cyclin-dependent kinases 1 and 2. Further studies into these and other hits identified in our unbiased proteomics approach will shed light on AAV biology and assembly mechanisms and can directly inform cell line and culture conditions toward maximizing AAV vector production in mammalian cells.

667. Genome-Wide CRISPR/Cas9 Screening Identifies GPR108 as a Highly Conserved AAV Entry Factor

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Adeno-associated virus is a highly promising vector for therapeutic gene transfer, yet cellular determinants of AAV entry are poorly understood. We previously identified a subset of AAVs that do not use the canonical AAV receptor, AAVR, leading us to question what other cellular factors are involved in the AAV entry pathway of different serotypes. We employed a genome-wide CRISPR/Cas9 screen and identified a second highly conserved AAV entry factor, GPR108, required for all AAV variants tested aside from the most highly divergent capsid, AAV5. GPR108 knockout in multiple human cell lines demonstrate a 10 to 1,000-fold decrease in transduction of all serotypes, which can be rescued by GPR108 cDNA. Cellular binding assays demonstrate similar levels of attachment for all serotypes tested in GPR108 KO, AAVR KO, or AAVR/GPR108 double KO cell lines, relative to WT, suggesting a major role in AAV entry that is post-attachment. We additionally generated chimeric proteins between GPR108 and the highly similar GPR107, which demonstrate that both the N and C terminal domains are required for optimum function as an AAV entry factor. Importantly, GPR108 KO in the mouse hepa cell line causes roughly a 100-fold decrease in transduction by hepa-permisive AAV variants, and mouse GPR108 is functional to rescue a GPR108 KO in human Huh7 cells, demonstrating a high conservation of this AAV entry factor across species. Chimeric AAV capsids generated from GPR108-dependent AAV2 and GPR108-independent AAV5 demonstrate that GPR108 usage is determined by the VP1u portion of capsid, suggesting a conformational change must occur in capsid before GPR108 engagement, and highlighting a potential role for GPR108 in endosomal escape, as VP1u contains a highly conserved phospholipase domain. We demonstrate that this uncharacterized 7-TM domain protein localizes to the golgi, as demonstrated by co-localization with TGN46. These and previous studies on AAVR demonstrate that all AAVs appear to require trafficking to the TGN for efficient entry, suggesting the vector is maintained within the endomembrane system until very late in the entry pathway. We therefore have identified the

second highly conserved cellular protein required as an AAV entry factor, and suggest a novel mechanism by which the AAV capsid is maintained within the endomembrane system and requires a cellular factor, primarily GPR108, to efficiently traverse the cellular membrane and gain access to interior of the cell. Our studies have significant implications for vector design and implementation of these AAV-based gene therapies.

668. Identification and Validation of Novel Host Factors for AAV Infection

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Adeno associated virus (AAV) is a non-pathogenic virus that has been used extensively as a vector for gene therapy. The mechanisms by which AAV enters the cell, is trafficked to the nucleus and establishes transgene expression are poorly understood. In this study, we used a pooled CRISPR/Cas9 library directed against all human genes to identify positive and negative factors modulating AAV2 transduction. U-2 OS cells with an integrated CRISPR library were transduced with AAV2 encoding GFP and sorted based on the intensity of GFP expression. DNA was isolated from sorted cells and the sgRNA barcodes were sequenced to identify genes which may enhance (GFP high) or reduce virus transduction (GFP low or negative). Genome-wide screens with 80K (1 module, 4 sgRNAs/gene) and 155K (3 modules, 8 sgRNAs/gene) libraries produced similar results. In addition to novel findings, we observed overlap with a previously published insertional mutagenesis screen in HAP1 cells for AAV host factors. A subset of screen hits were validated in flow cytometry and imaging studies using stable knockout cell lines (2 sgRNAs/ gene) and GFP encoding viruses. KIAA0319L (AAVR) knockout cells were used as a positive control for a gene known to inhibit AAV transduction. In these validation studies, we confirmed the role of several new genes in mediating virus transduction in 8 different AAV serotypes. Interestingly, we also identified at least one gene that displayed serotype selectivity. In conclusion, pooled CRISPR screens are an effective tool for dissecting complex biological processes such as viral infection. Our screens confirmed KIAA0319L as an AAV receptor and provided new insights into the events involved in AAV infection.

669. Discovery of a Novel Frameshifted ORF within the AAV Capsid Gene

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Annotation of the AAV genome and study of its many functional elements has enabled the use of AAV for therapeutic purposes. However, many aspects of AAV biology remain poorly understood. After foundational work studying the role of Rep and Cap isoforms, the discovery of Assembly Activating Protein (AAP), a frameshifted ORF within Cap, improved our understanding of capsid transport and assembly. Other frameshifted ORFs, such as the X-gene, have been proposed, but their significance is unclear. To better understand AAV function, we created a scanning library of all possible single codon substitutions, insertions and deletions at all positions in the AAV2 Cap gene. One advantage of this comprehensive and unbiased approach to library generation is the potential to detect previously unknown gene products and other genetic elements. In particular, functions independent of coding for Cap could manifest as fitness differences among synonymous codons. Toward this aim, we devised a Frameshift Score (FS) metric to detect the presence of frameshifted ORFs by comparing the differences in packaging fitness observed among synonymous mutants with the presence or absence of stop codons in alternative reading frames. As expected, we observed a highly significant FS for AAP. In contrast, we detected no significant FS for positions within the X-gene. Searching for additional frameshifted ORFs within the entirety of Cap, we observed a highly significant FS representing a novel frameshifted ORF within the +1 frame of the VP1 region. Guided by differences in fitness for synonymous codons of Cap, we identified Cap nt 80-439 (VP positions 27-147) as the most likely location of the new ORF. We hypothesized that the ORF starts with CTG, a non-canonical start codon (similar to AAP). This novel ORF's start and stop codons are conserved across most other serotypes, suggesting a real and conserved function. Dual sequence constraints from the capsid protein and this ORF help to explain the strikingly high degree of conservation observed for this region, even outside of the VP1 PLA2 domain. Notably, NCBI pBLAST and psiBLAST of the 119 amino acid ORF sequence against the non-redundant protein database returned no proteins with significant homology. We confirmed translation of this ORF in its native context via western blot using a version FLAG-tagged at the C-terminus. AAV2 capsids assemble in the nucleolus, but intriguingly, anti-FLAG immunofluorescence imaging revealed that the protein was excluded from the nucleus and appeared to be membrane-associated. Based on these observations, we propose the name "membrane-associated accessory protein" (MAAP). To confirm a functional role for MAAP, we repeated the original production experiment but this time expressed MAAP in trans. The addition of MAAP in trans rescued the packaging abilities of VP mutants containing stop codons within MAAP. Importantly, MAAP stop codon mutants were not rescued by MAAP negative controls. These experiments indicate a role for MAAP in virus production. Further characterization of this new gene's function will improve our understanding of AAV biology, while also enhancing our ability to engineer novel functional capsids and to produce highly potent recombinant AAV for therapeutic uses.

Neurological and Neurosensory Gene Therapy

670. Optimizing In Vivo NGLY1 Gene Delivery Towards Gene Therapy for NGLY1 Deficiency

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NGLY1 (*N*-glycanase 1, also known as peptide-*N*-asparagine amidase or PNGase) is a deglycosylating enzyme that removes *N*-glycans from glycoproteins. NGLY1 deficiency, caused by loss-of-function mutations in the *NGLY1* gene, is an ultra-rare genetic disorder. Patients suffer from developmental delay, low muscle tone, seizures, lack of tears, elevated liver transaminases in childhood, and movement disorder. There are currently no treatment options available other than supportive care. We hypothesize that a gene replacement therapy to restore NGLY1 function primarily in the central nervous system (CNS) could alleviate associated disease symptoms. In this study, we aim to optimize the *in vivo* gene delivery of *NGLY1* using recombinant adeno-associated virus (rAAV) as a stepping stone to the pre-clinical evaluation of therapeutic efficacy in disease rodent models. We generated two constructs expressing a codon-optimized human *NGLY1* cDNA, driven by either the CB or the JeT promoter, respectively. Although weaker, the small size of the JeT promoter allowed us to package JeT-NGLY1 as a self-complementary (sc) rAAV genome, a potential advantage for *in vivo* gene delivery using rAAV. Using *Ngly1*-null mouse embryonic fibroblasts (MEFs) and plasmid transfection, we showed that the plasmid carrying CB-NGLY1 yielded 5-fold higher NGLY1 expression than the one carrying JeT-NGLY1, as determined by western blot. We chose to test both the AAV9 and AAVPHP.B capsids due to their known ability to broadly target CNS tissues following systemic delivery in C57BL/6 mice. We next treated age- and sex-controlled young C57BL/6 adult wild-type mice with the same dose ($1x10^{12}$ vg total) of each vector by tail vein injections, and quantified rAAV genome biodistribution (gene delivery) and NGLY1mRNA expression (transduction) in various tissues by droplet digital PCR (ddPCR). The scAAV9.JeT and ssAAV9.CB vectors performed equally well with respect to gene delivery in all tissues examined. Similarly, in the CNS both vectors were comparable with respect to NGLY1 transgene expression, suggesting that the sc genome configuration compensated for the weaker JeT promoter strength. However, the scAAV9.JeT vector was 10-fold less efficient than the ssAAV9.CB vector in driving NGLY1 expression in the heart and skeletal muscle. In addition, consistent with previous reports, AAVPHP.B yielded 1-2 logs higher gene delivery and transduction than AAV9 in the CNS for ssAAVPHP.B.CB. In preliminary experiments using *Ngly1*-null mice, we detected rAAV-borne NGLY1 protein expression in peripheral tissues, but not in the CNS, likely due the suboptimal dose used in this pilot experiment. Although the presented results are encouraging, further optimization of experimental parameters and of preclinical assays to characterize NGLY1 protein expression will be useful. As improved *Ngly1*-null animal models, including mice and rats, are being developed and characterized by other investigators, we are continuing to develop the rAAV-mediated gene therapy for NGLY1 deficiency through a collaborative effort. This study is supported by a grant from the Grace Science Foundation. #Co-corresponding authors.

671. Therapeutic Rescue of Spinal Muscular Atrophy Mouse Models with AAV9-Exon Specific U1 snRNA

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Spinal Muscular Atrophy results from loss-of-function mutations in *SMN1* but correcting aberrant splicing of *SMN2* offers hope of a cure. However, current splice therapy requires repeated infusions and is expensive. We previously rescued SMA mice by promoting the inclusion of a defective exon in *SMN2*with germline expression of Exon-Specific U1 snRNAs (ExspeU1). Here we tested viral delivery of SMN2 ExspeU1s encoded by adeno associated virus AAV9. Strikingly the virus increased *SMN2*exon 7 inclusion and SMN protein levels and rescued the phenotype of mild and severe SMA mice. In the severe mouse, the treatment improved the neuromuscular function and increased the life span from 10 to 219 days. The rescued adult mice had normal neuromuscular junctions and a normal number of spinal cord motor neurons. ExspeU1 expression persisted for one month and was effective at around one five-hundredth of the concentration of the endogenous U1snRNA. RNA-Seq analysis revealed our potential drug rescues aberrant SMA expression and splicing profiles, which are mostly related to DNA damage, cell cycle control and acute phase response. Vastly overexpressing ExspeU1 more than one hundred-fold above the therapeutic level in human cells was not toxic and did not significantly alter global gene expression or splicing. These results indicate that AAV-mediated delivery of a modified U1snRNP particle improves defective *SMN2* splicing and is a safe and powerful therapeutic option against SMA.

672. Sustained Mutant Huntingtin Lowering in the Brain and Cerebrospinal Fluid of Huntington Disease Minipigs Mediated by AAV5-miHTT Gene Therapy

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HTT-lowering therapies hold great promise to slow-down or halt neurodegeneration in Huntington disease (HD). We have developed an engineered microRNA targeting human huntingtin (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 have used transgenic HD (tgHD) minipigs to assess the translatability of our approach in a large animal model. Animals were injected with AAV5-miHTT ($1.2x10^{13}$ gc/brain), bilaterally into striatum (caudate and putamen) and sacrificed 6 months post-treatment. Widespread brain biodistribution of vector DNA was observed, with the highest levels in target (striatal) regions but also in thalamus and cortical regions, in both grey and white matter. Expression of miHTT was highly correlated with vector DNA in all brain areas. Corresponding to the vector DNA and miHTT expression, a reduction of mutant HTT (mHTT) mRNA and protein was observed in AAV5-miHTT treated animals with respect to controls. mHTT protein lowering was on average more than 75% in the injected areas, and between 30-50% in most of the distal regions. Translational pharmacokinetic and pharmacodynamic measures in the cerebrospinal fluid (CSF) were in line with the effects observed in the brain. We detected miHTT in the CSF, and CSF mHTT protein lowering up to 50% at 3 and 70% at 6 months post-dosing. This study demonstrates widespread biodistribution and durable efficiency of AAV5-miHTT in disease-relevant regions in a large brain, and the potential of CSF translational measures to follow-up efficacy.

673. Investigating Dual AAV-Based Treatments for MYO7A Usher Syndrome in Myo7a-/- Mice and Macaque

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Purpose: Our goal is to develop a safe and effective dual Adeno-associated virus (AAV)- based gene therapy for the treatment of autosomal recessive Usher Syndrome 1B (USH1B). USH1B patients present with deafness/vestibular defects from birth, progressive retinal degeneration, and vision loss within the first decade. We previously showed that both hybrid (HY) and simple overlap (SO) dual AAV vector platforms drive full-length MYO7A expression in subretinally injected (SR) C57BL/6J (WT) mice. The HY system produced the highest levels of full-length *MYO7A,* and a truncated protein derived from the 'front-half' vector. A dose responsive loss of retinal structure/function was observed in mice SR with HY vectors that may have resulted from overexpression of MYO7A in wild-type animals. In contrast, the SO platform did not produce truncated protein or cause loss of retinal structure/function. The level of SO- mediated full-length MYO7A was significantly lower than that produced by the HY platform. The purpose of our study was to 1) investigate if SR of dual AAV-*MYO7A* vectors lead to loss of retinal structure/function in MYO7A knockout mice and 2) assess tolerability of dual AAV-*MYO7A* vectors in a species with ocular characteristics most similar to man (macaque). **Approach:** SO and HY dual AAV vectors containing either the smCBA or hGRK1 promoter were packaged into AAV5 and AAV8(Y733F) via triple transfection. The Cre-lox system was used to create a novel USH1B mouse (*Myo7a*$^{-/-}$) on the C57BL/6J background by deleting exons 10-11 of *Myo7a*. This deletion ablates expression of MYO7A. *Myo7a*$^{-/-}$ mice were SR injected with 1E12 vg/mL of either SO or HY dual AAV-*MYO7A* vectors (5E11 vg/mL each of front and back vector). Control eyes were injected with BSS. *Myo7a*$^{-/-}$ and WT mice were SR injected with 5E11 vg/mL of AAV8(Y733F) front-half SO or front-half HY vectors. Retinal structure and function were analyzed using optical coherence tomography (OCT) and electroretinogram (ERG) at 6 weeks post injection (p.i.). Two macaques were SR injected with 5e11 vg/mL of SO dual AAV5-*MYO7A* vectors (2.5E11 vg/mL each of front and back vector). Retinal structure and function were assessed with confocal scanning laser ophthalmoscopy, OCT and ERG at baseline, 1, and 2 months p.i. Retinas from all experiments were analyzed for the presence of dual vector- mediated MYO7A transcript and protein using qRT-PCR and western blot, respectively. **Results:** Our previous results indicate that back-half vectors alone do not produce MYO7A transcript. Therefore, we used transcript from the back-half vector as a measure of full-length MYO7A arising from recombined vector. Both SO and HY platforms produced full-length MYO7A transcript and protein in *Myo7a*$^{-/-}$ mice SR injected with either AAV5- or AAV8(Y733F)- based dual vectors. When comparing both platforms,

the HY platform resulted in higher levels of MYO7A expression. When comparing the capsids, AAV8(Y733F) was more efficient than AAV5. Analysis of retinal structure/function in *Myo7a*$^{-/-}$ mice treated with either dual- or front-half vector alone is ongoing. AAV5-based SO vectors were well tolerated in macaque, and did not result in any gross changes in retinal structure or function. MYO7A transcript, but not protein, was detected in treated macaque retinas. **Conclusion:** Our results support further development of dual AAV-*MYO7A* vectors for the treatment of USH1B. Future work will incorporate a more potent capsid and additional testing in macaque.

674. tIPE - a GMP-Grade Non-Viral Gene Therapy Medicinal Product (GTMP) to Treat Neovascular Age-Related Macular Degeneration (nvAMD

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Purpose: Gene therapy is nowadays a reality with 9 approved GTMPs. However, most of them treat rare congenital diseases. Additionally, all use viral vectors for gene delivery with inherent costs and immunological issues. Neovascular age-related macular degeneration (nvAMD) is the first cause of blindness in elderly patients of industrialized countries with >30 million patients worldwide. The TargetAMD consortium has designed a non-viral gene therapy for nvAMD, which offers efficient and safe integrative gene delivery using the random integration profile of the hyperactive Sleeping Beauty (*SB100X*) transposon system and a miniplasmid free of antibiotic resistance genes (pFAR4); the use of the pFAR4 miniplasmid also significantly diminishes production costs. In an 1 hour-long validated GMP procedure, autologous iris pigment epithelial (IPE) cells are isolated, transfected with the gene encoding pigment epithelium-derived factor (*PEDF*) and transplanted subretinally, where PEDF secretion inhibits choroidal neovascularization. The application for a phase Ib/IIa clinical trial has been submitted to Swiss regulatory authorities, Swissmedic. **Methods:** Transfected hIPE cells were cultured to confirm *PEDF* gene integration and elevated protein secretion. Safety and efficacy were established in mice, rabbits and rats. PEDF secretion was determined by western blot and ELISA. GMP validation was performed on human donor eyes (4 biopsies/eye). The tissue, prepared in the laboratory, was transported to the GMP facility, where IPE cells were isolated and transfected using GMP-grade consumables. After centrifugation, remaining DNA was measured in the supernatant and viability was determined on a 1000-4000 cells aliquot. The transfected IPE cells (tIPE) were loaded into a syringe for transplantation. The stroma of the iris and the supernatant were tested for microbiological and endotoxin contamination using Pharmacopoeia-compliant methods. Volume of tIPE, transport time and temperature were recorded. **Results:** 67% viable, transfected hIPE cells increased PEDF secretion to 12.1±51.4 PEDF ng/d/5x10^3 cells (80.5±18.2 days post-transfection); *PEDF* gene expression increased 5-fold. Rat ocular tissue showed an increased anti-angiogenic ratio (5.1±0.3 PEDF/VEGF). Neuronal tissues and fundi of 17 rabbits at 90 days post-transplantation showed no abnormalities. Analysis indicated ~1 *PEDF* gene copy integrated/genome, >300 kb distant from oncogenes. Production cost of GMP-grade plasmid is ~$4,000/patient (vs. ~$18,000/year/patient for anti-VEGF injections). GMP-grade production was validated in 104 biopsies from 26 eyes (time of death to cell isolation: 7.35±1.29 d) by 3 independent producers and compliant to the release criteria (not detailed here) of the GTMP. On average 17'882±11'725 cells were isolated/biopsy, whose viability did not differ from controls (tIPE: 31.9±19.0; Co-P: 29.0±11.6). Remnant DNA was removed (79.3±22.6% of DNA introduced in excess). tIPE volume was 81.5±12.7% of the maximal possible volume (≤50 µl), produced in <20 min; transport time and temperature were compliant (2.8±1.3 min; 11.3±3.6°C). Only 4 out 104 samples were microbiologically contaminated and 0 had increased endotoxin levels (≤2 EU/mL). **Conclusions:** The results confirm the feasibility and potential efficacy of transplanting subretinally transfected cells in nvAMD patients. The present GMP-grade *ex vivo* cell therapy approach offers a life-long treatment solution without documented side effects, at reasonable costs. The application for the planned clinical trial was submitted to Swissmedic in 2018 with the potential of the first patient being treated within the next 4 months.

675. CRISPR/Cas Based Evaluation of the Therapeutic Potential for USH2A Associated Diseases

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Mutations in *USH2A* gene account for most cases of Usher Syndrome type II (USH2), characterized by a combination of congenital hearing loss and progressive vision loss. In particular, approximately 30% of USH2A patients harbor a single base pair deletion, c.2299delG, in exon 13 that creates a frame-shift and premature stop codon, leading to a non-functional USH2A protein. The USH2A protein, also known as usherin, is an extremely large transmembrane protein (5,202 aa), which limits the use of conventional AAV-mediated gene therapy; thus development of alternative approaches is required for the treatment of USH2A patients. As usherin contains multiple repetitive domains, we hypothesize that removal of one or more of those domains encoded by mutant exon(s) in the *USH2A* gene may reconstitute the reading frame and restore the production of a shortened yet adequately functional protein. In this investigation, we used CRISPR/Cas9 to generate a mouse model *Ush2a-ΔEx12*, in which we deleted exon 12 of the mouse *Ush2a* gene (corresponding to exon 13 of human *USH2A*). Characterization of this *Ush2a-ΔEx12* mouse line showed expression of the anticipated form of Ush2a lacking exon 12 and with exons 11 and 13 fused in frame. Importantly, we reveal that this shortened form of Ush2a is expressed and localized correctly in the mouse retina and cochlea. When the *Ush2a-ΔEx12* allele was expressed on an *Ush2a null* background, the Ush2a-ΔEx12 protein successfully restored the impaired hair cell structure and auditory function present in *Ush2a*$^{-/-}$ mice. The expression of Ush2a-ΔEx12 also reversed the accumulation of GFAP and mislocalization of cone opsin in the retinas of 3-month-old *Ush2a* mull mice. A CRISPR/Cas9-based exon-skipping gene editing

strategy to restore the reading frame of *USH2A* by deleting exon 13 is technically feasible, but until now, it was unknown whether the resulting shortened protein would be functional *in vivo*. Together, our results support the development of a CRISPR/Cas9-based exon-skipping strategy for rescuing the vision and hearing loss in USH2A patients.

676. Optimized Surgical Approach Leads to Highly Efficient AAV Gene Transfer to Inner Hair Cells in Rhesus Macaque

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Cochlear and vestibular gene therapy holds significant potential to alleviate hearing and balance disorders. In genetic mouse models of disease, adeno-associated viral vectors (AAV) have been shown to partially rescue hearing and balance. As shown for other organ systems, the AAV serotype has been shown to be a primary driver of efficiency and tropism of the gene transfer. Our prior data in newborn mice established the benefits of Anc80L55 in targeting inner and outer hair cells following round window membrane injection (RWM) (Landegger et al., Nature Biotech, 2017). Here, we report the feasibility and efficiency of the surgical approach and vector delivery in a large non-human primate model. The primate model was identified to control for scale, species, host response, and pharmacokinetics within the fluid-filled cochlea. Prior to in life studies, extensive studies were conducted to optimize the surgical procedure to ensure appropriate distribution and minimize surgical morbidity. Briefly, in a trans-mastoid approach was taken to access the middle ear. Next, the vector solution was injected in a volume of 30ul through the RWM following a small fenestration in the oval window for pressure release. This study was designed to enroll rhesus macaques without detectable titers against any of the injected AAVs. Specifically, AAV1 (n=2) or Anc80L65 (n=3) expressing eGFP was injected in one ear while the contralateral ear was uninjected and served as a control. The procedure and vector injection was well tolerated with no clinical signs of discomfort or focal neurologic or systemic signs. Blood cell counts and chemistries were within normal range. AAV neutralizing antibodies were detected in serum but not in CSF following, but not prior to injection in all animals. Cochlear transduction was evaluated by histology 7 to 14 days following injection. All animals except one showed significant cochlear eGFP expression. Anc80L65 in 2 animals led to up to 90% inner hair cells transduction whereas AAV1 was markedly less efficient at equal dose. Interestingly, transduction for both vectors declined from apex to base without any evidence of vector-induced damage or toxicity at the base. Other cochlear and vestibular cell types were targeted at variable rates. These data from non-human primates demonstrate that Anc80L65 allows both safe and efficient gene transfer to the inner ear via round window membrane injection. Our results motivate future translational studies in large animal models of auditory and vestibular dysfunction affecting hair cells, prior to human clinical trials.

Cancer Gene Therapy

677. First in Human Phase I Trial of the Combination of Two Adenoviral Vectors Expressing Either HSV1-TK or FLT3L for the Treatment of Newly Diagnosed Resectable Malignant Glioma at First Diagnosis: Preliminary Results

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This is the first report on a novel therapeutic approach for treating newly diagnosed high grade malignant gliomas. Namely the first in human phase I trial of the combination of two adenoviral vectors expressing HSV1-TK and Flt3L. Treatment of Grade IV malignant brain tumors has so far been recalcitrant to further improvements. Surgery and radio-chemotherapy have improved survival to 18-21 months. There is thus a great need to introduce new treatments for this highly aggressive disease. In 2005 we postulated that the reason for poor immune responses to brain tumors was that the brain lacks dendritic cells that take up antigens, and transport them to the lymph nodes to initiate a systemic anti-brain tumor immune response. We reasoned that if such cells can be recruited to the brain they would be able to pick up antigens from dying tumor cells to raise an anti-tumor immune response. We chose Flt3L to attract dendritic cells to the brain. HSV1-TK together with ganciclovir will kill tumor cells to provide antigens to dendritic cells. The strategy works in animal models of glioma (both implantable and GEMM). We showed infiltration of the brain by dendritic cells and other immune cells, the generation of immune memory to gliomas mediated by CD8 and CD4 T cells, the capacity of the immune system to recognize neoantigens, the need for HMGB1 to stimulate TLR2 on dendritic cells to initiate the anti-tumor immune cascade. With strong basic science background,

following the granting of the IND and approval by the FDA, a Phase I clinical trial using first generation adenoviral vectors expressing HSV-1 TK, and Flt3L was started in 2015. The treatment was administered intraoperatively following complete glioma resection. Valacyclovir was provided to activate the cytotoxic activity of HSV1-TK. Two cycles of valacyclovir were given. One, from Day 1-3 post surgery for 14 days. The second cycle was administered from Week 10-12. Lengthening of administration of valacyclovir was motivated by our earlier work demonstrating expression of HSV1-TK up to 12 months post-adenovirus administration in rodents. Radiation was started 15-35 days post-vector administration and proceeded usually for 6 weeks. Temozolamide treatment was started simultaneously with radiation. Four weeks after completion of the radiotherapy the maintenance phase of temozolomide, i.e. standard of care, was started and continued according to clinical decisions. The primary outcome of the trial was toxicity of the novel treatment; a secondary outcome is overall survival. The trial administered a dose escalation of both vectors, starting at 1x10^9 and up to 1x10^11 of each vector. Dose escalation proceeded by increasing the dose of one vector at the time through a total of 6 combinations. The trial thus treated 6 cohorts of 3 patients each, a total of 18 patients. The experimental treatment was well tolerated. Preliminary data on the toxicity, adverse events, severe adverse events, of the combined gene therapy will be presented. Data are currently being analyzed. Progression Free Survival and Overall Survival will be compared to contemporary controls, historical controls, other gene therapy trials, and other non-gene therapy trials for the treatment of glioblastoma.

678. CRISPR/Cas9-Mediated Ablation of the SHP-1 Gene Enhances the Therapeutic Efficacy of IL-13Ra2 CAR T Cells in Glioma Xenograft Models

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Background: Advances in oncology have led to significant improvements in survival rates for a wide variety of hematological and solid cancers. In stark contrast to this, however, the survival rates for high grade gliomas (HGG) have not seen significant increases in several decades. As an alternative to traditional chemotherapeutics and radiation, chimeric antigen receptor (CAR) T cell therapy has recently emerged as one of the most promising therapies for high grade gliomas. We have previously demonstrated that gene modifications in CAR T cells targeted to the glioma antigen IL-13Rα2 significantly enhanced their therapeutic efficacy. However, tumor recurrence posed a significant issue due to poor *in vivo* T cell expansion. These results indicated the need to explore alternative modifications of IL-13Rα2 CAR T cells to combat the issue of tumor recurrence. In this study, we evaluated a gene editing strategy that utilized CRISPR/cas9 technology to eliminate SHP-1, a potent negative T cell regulator, from IL-13Rα2 CAR T cells. The effects of this genetic modification on the therapeutic efficacy of IL-13Rα2 CAR T cells was then evaluated in murine glioma xenograft models. **Methods:**We generated IL-13Rα2 CAR T cells deficient of SHP-1by first transducing T cells with a retrovirus containing expression cassettes encoding IL-13Rα2-CAR.CD28.ζ and then electroporating transduced cells with single guide RNA-cas9 RNP complexestargeting SHP-1. Guides targeting mCherry served as a control. We determined the effector function of IL-13Rα2.SHP-1 KO CAR T cells *in vitro*using standard assays, and in orthotopic and heterotopic U373 glioma xenograft models. **Results:** Using CRISPR/Cas9 technology, SHP-1 protein levels were dramatically reduced in IL-13Rα2 CAR-transduced T cells as assessed by Western blot. Importantly, knockout of SHP-1 had no effect on IL-13Ra2-specific CAR transduction efficiency, T cell viability, or phenotype of the CAR T cell product when compared to control knockout CAR T cells. Silencing SHP-1 in CAR T cells endowed T cells with enhanced proliferative abilities as determined by CFSE dilution. In addition, IL-13Rα2.SHP1-KO CAR T cells were as efficient as IL-13Rα2.Control-KO CAR T cells in killing IL-13Rα2-positive glioma cells *in vitro*.Finally, administration of IL-13Rα2.SHP-1 KO CAR T cells to mice bearing subcutaneous U373 glioma resulted in delayed tumor growth when compared to mice that received IL-13Rα2.Control-KO CAR T cells. This resulted in a significant increase in overall survival of treated mice ($p<0.05$). A similar delay in tumor growth following infusion of IL-13Rα2.SHP-1 KO CAR T cells was also observed in mice bearing intracranial U373 tumors. Survival studies using this orthotopic intracranial model are ongoing. **Conclusions:** Our results demonstrate both the feasibility of generating and the increased efficacy of CAR T cell products modified using CRISPR/cas9 to remove negative regulators of T cell function. In particular, SHP-1 has shown to be an attractive target to remove from CAR T cell products to enhance their anti-glioma activity.

679. A Phase I/IIa Dose Escalation Study Evaluating the Safety and Efficacy of Autologous CD34+ Enriched Hematopoietic Progenitor Cells Genetically Modified for Human Interferon-Alpha2 in Patients with Glioblastoma Multiforme and an Unmethylated MGMT Promoter (TEM-GBM-001)

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Glioblastoma (GBM) remains the most common malignant primary brain tumor in adults. The median survival with multidisciplinary approaches to treatment including neurosurgery, radiotherapy and temozolomide (TMZ) is 15 months. However, most patients with GBM and an unmethylated O-6-methylguanine DNA methylase (MGMT) gene promoter, which predicts patient response to TMZ, have a worse prognosis with approximately 20% of patients surviving to 2 years. Poor prognosis in GBM is likely related to tumor heterogeneity, diffuse tumor cell infiltration and the presence of a highly immunosuppressive tumor microenvironment (TME). The TME in GBM is mainly composed of tumor-associated macrophages (TAMs) and microglia. M2-

macrophages, which are more prevalent in GBM tissue than M1 types, exhibit immunosuppressive properties. A subset of tumor-infiltrating bone marrow derived macrophages characterized by expression of the angiopoietin receptor Tie2 (TEMs) have features of M2-TAMs, promote tumor's angiogenesis and are infrequently found in normal organs. The Tie2 receptor is weakly expressed by circulating monocytes, but is significantly upregulated upon homing to tumors. Studies showed that tumor infiltration by M2-TAMs (including TEMs) is a negative prognostic factor, fostering tumor invasion and growth by promoting angiogenesis and suppressing anti-tumor immunity. TEMs represent a suitable carrier for the local and tumor restricted release of an anti-tumor molecule thus enabling maximum therapeutic efficacy combined with a reduced systemic toxicity. Interferon alpha (IFN) has antitumor effects, inhibits angiogenesis and modulates the immune system. Cell-based delivery of IFN into the TME by TEMs is expected to provide efficacy, taking advantage of its pleiotrophic anti-tumor activities and overcoming the tolerability issues associated with systemic IFN treatment. The antitumor activity of this novel strategy of tumor-selective IFN delivery by TEMs, has been successfully demonstrated in several orthotopic, spontaneous, and metastatic pre-clinical animal models of oncogenesis. Autologous human hematopoietic stem and progenitor cells (HSPCs) have been transduced e*x vivo* with a 3rd generation lentiviral vector encoding the human IFN-α2 gene under the control of the human Tie2/TEK enhancer/promoter sequence and a post-transcriptional regulation layer of microRNA-126 target sequences. These sequences allow targeted suppression of transgene expression in HSPCs, while maintaining IFN transgene expression in TEMs. We are currently conducting a Phase I/IIa clinical study in Milan to evaluate this therapeutic approach (Temferon) in 21 patients with GBM and unmethylated MGMT promoter (EudraCT Number 2018-001404-11). In Part A, 9 patients will participate in dose escalation cohorts and in Part B, a single dose selected from Part A will be evaluated in an additional 12 patients. The study design and progress will be discussed at the meeting.

680. Mullerian Inhibiting Substance Type 2 Receptor (MISIIR)-Specific CAR T Cells for the Treatment of Ovarian Cancer and Other Gynecologic Malignancies

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Ovarian cancer (OC) ranks 5th among cancer-related deaths in women and is the most lethal of all gynecologic malignancies, with an overall 5-year survival rate of 46%. The recent success and FDA approval of Chimeric Antigen Receptor (CAR) T cell therapy targeting CD19 for the treatment of hematologic malignancies rationalizes the development of similar strategies. Despite its promise, effective CAR T cell therapy for OC has not yet been achieved. One of the challenges that may contribute to this lack of success is the appropriate selection of a target antigen that is homogeneously and highly expressed in malignant cells while absent in normal cells, in order to ensure an efficacious and safe treatment. The Mullerian inhibiting substance type 2 receptor (MISIIR) is a member of the TGF-β receptors family involved in the regression of the primordial female reproductive tract in male embryos. This action is exerted through the interaction with a soluble ligand, Mullerian inhibiting substance (MIS) that triggers a downstream signaling cascade inducing apoptosis. In fact, MIS signaling through MISIIR has been shown to cause growth inhibition in ovarian, breast, prostate and endometrial cancer cell lines *in vitro* and *in vivo*, indicating the relevance of this pathway in cancer. In humans, MISIIR is expressed in a restricted set of reproductive tissues and is overexpressed in the majority of OCs as well as in other gynecologic tumors, making it a sound candidate target antigen. Here, MISIIR-specific CAR T cells comprised of 4 different human MISIIR-specific single-chain antibody variable fragments (scFv) isolated from a phage display library and coupled to CD27 and CD3ζ intracellular signaling domains were generated. The distinct CAR T cell variants were compared *in vitro* in terms of antigen-specific cytokine secretion and cytotoxicity when co-cultured with antigen-deficient tumor cells (C30) or with target cells engineered to overexpress MISIIR (C30.MISIIR) in order to select the best in class CAR. The selected CAR, namely GM7-27Z, also showed antigen-specific reactivity against a broad range of human tumor cell lines including ovarian and endometrial cancer expressing variable levels of endogenous MISIIR. Importantly, GM7 CAR T cells didn't show any cytotoxicity when co-cultured with a broad panel of normal primary human cells, suggesting a safe profile of targeting MISIIR with CARs. When tested *in vivo*, GM7 CAR T cells showed potent antitumor effect in immunocompromised mice bearing large established subcutaneous C30.MISIIR tumors, completely clearing 60% of the tumors and significantly prolonging mice survival. Nevertheless, while statistically significant, antitumor effect was mild when assessed in more aggressive tumor models which expressed lower endogenous levels of MISIIR. Finally, to validate the applicability of this therapy to actual patients, GM7 CAR T cells were tested *in vitro* with patient-derived OC cells. Importantly, MISIIR-specific CAR T cells showed antigen-specific upregulation of T cell activation markers as well as specific cytokine secretion and tumor cell lysis when co-cultured with tumor digests. Overall, the data obtained so far supports the potential of MISIIR as a novel target for the efficacious and safe treatment of OC and other gynecologic malignancies using CAR T cell therapy.

681. γδ CAR-T Cells Show Dual CAR- and TCR-Mediated Mechanisms of Tumor Cell Recognition against Bone Metastatic Castrate Resistant Prostate Cancer

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Bone metastatic castrate resistant prostate cancer (mCRPC) remains a disease with limited therapeutic options. Current approaches include hormone ablation therapies, which target cancerous cells, and the use of bisphosphonates, such as zoledronate (ZOL), as a palliative approach to prevent cancer-induced bone disease. In spite of these treatments, the malignancy progresses and patients succumb to the disease

within 2-3 years, highlighting the need for new therapeutic strategies. Immunotherapies based on chimeric antigen receptor-T cells (CAR-T) have generated impressive results in hematological malignancies, however their efficacy against solid tumors is in need of improvement. In this work, we tested the therapeutic potential of a subset of T cells expressing the γδ TCR as a platform for CAR-T cell immunotherapy for mCRPC and evaluated their therapeutic effects in combination with ZOL. We first engineered both αβ and γδ CAR-T cells specific for the prostate stem cell antigen (PSCA) and studied their *in vitro* cytolytic capacity using the xCELLigence Real-Time Cytotoxicity Assay (RTCA). Three target:effector ratios were used (1:1, 1:2.5, and 1:5). In each of the three conditions, γδ CAR-T cells induced a faster decrease in cancer cell viability than αβ CAR-T cells, suggesting that they are more potent and/or faster cytotoxic effectors. Next, we assessed the anti-tumor activity of γδ T-cells on established bone metastatic CRPC lesions *in vivo*. Luciferase tagged, PSCA overexpressing C4-2B prostate cancer cells (C4-2B -PSCA) were injected into the tibias of 6-week old, male NSG mice (n=20). Once tumors were detected by bioluminescence, one group received a high dose of γδ CAR-T cells ($1.5x10^7$, i.v.) while the control group received vehicle (saline). The γδ CAR-T-treated group experienced a rapid reduction in tumor burden, which resulted in significantly improved survival of the mice. Specifically, γδ T-cells recognize phosphoantigens, such as isopentenyl pyrophosphate (IPP), which can be induced pharmacologically by bisphosphonates. In order to measure the accumulation of IPP in C4-2B-PSCA cells, tandem LC-MS/MS was performed after incubation with 10μM or 25μM of ZOL, for 1h or 18h. We observed that C4-2B-PSCA cells produce high levels of IPP in response to this bisphosphonate. In addition, we evaluated the CAR- and TCR-mediated mechanisms of tumor cell recognition by γδ CAR-T. Parental C4-2B, or C4-2B-PSCA cells were challenged with untransduced (UT) or CAR-transduced γδ-T cells, in the presence or absence of ZOL, and the viability of tumor cells was monitored over time using the RTCA approach. We showed that γδ-T cells, regardless of CAR expression, induced a significant reduction in the viability of C4-2B cells when given in combination with ZOL. As expected, CAR-expressing γδ-T cells eliminated PSCA-expressing targets. Importantly, when γδ CAR-T cells were used in combination with ZOL, maximum cytotoxicity was achieved, suggesting that the CAR and the endogenous TCR may act in concert to maximize antitumor efficacy. Our data show that γδ CAR-T cells have superior *in vitro* cytotoxic activity compared to αβ CAR-T cells, and this activity can be further enhanced by ZOL. This bisphosphonate can also induce accumulation of IPP by prostate tumor cells which promotes γδ T-cell activity. Moreover, γδ CAR-T cells were able to eradicate intratibial C4-2B-PSCA tumors, proving their *in vivo* anti-tumor efficacy. By exploiting both the CAR- and TCR-dependent mechanisms of action, we propose γδ CAR-T cells as a new alternative to improve the clinical outcome of patients with mCRPC being treated with bisphosphonates.

682. Development of an Allogeneic Universally Tolerated NKT Cell Platform for Off-the-Shelf Cancer Immunotherapy

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Production of autologous or HLA-matched allogeneic T cells for cancer immunotherapy generates substantial variability and high treatment costs. We employed CD1d-restricted natural killer T (NKT) cells to address the shortcomings of allogeneic T cells for immunotherapy applications. Unlike T cells, NKTs are inherently non-alloreactive and could be used as an off-the-shelf product to treat multiple patients without the risk for graft-versus-host disease. Nevertheless, NKTs may still be recognized as foreign and eliminated by an allogeneic host's immune system. To extend the longevity of therapeutic NKTs *in vivo,* we engineered a dual function chimeric antigen receptor (CAR) construct that: 1) targets B-cell antigen CD19 via a clinically validated single chain variable fragment combined with CD28 co-stimulation, and 2) reduces expression of HLA class I and II using anti-β2-microglobulin (B2M) and -MHC class II-associated invariant chain (Ii) shRNA sequences to reduce the allogenicity of transduced NKTs to the host immune system. To develop off-the-shelf banks of NKTs expressing our optimized construct, we *ex vivo* expanded NKTs from 14 healthy donor candidates and evaluated their rate of expansion, immunological phenotype, and *in vitro* functionality. We regenerated CAR-shRNA NKTs from six selected donors in order to evaluate their *in vivo* anti-tumor activity in NSG mice carrying human lymphoma xenografts. We observed a range of tumor control capacities across all donors. NKTs from the top three donors mediated curative anti-lymphoma activity in the majority of treated mice up to 12 weeks post-injection, while most mice treated with NKTs from the remaining three donors succumbed to or showed major tumor growth by 7-9 weeks post-injection. Importantly, NKTs that mediated the best anti-tumor effect expressed higher levels of CD62L prior to injection than their worse performing counterparts. Based on these findings, CAR-shRNA NKTs from the top three donors will be produced and banked under cGMP conditions for clinical testing in patients with B-cell malignancies. In all, we have generated healthy donor NKT cells with potent CAR-directed anti-CD19 activity and molecular machinery that limits their allogenicity. Our findings have also demonstrated substantial variability in the capacity of healthy donor NKTs to support CAR-directed anti-tumor functionality, which is a crucial consideration in developing off-the-shelf cell banks for cancer immunotherapy.

683. CAR T Persistence and Anti-Leukemic Efficacy In Vivo are Dependent Upon Lentiviral Vector Internal Promoter: MSCV vs EF-1 Alpha

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Objective: The role of the internal promoter encoded within the lentiviral vector gene expression cassette has not been extensively studied. We sought to understand if two of the internal promoters commonly employed in clinical gene vectors, one derived from the murine stem cell virus (MSCV) and another from the human elongation factor 1 gene (EF-1α), were equal in the long-term functioning of CAR T cells in three separate *in vivo* models of malignancy. **Approach:** CAR T cells were generated by lentiviral transduction of activated primary human T cells with either a CD19 CAR or a TSLPR CAR, driven by either an EF-1α or a MSCV internal promoter. **Results:** TSLPR-CAR activity was tested in two different models of TSLPR(+) ALL in NSG mice: 1) JH331-GL, a PDX which naturally overexpresses TSLPR due to *CRLF2* rearrangement, and 2) a REH-TSLPR leukemia xenograft. In these models, both the MSCV-TSLPR CAR and the EF-1α-TSLPR CAR initially rejected TSLPR(+)-leukemia. However, the MSCV-TSLPR CAR groups subsequently relapsed, whereas the EF-1α-CAR groups remained in durable remission. In a third setting, $CD19^+$ Raji lymphoma xenograft in NSG mice was treated with a low dose of $2x10^6$ CAR19 T cells/mouse. The EF-1α CAR19, but not the MSCV CAR19, fully controlled tumor growth. Importantly, day 14 circulating MSCV CAR19 cells had an elevated expression of PD-1, and there was an increase in lymphocytes with a T_{EM} phenotype ($CD45RO^+CD62L^-$), and reduced T_N ($CD45RO^-CD62L^+$) as compared to EF-1α CAR19, indicating greater T cell exhaustion and terminal differentiation of MSCV CARs. In long-term *in vitro* co-culture/re-challenge assays, in which CAR T cells were combined with Raji cells at effector to target ratio of 0.3 on day 0, and again on day 7, the MSCV-driven CARs showed an earlier increase of exhaustion markers (elevated PD-1, Tim-3, Lag-3 and decreased CD69), and increased T cell memory subsets ($CD45RA^-CD45RO^+$), lower long-term tumor clearance and proliferation, and rapid down-regulation and delayed recovery of CAR surface expression, as compared to EF-1α CARs. **Conclusions:** We have demonstrated in two independent CAR systems, one targeting the CD19 antigen and another the TSLPR antigen, that the EF-1α CARs were superior to MSCV CARs in both persistence and anti-tumor activity. Studies are underway to define the molecular mechanisms responsible for the differential activity of MSCV-and EF-1α-driven CAR T cells.

RNA Virus Vectors for Therapeutic Applications

684. β-Globin Locus Control Region Core Sequences Driving Expression of Anti-Sickling Globin Ameliorates Disease Phenotype in a Mouse Model of Sickle Cell Disease

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Objective: Clinical translation of gene therapy vectors to the clinic for treatment of Sickle Cell Disease (SCD) has historically been hindered by low titer and sub-optimal gene transfer to human $CD34^+$ hematopoietic stem and progenitor cells (HSPCs). The relatively large size of human genomic elements within β globin lentiviral vectors (β-LVs) are suspected to diminish titer and transduction efficiency. Thus, a strategy was developed to reduce the length of the β-globin Locus Control Region (LCR) DNase 1 hypersensitive site (HS) elements knowing that the high-level expression of anti-sickling βAS3-globin required to reverse the sickle phenotype of red blood cells could be jeopardized. **Methods:** Current genomic and epigenomic databases were deployed to elucidate boundaries for the functional elements within the HS sites to produce a composite LCR of reduced length. When designing the new "core" elements, multiple variables were considered: 1). histone modification and displacement, 2). transcription factor binding, 3). DNase1-accessibility, and 4). sequence conservation. The overall lengths of combined HS elements (2, 3, 4) were reduced from 3.6kb to 1.2kb. Supplementary elements of minimal length were added to aid in position-independent expression. **Results:** This novel construct, termed CoreGA-AS3-FB, was produced at nearly three-fold higher titer ($p < 0.0001$), possessed nearly three-fold higher gene transfer to HSPCs cultured under myeloid conditions ($p < 0.01$), and expressed ~60% the amount of βAS3-globin per vector genome in HSPCs cultured under erythroid conditions ($p<0.001$), when compared to a standard β-LV at equal multiplicity of infection (MOI). When evaluated at an average bone marrow (BM) vector copy number (VCN) of 2 in a mouse model of SCD, average (AVG) levels of %Hb βAS3/total hemoglobin tetramers were found to be 18.5%. A methylcellulose-based colony-forming unit assay demonstrated long-term and persistent expression of βAS3-globin by CoreGA-AS3-FB transduced bone-marrow cells acquired at time of euthanasia. While percentages of PCR-positive colonies seen for CoreGA-AS3-FB were 81% when compared to 93% of PCR-positive colonies seen for the standard β-LV, 17% of colonies from the CoreGA-AS3-FB experimental arm possessed >4 integrants per cell while only 1% of colonies from the standard β-LV experimental arm possessed >4 integrants per cell. This result highlights CoreGA-AS3-FB's superior transduction efficiency despite the fact it was constrained by using less vector to achieve equivalent bulk VCNs. Significant increases in Hb levels and red blood cell (RBC) counts were observed at time of euthanasia for CoreGA-AS3-FB when compared to mice that received mock transduced BM. The AVG Hb levels for mice that received BM transduced with a standard β-LV (n=6/arm), CoreGA-AS3-FB, or no vector (mock), were on AVG 11, 10, and 8 g/dL, respectively. The AVG

RBC counts for mice that received BM transduced with a standard β-LV, CoreGA-AS3-FB, or no vector, were 8, 8, and 6, (x10^6) cells/uL, respectively. **Conclusion:** Case reports describing patients afflicted with both SCD and hereditary persistence of fetal hemoglobin often describe the clinical course as benign when HbF levels are 10% or higher (likely due to pancellular distribution of HbF). Thus, *in vivo* percentages of βAS3-globin seen for CoreGA AS3-FB at a VCN of 2 are at levels of expression expected to be therapeutic. The higher titer CoreGA-AS3-FB LV with improved transduction efficiency ameliorated hematologic parameters defining the pathological phenotype of SCD in the mouse model of the disease. This new lentiviral vector design should have advantages for clinical-scale production providing the highest level of gene transfer for the lowest amount of vector.

685. Liver-Directed Gene Therapy with Lentiviral Vectors in Newborn Mice and Dog Puppies

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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 cell division in liver growth, thus challenging their proficient use in pediatric patients. In contrast, lentiviral vectors (LV) integrate into the target cell chromatin and are maintained as cells divide. 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, reaching up to 300% of normal activity of a human coagulation factor IX (FIX) transgene, without signs of toxicity or clonal expansion of transduced cells. These studies support further pre-clinical assessment and clinical evaluation of liver-directed LV gene therapy in adults with 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. Note that LV protection from phagocytosis is species-specific and is not active in the dog model, thus efficiency of hepatocyte transduction is lower in dogs than NHP. Reconstituted FIX activity initially peaked at 15-30% of normal then decreased over the first months post LV and stabilized at 3-5% of normal up to 3.5 years post LV. To investigate this outcome, we turned to mice and monitored LV-modified liver cells during growth. We administered increasing doses of LV expressing marker genes (GFP or luciferase) under the control of a hepatocyte-specific expression cassette to newborn mice. Exploiting 3D imaging of cleared livers and bioluminescence, we showed an initial decrease in transgene expression and dilution of transduced hepatocytes, followed by stabilization and maintenance of transgene expression, accompanied by expansion of transduced hepatocytes. We have reproduced this finding using LV with different hepatocyte-specific promoters. These data suggest different growth phases supported by different hepatocyte populations within the mouse liver. Moreover, we are investigating the clonal composition of hepatocytes during post-natal liver growth, in Alb-Cre/Rosa26-confetti mice. We also have evidence for *in vivo*LV transduction of bile duct cells and their capacity to generate LV-positive liver organoids *in vitro*. Overall, our work will inform about the extent and mechanism underlying long-term maintenance of LV-transduced hepatocytes in newborns, provide a rationale for application of LV-mediated liver gene therapy to pediatric patients and will also address the role of different cell populations involved in post-natal liver growth. *These authors share senior authorship.

686. Evaluation of the Efficacy and Safety of Intraosseous Delivery of Platelet-Specific Factor VIII-Lentiviral Vectors as an In Vivo Gene Therapy for Hemophilia in Humanized NSG Mice

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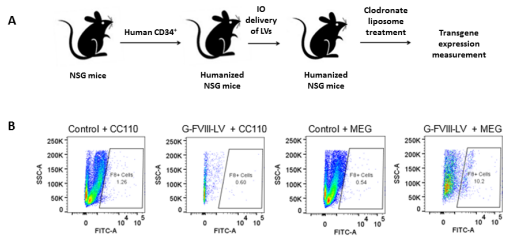
Hemophilia A is a genetic disorder resulting in a deficiency in functional factor VIII (FVIII) secreted in plasma and presents as an ideal disease model for gene therapy. Our lab has successfully developed an *in vivo* intraosseous injection (IO) therapy to deliver FVIII/N6 lentivirus (G-FVIII-LV) driven by the platelet-specific GpIbα promoter. Viral transduction of hematopoietic stem cells (HSCs) ensures long-term maintenance of the gene therapy. FVIII is produced and then stored in the α-granules of platelets, avoiding detection by the immune system, making this therapy accessible to patients who have already developed inhibitory antibodies to FVIII. We have previously shown that IO delivery of G-FVIII-LV partially corrected the hemophilia phenotype in mice for at least 5 months. The aim of the current study was to assess the safety and efficacy of IO delivery of G-FVIII-LV in humanized mice to assure the feasibility of translating this therapy to clinical models. We first conducted *in vitro* studies to confirm successful transduction and expression of G-FVIII-LV and G-GFP-LV in human $CD34^+$ cells isolated from G-CSF mobilized donors. Cells were cultured in StemSpan™ Serum-Free Expansion Medium supplemented either with cytokine cocktail (CC110) to expand HSCs or with Megakaryocyte expansion supplement (MEG) following transduction to promote differentiation into megakaryocytes. After 7 days of growth, cells cultured in MEG had a higher percentage of both $CD41a^+$ and late-stage differentiated $CD41a^+CD42b^+$ megakaryocytes than those cultured in CC110, indicating preferential differentiation into megakaryocytes in this media. We also observed higher FVIII and GFP expression in MEG cultured cells, suggesting successful megakaryocyte-specific expression.

We then tested these LVs *in vivo* in humanized NSG mice. NSG mice with and without the W-41 mutation in the *Kit* gene were preconditioned with busulfan (25mg/kg) 24 hours before the transplantation of hCD34$^+$ cells via RO injection. Eight weeks later, we observed higher engraftment of hCD34$^+$ cells in the W-41 NSG cohort compared with NSG mice, averaging greater than 80% engraftment in bone marrow. Human platelet count was also higher in the W-41 NSG mice. We next performed IO delivery of LVs in cohorts of transplanted mice. Clodronate liposome treatments increased human platelet counts, allowing for detection of notable FVIII expression in platelets isolated from PBMCs of mice treated with G-FVIII-LV. Bone marrow was isolated several weeks after the IO injection and engrafted human HSCs were isolated. The isolated cells were subsequently cultured in MEG and expressed significant levels of FVIII and GFP after 6 days. We isolated the gDNA of hCD34$^+$ cells transduced by G-GFP-LV and G-FVIII-LV respectively at various MOIs from the bone marrow of two groups of mice that had undergone IO injections with these two viral vectors respectively. Currently, we are assessing marking by qPCR and using MGS-PCR to analyze integration sites of the lentivirus to assess the safety of this therapy. Given its efficacy in humanized mouse models, IO delivery of platelet-specific FVIII presents as a promising *in vivo* gene therapy for correcting the hemophilia phenotype.



A. Schematic of IO delivery of LVs into humanized NSG mice
B. Isolated hCD34$^+$ cells from G-FVIII-LV treated humanized mice were cultured with CC110 or MEG for 6 days and FVIII expression was measured by flow cytometry

687. Vectorised 'Mini' hU6 Pol III Promoter Exhibits Nucleosome Redundancy and Supports Multiplexed CRISPR Guide Expression

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RNA polymerase III (Pol III) promoters naturally express short non-coding RNA making them ideal for the expression of microRNA, interfering or short-hairpin RNA, and CRISPR single guide RNA (sgRNA). Mammalian Pol III promoters are being widely investigated for genome editing applications, especially where vector based delivery and subsequent expression is desirable. The classical DNA structure of a Pol III promoter comprises a proximal sequence element (PSE) and a TATA box, with an upstream distal sequence element (DSE) incorporating octamer (Oct) and SPH sequences. Despite the requirement for protein-protein interaction between DSE and PSE bound transcription factors, these elements are classically separated by a relatively large spacer sequence. The current model for Pol III transcriptional activity indicates that spacer sequence looping around a functional nucleosome brings the DSE and PSE into proximity allowing transcription factor co-localisation. Whereas minimal H1 Pol III promoters, devoid of nucleosomal DNA spacer sequence, have been widely described and incorporated into both viral vectors and DNA expression plasmids, nucleosomal function incorporated within spacer sequences has been considered critical for activity of hU6 in the native context of chromosomal expression. We investigated truncated variants of hU6 and confirmed the essential roles of the TATA, DSE and PSE elements but uncovered nucleosome redundancy when incorporated into lentiviral 3'LTR configurations for genomic disruption of T cell receptor genes. Robust CRISPR/Cas9-mediated editing of the T cell receptor alpha constant (TRAC) locus in primary T cells was achieved using a 'mini' hU6 devoid of nucleosomal spacer sequence, for expression of sgRNA. Interestingly, no significant difference was observed in knockout efficiency when compared to wild type hU6. Removal of this 138bp nucleosomal spacer region reduced the size by >50% of the original promoter and therefore is favourable for incorporation into size restricted elements. The incorporation of both our mini hU6 and a mini H1 Pol III promoter within the 3'LTR of a lentiviral vector, employing alternative CRISPR scaffold designs; supported high titre vector production and simultaneous modification of additional loci including Beta-2-microglobulin (B_2M) for HLA class I disruption and CD52, the target antigen for Alemtuzumab. In summary, we describe a mini hU6 promoter, devoid of the nucleosomal DNA spacer sequence, which supported efficient genome editing effects, alone and in combinational CRISPR mediated applications.

688. Systemically Delivered Zinc Finger Protein Directed Silencing of HIV

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Human Immunodeficiency Virus type 1 (HIV) causes a persistent latent infection. Control of HIV using antiretroviral therapy (ART) comes at the cost of life shortening side effects and drug resistant HIV. A means to an adjunctive therapy to ART, that may be resistant to emerging drug resistant variants of HIV, and can stably repress viral infection in the absence of ART could prove valuable. Towards this goal we developed and report here a HIV promoter targeted Zinc Finger Protein (ZFP-362) which binds to unique NFκB binding site on HIV-1 LTR promoter. ZFP-362 was fused to DNA methylferase DNMT3a (ZFP-362-3A) and screened for antiviral properties. The resultant ZFP-362-3A was able to suppress HIV-1 expression from chronically HIV-1 infected Jurkat cells to a significant extent for a long period of time including in presence of potent viral activators. In an attempt to further boost the efficiency of targeted repression, functional domains from DNMT3a or repressor domains from other proteins were combined, yielding several fusion constructs. Out of the 11 candidate fusion constructs screened, 2 showed approximately 90 percent long

term suppression of HIV p24 expression. As expected, the top two repressive ZFP-362-3A fusion constructs were specifically enriched on LTR promoter due to direct binding of ZFP-362. This binding resulted in efficient CpG methylation and epigenetic repression at LTR. The top candidate ZFP-362-3A constructs were found to be efficiently delivered to HIV infected cells when packaged into exosomes. Our results pave the way for exosome mediated systemic delivery of therapeutic cargo of ZFP-362-3A to epigenetically repress HIV-1 infection without the perils of ART drugs.

689. High-Efficiency Lentiviral Transduction of Human CD34+ Cells in High-Density Cell Culture with Poloxamer and Prostaglandin E2 Supplementation

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Hematopoietic stem cell (HSC)-targeted gene therapy has been reported to cure several hereditary immunodeficiencies, and it is under development for hemoglobin disorders, including β-thalassemia and sickle cell disease (SCD). More effifcient lentiviral transduction of human CD34+ HSCs remains crucial to improve gene therapy. We have previously optimized lentiviral transduction in human CD34+ cells by using serum-free media including minimal cytokines (stem cell factor, fms-like tyrosine kinase 3 ligand, and thrombopoietin) with 1-day prestimulation, and demonstrated efficient transduction and high-level engraftment in humanized mouse and rhesus transplantation models. This standard human CD34+ cell transduction method in current gene therapy trials achieves transduction in around 50% of CD34+ cells. In this study, we further improved transduction efficiency in human CD34+ cells by using high-density CD34+ cell culture with adjuvant supplementation, while maintaining the previous conditions used in our standard method. First, we transduced human CD34+ cells at increasing cell-densities, and observed ~4-fold more efficient lentiviral transduction (GFP-positive (%GFP) 88±1%) and lower cell proliferation in the highest cell-density (4e6/ml) as compared to a standard cell-density (1e5/ml) (%GFP 21±1%) ($p<0.01$). We then transduced half of the CD34+ cells with a GFP-expressing lentiviral vector for 1 hour (sufficient for internalization) and stained the other half of the cells with a far-red dye. After washing cells with PBS, we co-cultured transduced CD34+ cells with non-transduced CD34+ cells (far-red-stained) at various cell-densities. Three days later, higher GFP-marking was observed in non-transduced cells (far-red-positive cells) in high-density CD34+ cell culture (%GFP 5.3±0.5%) compared to low-density culture (%GFP 0.8±0.3%) ($p<0.01$). These data demonstrate that high-density CD34+ cell culture enhances lentiviral transduction by increasing cell-to-cell contacts rather than by direct vector exposure. We then added poloxamer 407 (P407) and prostaglandin E2 (PGE2) in high-density CD34+ cell transduction, since P407 may improve vector-to-cell binding due to surfactant properties and PGE2 may improve vector replication (reverse transcription) by stimulating CD34+ cells. Traditional polycation-based adjuvants (polybrene and protamine) decrease transduction efficiency in CD34+ cells due to their toxicity; however, addition of P407 (100μg/ml) and PGE2 (10μM) resulted in ~4-fold (overall ~15-fold) improvement of transduction efficiency in high-density CD34+ cell culture (%GFP 93±0%, vector copy numbers (VCN) 14±0) without significant toxicity as compared to a no adjuvant control ($p<0.01$) (%GFP 45±2%, VCN 4±0). In escalating doses of lentiviral transduction, P407 more strongly improved transduction compared to PGE2, but the combination of P407 and PGE2 resulted in the highest transduction efficiency in high-density CD34+ cell culture, perhaps because the adjuvant effects of P407 and PGE2 have different mechanisms. We have evaluated CD34+ cell engraftment following high-density cell trandsuction with P407 and PGE2 in a xenograft mouse model, and results will be reported. In summary, we developed a high-efficiency lentiviral transduction method in high-density human CD34+ cell culture with adjuvant supplementation (P407 and PGE2), resulting in ~15-fold improvement in transduction efficiency and consistently achieving >90% transduction and VCN ~10 in human CD34+ cells without significant toxicity. Our optimized transduction method should allow us to improve HSC-targeted gene therapy.

690. Gene Delivery to the Corneal Limbal Stem Cells Using AAV and Lentiviral Vectors

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Limbal stem cell (LSC) transplantation is a promising treatment of ocular surface diseases especially limbal stem cell deficiency (LSCD). Genetic engineering represents an attractive strategy to increase the potential for success in LSC transplantations either by correcting autologous diseased LSCs or by decreasing the immunogenicity of allogeneic LSCs. However, no study has been performed to comprehensively compare the two most popular viral vector formats (Adeno-associated virus vector, AAV and Lentiviral vector, LV) in human LSCs. Therefore, eighty human corneas with selected donor age, death-to-preservation time, and endothelium/epithelium integrity were chosen for investigation. Transduction efficiency was evaluated by flow cytometry, viral genomes, and fluorescence microscopy after introducing self-complementary AAV serotypes 1-6, 8 and 9 or LV carrying a GFP cassette to fresh limbal epithelial cells, cultivated LSC colonies, or after corneal intrastromal injection. Colony formation efficiency (CFE) was calculated and immunofluorescence staining was performed to colocalize GFP expression with LSC markers in colony cross-sections and in corneas. Viral genomes and LSC markers were analysis by Q-PCR or RT-Q-PCR utilizing probe detection. For fresh limbal epithelial cells, AAV6 showed the highest transduction efficiency, followed by LV and AAV4 at 24h post vector incubation. The CFE showed no significant differences among AAV serotypes, LV, and non-treated controls. The percentage of GFP+ colonies at 14 days post-seeding was significantly higher in the LV group of which the majority remained GFP+ on serial passages, while the AAV6 treated colonies showed an abnormal GFP+ phenotype with no GFP+ colonies in passage 2 or thereafter. Interestingly, colony cross-section immunofluorescence showed GFP was uniform throughout the LV

treated cell colonies while remarkably limited to superficial layers in most colonies following AAV6 treatment. Cultivated LSC colonies contained multiple cell types with a subset demonstrating LSC markers and responsible for serial colony formation. Interestingly, AAV6 vectors, not LV, did not elicit productive transduction in LSCs despite vector uptake. Following AAV6 or LV intrastromal injections, strong GFP fluorescence was noted in the stroma while minimal expression was observed in the limbal epithelium with no GFP+ derived LSC colonies. The collective results demonstrate the superiority of LV for stable LSC genetic engineering and an unreported phenomenon of AAV restriction to apparently more differentiated cells derived from the human limbus.

Cell Therapy for Metabolic Disorders

691. Selective Expansion of Gene-Targeted Hepatocytes Using Acetaminophen Leads to Reproducible Long-Term Liver Repopulation

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Selective in vivo expansion of gene-targeted hepatocytes offers promise for overcoming low targeting efficiencies inherent with many gene therapy systems. In certain genetic disorders, such as methylmalonic acidemia, therapeutic transgene expression confers a growth advantage to targeted hepatocytes, leading to expansion of these cells and enhanced therapeutic efficacy. However, this phenomenon is not commonly observed in other diseases for which gene therapies are being developed. Thus, we developed a universal system for expanding gene-targeted hepatocytes in vivo. Previously we described a system for expanding gene-targeted hepatocytes by blocking expression of cytochrome P450 reductase (Cypor). Cypor-deficient hepatocytes are resistant to acetaminophen-induced liver toxicity as these cells are unable to convert the drug to its toxic intermediate, NAPQI. We showed that hydrodynamic tail vein injection of a CRISPR plasmid targeting the mouse Cypor gene followed by bi-weekly injections of a high dose of acetaminophen results in expansion of Cypor-negative hepatocytes from a starting rate of ~1% to an average of 34% of the liver, as shown by immunofluorescent staining and indel analysis. This result is highly reproducible (n = 45). Here, we report data on the safety and efficacy of this approach in consideration for clinical use. To test whether Cypor-negative hepatocytes are stable in the absence of selective pressure, mice that had received complete acetaminophen selection were divided into two groups. One group received continued selective pressure in the form of weekly acetaminophen doses while the other group received no acetaminophen. No significant difference in frequency of Cypor-negative hepatocytes was seen between the groups up to 35 weeks after stopping acetaminophen treatment. Furthermore, no negative health impacts were evident in either group. Additionally, we have shown that selection can be achieved using an acetaminophen-containing diet in lieu of injections. This avoids the potentially dangerous spikes in liver enzyme levels produced by acute acetaminophen liver injury, and we have seen no mortality associated with the acetaminophen diet. These results are promising for the clinical feasibility of this system. Acetaminophen-mediated expansion of gene-targeted hepatocytes can be achieved using mild liver injury and results in stable repopulation of the liver. In addition, no negative health effects of this system have been observed, though additional animal models should be tested.

692. Correction of a Metabolic Liver Disease after Ex Vivo Gene Editing of Human Hepatocytes

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Background and Aims: Urea cycle disorders (UCD) are inborn errors of ammonia detoxification in the liver. The most common UCD is ornithine transcarbamylase deficiency (OTCD). The only definitive treatment for severe UCDs is orthotopic liver transplantation. Partial and temporary corrections of OTCD have been reported with allogeneic hepatocyte transplantation. An attractive approach for treating inborn errors of metabolism is the direct genetic correction of patient's own cells and autologous transplantation, avoiding the need for immunosuppressive drugs and improving the prospects for robust hepatocyte repopulation. However, the efficiency and safety of *ex vivo* CRISPR gene editing of human primary hepatocytes from patients with genetic liver diseases has not been established. **Methods:** Human hepatocytes were isolated from non-UCD patients (proficient in urea cycle activity) and from the liver of an 8-month old male patient who received a liver transplant for severe OTCD. Human primary hepatocytes were genetically corrected, *ex vivo*, through deletion of a mutant intronic splicing site by Cas9 nuclease delivered as ribonucleoprotein (RNP). The corrected hepatocytes were transplanted into the liver of FRGN mice which are Fah-deficient and severely immunodeficient (Rag2 -/-, Il2rg-/-, NOD). When the protective drug, NTBC, is withdrawn, the mice can be re-populated to high levels (>80%) with human hepatocytes. The degree of contribution of repopulated Cas9-corrected cells was measured. Disease phenotype in animals transplanted with gene-edited cells was compared to animals repopulated to similar levels with uncorrected patient cells, or hepatocytes from normal individuals. All animals were maintained on a normal (19%) protein diet. **Results:** DNA sequencing of patient cells revealed a point mutation in a normally intronic region of the OTC gene that creates a novel splice acceptor site that causes the inclusion of a 135bp intronic region in OTC mRNA between exons 5 and 6 and a premature stop codon after exon 5, resulting in an essentially null OTC allele. OTCD primary hepatocytes were corrected with Cas9 RNP and dual gRNAs, *ex vivo,* achieving editing efficiencies >60%, after verification of DNA , mRNA, and protein. The metabolism of ^{15}N- ammonium chloride into urea was significantly enhanced in the corrected gene-edited hepatocytes assayed *ex vivo*. Mice that were highly repopulated with uncorrected, mutant OTC human

hepatocytes displayed elevated ammonia levels and a significant delay in the clearance of an ammonia challenge. Animals transplanted with approximately 60% corrected hepatocytes displayed normal ammonia levels and an enhanced clearance of an ammonia challenge consistent with both gene correction and normal function of the edited hepatocytes. Investigations of amino acid levels and off target liabilities using VIVO (Ackakaya, Nature 2018) are ongoing. **Conclusions:** FRGN mice repopulated with mutant OTC human hepatocytes display characteristic symptoms of the human genetic liver disease. The OTCD phenotype can be reversed by *ex vivo* Cas9 gene editing of the patient's hepatocytes. This study provides proof-of-concept evidence of the efficiency and safety of gene editing and the correction of a metabolic liver disease and points the way to future translatable therapeutic genome editing for OTC and other genetic diseases of hepatocytes.

693. Characterization of Hematopoietic System Reconstitution In Vivo in Metachromatic Leukodystrophy Gene Therapy Patients

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Here we report the molecular analysis of hematopoietic reconstitution in 20 patients enrolled in a self-inactivating lentiviral vector-based hematopoietic stem cell (HSC) gene therapy clinical trial for metachromatic leukodystrophy conducted at SR-Tiget (up to 7 years' follow-up) and in 7 additional patients treated via early access programs. We retrieved integration site (IS) from CD34+, myeloid and lymphoid cells purified at different time points after therapy (in the first year at 1, 3, 6, 9, 12 months whereas after the first year every 6 months) from bone marrow and/or peripheral blood using PCR protocols. From each patient, we retrieved from 6,000 to 65,000 IS, many of which persisted long term with multi-lineage potential. Regarding potential implications for the safety of the treatment, we did not observe clonal dominance events, no bias to integrate near cancer genes and no common insertion sites generated by genetic selection in any patient. The clonal dynamics of hematopoietic reconstitution of the different lineages showed that circulating lymphoid cells were oligoclonal at early time-points and progressively switched to polyclonal after 6 months, whereas myeloid cells were polyclonal from the first time points. Estimations of the HSC activity, obtained by mark-and-recapture statistics of IS observed over time in short-lived cells, showed that at earlier time points the population size was >26,000 cells that then progressively stabilised to ~10.000 from 9 months post-transplantation, suggesting that the initial waves of reconstitution are sustained by short-lived progenitors. Our data indicate that the treatment results in a highly diversified polyclonal and multilineage reconstitution of hematopoiesis without signs of genotoxicity.

694. Ex Vivo and In Vivo Hematopoietic Stem Cell Gene Therapy of Hemophilia A in Mice

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While hemophilia A gene therapy based on rAAV-mediated liver transduction and expression of factor VIII (FVIII) from hepatocytes has shown first promising clinical outcomes, it also has a number of disadvantages including the gradual loss of transgene expression due to hepatocyte turnover, induction of FVIII inhibitor antibodies, the high cost of rAAV vector manufacturing, and the risk of rAAV genotoxicity due to preferential integration into genes, specifically in patients with underlying chronic hepatitis virus infection, which is still a large fraction of hemophilia patients. These problems could potentially be overcome by in vivo hematopoietic stem cell (HSC) gene transfer and FVIII expression from peripheral red blood cells. Our proposed approach has the potential for live-long therapeutic correction after a single intravenous intervention. The enormous amplification of gene modified HSCs upon differentiation into red blood cells and the high-efficiency protein synthesis machinery of these cells create a basis for FVIII production at curative levels. Furthermore, the genetic modification of only a fraction of HSCs could result in tolerance against the transgene product. We have developed a new approach for *in vivo* gene delivery into HSCs that does not require myeloablation and HSC transplantation. It involves 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 (HDAd5/35++) vector system. HDAd5/35++ vectors target CD46, a receptor that is expressed on primitive HSCs. Transgene integration is achieved (in a random pattern) using a hyperactive Sleeping Beauty transposase (SB100x). After in vivo HSC transduction/selection in CD46-transgenic mice, we demonstrated supraphysiological serum concentrations and activity of a bioengineered human factor VIII version (ET3). The ET3 gene was under the control of a mini-beta globin LCR which restricted ET3 expression to erythrocytes. Despite high-level ET3 production from erythroid cells, no effects on hematopoiesis were observed. After initial development of inhibitory anti-ET3 antibody, serum antibody levels greatly decreased in ~50% of treated mice most likely due to low level ET3 expression in the thymus and development of tolerance. After ex vivo transduction of HSCs from CD46-tg/hemophilia A mice and subsequent transplantation into lethally irradiated hemophilia A mice, a phenotypic correction was achieved based on physiological factor VIII serum activity, normal aPTT, and normal bleeding time after tail clipping. In vivo HSC transduction/selection studies in hemophilia A mice as well as studies towards a more efficient induction of tolerance to factor VIII are ongoing and results will be reported.

695. HSPC Gene Therapy for Cystinosis Remains Effective in Patients Carrying the Large Deletion abolishingSHPK expression

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Cystinosis is a rare lysosomal storage disorder caused by loss-of-function mutations in the *CTNS* gene leading to lysosomal cystine accumulation, crystallization and ultimately multi-organ failure and lethality. Current therapies merely ameliorate symptoms and delay progression of the complications, so our group has pioneered the use of hematopoietic stem and progenitor cell (HSPC) transplantation as a new therapy for cystinosis. In mice, a single transplantation of heathy HSPCs into irradiated *Ctns*$^{-/-}$ recipients permanently restores murine *Ctns* expression in all tissues and preserves tissue architecture and biochemical function. Translation of this approach into a human autologous transplantation of gene-corrected HSPCs using a lentivirus vector Phase I/II clinical trial is underway, with enrollment of the first cohort of cystinosis patients in 2019. The most common cystinosis-causing mutation in humans is a 57 kb deletion which eliminates the *CTNS* gene as well as the neighboring *SHPK* (a.k.a. *CARKL*) locus. This gene encodes an enzymatic intermediary of the Pentose Phosphate Pathway of glucose metabolism and was recently found to regulate macrophage polarization and differentiation. As the mechanism of action in cystinosis HSPC therapy involves delivery of functional lysosomes from HSPC-derived macrophages to diseased tissue and roughly 40% of all human cystinosis patients carry homozygous deletions of *SHPK,* we sought to determine if *Shpk* expression was necessary for effective HSPC therapy. This will establish if these patients are likely to benefit from an autologous transplantation of their HSPCs with lentiviral-induced *CTNS* but not *SHPK*. We generated and characterized the first two *Shpk* knockout (KO) models by introducing genomic deletions using CRISPR-Cas9 guides targeted at either the *Shpk* start codon or second exon. Edited offspring were characterized by PCR genotyping and Sanger Sequencing and then bred to homozygosity. We observed dramatic reduction or outright elimination of *Shpk* mRNA and protein expression across multiple tissues. In addition, bone marrow derived macrophages (BMDMs) from *Shpk*$^{-/-}$ mice shifted towards an anti-inflammatory phenotype - confirmation that our novel mouse models recapitulate published *in vitro* findings. *Shpk*$^{-/-}$ HSPCs along with WT and *Ctns*$^{-/-}$ controls were harvested from bone marrow of 2-month old donors by Sca1 immunomagnetic separation and injected via tail vein into DsRed-*Ctns*$^{-/-}$ mice lethally irradiated with 6.5 Gy of whole-body radiation. Four to six months post-transplantation, recipient mice were sacrificed with tissue samples analyzed by qPCR and Mass Spectrometry for murine *Ctns* expression and overall cystine load. Kidney *Ctns* expression was restored and cystine levels were significantly reduced across multiple tissues in mice receiving *Shpk*$^{-/-}$ but not *Ctns*$^{-/-}$ HSPCs - demonstrating that transplantation therapy remains effective despite elimination of *Shpk* in donor HSPCs. These data represent a promising indication that human cystinosis patients lacking *Shpk* can benefit from *ex vivo* gene therapy that restores only *CTNS* expression and need not be disqualified from the ongoing clinical trial on the basis of Shpk genotype.

696. Hepatocyte Transplantation into Lymph Nodes is Curative in the Pig Model of Tyrosinemia

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The effectiveness of cell based therapies to treat liver failure is often times limited by the inflamed and fibrotic environment frequently found in the diseased liver. In this study, we provide pre clinical proof of concept for the treatment of liver failure through ectopic hepatocyte transplantation into lymph nodes in a large animal model of hereditary tyrosinemia type 1 (HT1), a metabolic liver disease caused by deficiency in the fumarylacetoacetate hydrolase (FAH) enzyme. Five FAH deficient pigs received autologous hepatocyte transplantation into mesenteric lymph nodes after *ex vivo* transduction with a lentiviral vector carrying the pig *Fah* gene. Transplanted hepatocytes showed early (6 hour) and durable (8 month) engraftment in mesenteric lymph nodes, with reproduction of vascular and hepatic microarchitecture within transplanted nodes. In addition, hepatocytes transplanted into lymph nodes were also able to migrate into the native liver, repopulating the diseased organ. The corrected cells generated enough liver mass to clinically ameliorate the metabolic disease as early as 97 days post transplantation, with complete normalization of tyrosine levels and liver function tests. Integration site analysis indicated that the population of corrected hepatocytes in the liver was a subpopulation of the cells present in the lymph nodes, indicating that the lymph nodes can serve as a source for healthy hepatocytes to repopulate a diseased liver. Ectopic transplantation of hepatocytes into lymph nodes is curative in the pig model of HT1 and is a promising approach to the treatment of liver disease in patients with pre existing liver damage and fibrosis.

697. Genome Edited Human Hematopoietic Stem Cells Correct Lysosomal Storage Disorders: Proof-of-Concept and Safety Studies for Mucopolysaccharidosis Type I and Gaucher Disease

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Lysosomal storage diseases (LSDs) are a group of more than 50 genetic disorders involving lysosomal dysfunction, most of which lack effective treatments. The need to intervene and improve outcomes has driven the establishment of newborn screening programs for some LSDs, like MSPI, though the real benefit of these programs is bound by the limited efficacy of the therapies. Current interventions available for a minority of LSDs are enzyme replacement therapy (ERT) and allogeneic hematopoietic stem cell transplantation. Generally, these approaches have limited efficacy and at best slow the progression of these diseases. A potentially safer, more effective approach is to engineer the patient's own hematopoietic system to secrete high levels of the relevant lysosomal enzyme. We have established an efficient genome-editing strategy where lysosomal enzymes are specifically targeted to the human *CCR5* safe-harbor locus. Such an approach constitutes an adaptable "one size fits many" platform that is independent of specific lysosomal enzymes or patient mutations. We used this strategy to engineer human hematopoietic stem and progenitor cells (HSPCs) to express iduronidase (IDUA) and glucocerebrosidase (GBA), the deficient enzymes in MPSI and Gaucher disease, the two most common LSDs. In cells derived from cord and adult peripheral blood, the mean modification rates were 34% ± 7 with 4.5Kb and 54% ± 10 with 3.7Kb expression cassettes. The modified cells produce supra-physiological enzyme levels: 25-fold for IDUA driven by the constitutive PGK promoter and 5-fold for GBA using the monocyte/macrophage promoter CD68. We find that neither the genome editing approach nor the enzyme overexpression affect the cells' potential to differentiate into multiple hematopoietic lineages. Specifically, HSPCs expressing GBA generated functional macrophages; the affected cells in this disease. Long-term and serial engraftment studies in immunocompromised mice demonstrate modification of cells with long-term repopulating capacity, albeit at lower efficiency than hematopoietic progenitors. The potential for these cells to improve the symptoms of an LSD was demonstrated using an MPSI mouse model for human cell engraftment. Transplantation of cells overexpressing IDUA in these mice showed improvements in biochemical, bone, and neurobehavioral pathology. Genotoxicity was examined by characterization of the off-target repertoire of our *CCR5* guide using COSMID and deep sequencing. Off-target activity initially found at 4 intronic sites was abolished using a higher fidelity Cas9. Edited HSPCs expanded in culture had normal transcriptional profiles in response to doxorubicin, suggesting normal p53 activity. Sequencing of 198 genes frequently mutated in tumors did not identify any recurrent mutations. Our studies provide proof-of-concept evidence of the safety and efficacy of using genome-edited human HSPCs modified to express a lysosomal enzyme to correct the biochemical, structural, and behavioral phenotypes in MPSI. The potential for this approach to become a platform for treating other LSDs is supported by our studies in Gaucher disease.

Advances in Cell Product and Gene Vector Manufacturing

698. A Robust Commercial rAAV-Vector Platform Process Using the iCELLis® 500 Fixed-Bed Bioreactor

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Adeno-associated virus (AAV) gene therapy has attracted a significant amount of attention in the field and recombinant AAV (rAAV) vectors have been used in more than 130 clinical phase gene therapy trials around the world, with first products approved. rAAV vectors are commonly manufactured using adherent HEK293 cells in 10-layer cell stack vessels (CS10) to supply the needs of early clinical stage studies. However, the CS10 process is expensive, work intensive and lacking the quantity and quality requirements needed for commercial manufacturing. To overcome these limitations, we have developed a new process relying on a packed bed bioreactor system (iCELLis® 500; Pall). Following development and optimisation studies, the iCELLis® 500 process run at full scale is now capable of generating product of quality comparable to the CS10 system but at a scale which can easily meet commercial demands and with significantly reduced cost of goods. To develop a transient transfection-based large-scale manufacturing process using the iCELLis® 500 system, a scale-down model in the iCELLis® Nano bench scale bioreactor was implemented. Process optimisation was focused not only to maximize the total yield but importantly also to improve the quality and consistency of vector batches. This was also achieved by optimisation of our rAAV plasmid packing system (see abstract: "Improved two-plasmid packaging system for manufacturing of AAV vectors with high quality and consistency"). By running several iCELLis® Nano systems in parallel transfection and production conditions were optimised using DoE-approaches. A scale-up of the new process to the iCELLis® 500 commercial manufacturing scale bioreactor was successfully accomplished by adjusting process steps where required due to the different geometries of the bench-scale model and full-scale bioreactor system. The final process was successfully transferred to, and tested at, three GMP manufacturing sites. Based on three iCELLis® 500 runs at full scale, the reproducibility

of the process was demonstrated by analysing the biomass reads together with the metabolites, impurities and final upstream process yield data. Process robustness was further investigated in both small and large scale by variation of several key process parameters. In the meantime, a variety of different rAAV vectors were successfully manufactured using the iCELLis® platform. With almost a dozen of successful iCELLis® 500 large-scale runs at various production sites, the process has demonstrated its robustness and ability to serve as a cost-effective platform suitable to accommodate any of the upcoming Freeline gene therapy targets.

699. Utility of Diatomaceous Earth in Lysate Clarification Simplifies AAV Production

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Adeno-associated virus (AAV) purification is a time- and resource-consuming process, which involves multiple steps of clarification, isolation, concentration and final formulation. Clarification of the producer cell lysate is critical step in which particle recovery can be challenging. Many research-grade processes involve centrifugation and filtration steps to accomplish lysate clarification, and pose a major barrier to scaling as well as cost-efficiency. Here, we evaluated the impact of depth filtration and pre-conditioning the lysate with diatomaceous earth (DE) prior to filtration of the lysate. Evaluation metrics were the recovery of genome-containing viral particles, purity, process time, robustness, and simplicity. Specifically, we performed purifications of AAV2/8 vector preparations at 650 mL lysate scale. Briefly, the overall process of AAV production involved triple transfection of HEK293, high salt harvest, the 3 conditions of clarification under study, tangential flow filtrations (100K MWCO) (TFF), iodoxinal density gradient ultracentrifugation, and a polishing and buffer exchange step using molecular weight cut off filters with final formulation (1xPBS+35mM NaCl+0.001%PF68). Clarification conditions were (a) high speed centrifugation followed by 0.45um vacuum filtration, (b) depth filtration, (c) pre-conditioning with DE followed by 0.22um vacuum filtration. Results demonstrate that recovery rate and purity of the final product were similar in all conditions tested. However, the viral solution following depth filtration turned viscous after concentration by tangential flow filtration (TFF), complicating and delaying further steps. DE filtration was the most time-efficient process while retaining purity and overall recovery, reducing processing time by approximately 90min per liter preparation, and making larger volume preparations feasible. In-process monitoring of purity via SDS PAGE, indicates a reduction of impurities by 70% in the lysate following DE-filtration, which is a vast improvement compared to the other tested methods. Given the utility of DE to clarify the AAV lysate to such degree early in the process, we aimed to simplify the purification protocol further by eliminating the ultracentrifugation step in the process described above. This methodology allowed us to generate high titer and high concentration AAV preparation with comparable purity yet at a highly reduced cost and effort. Ongoing studies further aim to evaluate empty versus full ratio, the impact of salt concentration, PH of viral solution, and other steps to increase further recovery and efficiency.In summary, our results indicate the utility of DE in research grade AAV process, particularly for high-throughput core facilities or when larger yields are required toward scaling of the process in order to support large dose studies. Further work is needed to evaluate the use of DE in processes for clinical grade AAV manufacturing.

700. Development of an NGS-Based Assay and Bioinformatics Workflow for CMC Characterization of Contaminating DNA in AAV Products

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All rAAV products contain varying quantities of non-vector DNA derived from the DNAs present in the host cells used for manufacturing the product. Some rAAV contaminants derive from the production host cell genome, leading to as-of-yet unsubstantiated concerns that oncogenes might be transmitted to patients and stimulate oncogenic transformation. Although qPCR methods commonly assess the degree of contamination by adenoviral E1A and E1B, assessment of contamination by one of the many hundreds of cellular proto-oncogenes has not been performed. Additionally, other sequence contaminants (e.g. immune regulatory genes) may be more likely to have a greater impact on the quality of rAAV vectors. To support further development of rAAV vectors and CMC characterization of rAAV associated contaminating DNA, we have utilized deep sequencing and statistical approaches to advance our understanding of factors governing contaminating sequence incorporation in rAAV. A robust rAAV particle associated DNA sequencing assay would provide quantitative representation of all the DNA present, which typically includes both single stranded and double stranded species of diverse size, structure, and sequence. We optimized an off-the-shelf library preparation kit (Swift Accel-NGS 1S Plus) normally used for degraded and damaged human DNA to generate strand-specific libraries from AAV8 encapsidated DNA and then developed a bioinformatic workflow to identify and quantitate the source of DNA. The bioinformatic analysis focuses on the major sources of DNA present during AAV production for our HEK293 transient transfection manufacturing system, and sequentially aligns reads against different reference sequences in a strand-specific manner in multiple rounds, where only unmapped reads are used after the initial round. This subtractive mapping strategy decreases processing time, reduces the likelihood of errant cross-reference multi-mappings, and allows the workflow to be quickly updated to accommodate alternative plasmid sequences. Our workflow strives to categorize contaminants into groups that are relevant to the interpretation of their potential impact on vector quality and provide quantitative information to enable eventual modeling of the mechanisms of sequence selection. Repeated simulations of a random sequence selection model were generated to compare with groups of actual contaminants and estimate the degree of nonrandom selection. We analyzed patterns of residual DNA across 35 samples including technical replicates and controls with a median sequencing depth of 1.26 million reads per sample. We observed that our method was highly reproducible and was strand specific when

tested on single stranded M13 phage DNA spiked-in at different quantities. Additionally, there was minimal reduction in read coverage across the hard-to-sequence ITR regions. As previously described, we observed the highest levels of contaminants from sequences near Rep binding sites on transfected plasmids (frequency ~$1.4x10^{-2}$). Of the host genomic sequences, the AAVS1 locus was frequently amongst the most abundant contaminant, consistent with the known presence of a strong Rep binding site in that location. The adenoviral E1A and E1B genes and cellular proto-oncogenes (as defined in COSMIC) were either undetected or present at low frequencies at this sequencing depth. Continued implementation of this analysis method on deeper sequencing libraries are needed to confirm this finding and expand our ability to engineer production cells and plasmids with optimal vector production properties.

701. Lentiviral/Retroviral Vector Large Scale Manufacturing

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MolMed is a medical biotechnology company focused on research, development and clinical validation of innovative therapies to treat cancer and genetic rare diseases. Lentiviral vectors (LVV), produced with transient quadri-transfection in 293T cell line and retroviral vectors (RVV), produced with a stable packaging cell line, are used in gene therapy studies. In the context of advanced clinical studies or commercial phase, an important manufacturing challenge is the optimization of vector production in large-scale platforms. To address this issue and to obtain a scalable and robust process, we are developing two different systems with cells in adhesion using Pall iCELLis® fixed-bed disposable bioreactors and with suspension cells in a culture system without animal derived reagents in stirred tank bioreactor. Transfection agent of choice is PEI-PRO HQ by Polyplus that allow efficient transfection of packaging cells without toxicity issues. The optimized parameters included seeding cell density, DNA concentration, timing and volumes of harvest. Final vectors were characterized for infectious viral titre (TU/mL), particle content (ng p24 or ng p30), process related impurities and then compared to vectors produced by current GMP processes based on Cell Factories (CFs). Downstream process was appropriately scaled up and designed in order to accommodate larger volumes of vectors from bioreactor. Single use Tangential flow Chromatography using AKTA Ready Flux and DEAE chromatography step using AKTA Pilot and Axichrom columns were successfully applied both in scaled down model and in final full scale with good removal of process related contaminants and no detrimental impact on vector infectivity and stability. Two full scale run (200L) in Pall iCELLis® bioreactor system have been already performed and results will be presented.

702. Optimization of Transfection and Culture Conditions to Maximize AAV Production in Suspension 293-Based Viral Production Cells

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Introduction: Production of AAV for early-phase clinical use requires manufacture utilizing hundreds of liters of upstream cell culture. Production facilities for early-phase trials have often relied upon adherent cell-based processes, though suspension cells offer greater flexibility and far superior scalability. Here, we have identified a variety of transfection and culture conditions to help maximize production of an AAV8 vector packaged with a FIX genome in 293-based suspension cells. **Methods**: AAV8-FIX was produced in Viral Production Cells (VPCs; Gibco), a derivative of the HEK-293F suspension cell line. Cells were maintained and transfected in shake flasks, ambr 15 micro bioreactors, and in 1 L stirred-tank reactors using serum-free LV-MAX Production Medium (Gibco). Transfection was performed with PEIpro (Polyplus). Conditions tested included cell seeding density and the amount of DNA per cell; the use of a two or three-plasmid transfection system; the use of a single-stranded or self-complementary genome; and the pH and dissolved oxygen setpoints in culture post-transfection. Titers were assessed in crude fractions (combined culture media and lysate) using AAV8 capsid ELISA (Progen) and digital droplet PCR. **Results**: Varying cell seeding density from 1×10^6 to 2.25×10^6 cells/mL while keeping DNA/cell constant led to a 1.4-fold difference in genome titers between the highest and lowest producing groups. Interestingly, using more DNA per cell has a greater effect on capsid production than on genome production, with up to 8×10^{11} capsids/mL produced with 2.25 ug DNA/1×10^6 cells, versus 1.9×10^{11} capsids/mL produced with 1 ug DNA/1×10^6 cells, a four-fold difference. Using three-plasmid transfection led to 1.12-1.15-fold greater production than two-plasmid transfection while maintaining equivalent molar ratios. Experiments comparing single-stranded to self-complementary AAV, with the mutated ITR as the only difference in plasmid sequence, showed that a single-stranded FIX genome packaged with an efficiency of 1.4-1.9-fold higher than the self-complementary equivalent. Finally, control of pH and dissolved oxygen showed that a pH of 6.9 led to higher titers than pH setpoints of 6.7 or 7.1, and that a low dissolved oxygen level had a neutral-to-positive impact on overall AAV production, allowing for the elimination of antifoam reagent in stirred-tank reactors. **Conclusions**: The wide variety of conditions tested here show how many factors can affect AAV production - culture conditions, plasmid configuration, recombinant genome, and the pH and dissolved oxygen levels post-transfection. Process development efforts for clinical AAV manufacturing, therefore, often need go back to the very basics of AAV production to optimize titers. The production levels we have achieved in VPCs are approximately two-fold lower, on a volume basis, than adherent 293T/17 cells. But while more process parameters need to be considered with suspension cells, VPCs are free of any regulatory concerns surrounding the SV40 Large T antigen, and provide a much more viable option for large-scale production.

703. A Scalable Microfluidic Platform to Enhance Transduction Efficiency and Reduce Viral Vector Usage

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Introduction: Manufacturing complexity and limited viral vector availability hampers manufacturing capacity of novel gene and cell therapies, slowing widespread clinical implementation and commercialization. Our scalable microfluidic platform significantly reduces viral vector usage and transduction times. We previously showed that microfluidic transduction leverages enhanced mass transport to reduce lentiviral vector (LV) usage up to 4 fold and transduction times by half in primary human T cells and hematopoietic stem cells of both human ($CD34^+$) and murine origin. In this study, we demonstrated that our microfluidic platform can be further scaled up to accommodate clinically relevant numbers ($\geq 10^9$ cells) using a modular approach. *Methods:* Scaled up 10^8 and 10^9 cell capacity devices were made by bonding individual channel layers connected in parallel for simultaneous loading. Single layer devices were also tested for scalability between 10^7 and 10^8 cell devices. 9-11 µm polystyrene beads were loaded into devices, incubated for 8 hrs, flushed with buffer, collected, and counted to mimic microfluidic transduction procedure. Jurkat cells were loaded and transduced at MOI 0.2 using GFP-LV. Cells were not transduced in the 10^9 cell devices due to LV constraints. Cell viability was assessed via trypan blue following collection. All microfluidic characterizations were compared to 6-well controls. *Results:* **Fig. 1A** shows a 10-layer device capable of holding $4x10^8$ cells. Collection efficiencies were >99% **(Fig. 1B)**. High cell collection **(Fig. 1C)** and viability was maintained **(Fig. 1D)**. MOI 0.2 transductions showed that scaling from $4x10^7$ cells in a single layer to $4x10^8$ cells in a 10-layer device yielded comparable transduction percentages **(Fig. 1E).** Both devices improved transduction over a 6-well control. **Fig. 1F** shows a clinical-scale prototype comprised of 17 layers to accommodate 10^9 cells. Characterization was performed as in **Figs. 1B-D**. Bead collection **(Fig. 1G)**, cell collection **(Fig. 1H)**, and cell viability **(Fig. 1I)** were all comparable to the 6-well control and showed no indication of trapping or negatively impacting cells. **Thus, microfluidics can be scaled up to accommodate >10^9 cells for clinical gene and cell therapy**. *Conclusion:* In addition to reducing transduction times and LV usage, our microfluidic platform readily scales up to accommodate clinical cell numbers. Furthermore, the device can be optimally configured (i.e. modulating the number of layers) to accommodate variable patient cell numbers or alternative manufacturing approaches. Ongoing work will investigate combination strategies such as HSC agonist UM171 to further enhance transduction and reduce LV usage, device optimization, and transduction of 10^9 primary human cells.

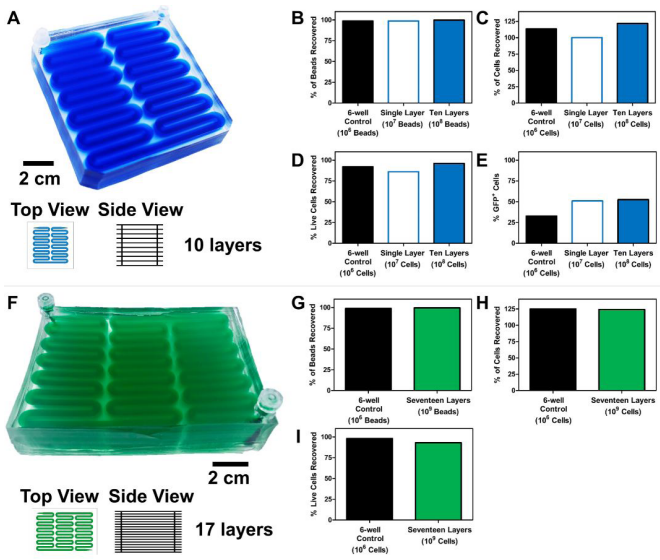

Figure 1: Microfluidics can be scaled up to simultaneously transduce clinically relevant cell numbers ($\geq 10^9$ cells) (A) Example of a microfluidic capable of transducing $\geq 4x10^8$ Jurkat cells. **(B)** Recovery of 9-11 µm polystyrene beads collected from the microfluidics. **(C)** Cell collection and **(D)** viability compared to a 6-well control. **(E)** Comparison of transduction at MOI 0.2 demonstrates scalability and improvement over 6-well. **(F)** Example of a clinical 10^9 cell scale device. **(G)** Bead and **(H)** cell collection compared to 6-well controls **(I)**.

704. High-Throughput CRISPR/Cas9 Genome-Wide Screens to Enhance AAV Manufacturing

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Over the past three decades, adeno-associated virus (AAV) has become an increasingly effective vector for delivering therapeutic DNA in the clinic. As efforts have progressed towards larger tissues and systemic routes of administration, as well as diseases with larger patient populations, manufacturing constraints are currently limiting the clinical development and, in the future, will hinder the commercialization of AAV gene therapies. To address this issue, we have developed a genome-wide CRISPR/Cas9 library screening technology to increase the capacity for HEK293T cells to manufacture AAV. Using the Synergistic Activation Mediator (SAM) and Genome-wide CRISPR Knock-Out (GeCKO) libraries, a library of guides designed to either knock-out (GeCKO) or over-express (SAM) each gene in the human genome was cloned into AAV for high throughput selection of guides that conferred higher AAV viral titers. After up to eight rounds of selection, the resulting pool of guides was subjected to Illumina sequencing, and the relative frequency of each was compared to that in the original library. Targets were identified by fold increase, and stable HEK293T cell lines were developed based on the most promising hits. We found that cells over-expressing the first two hits investigated increased infectious titer by 2.3-fold and 3.3-fold, respectively. In addition, this improvement is translated to

multiple AAV serotypes, and combinations of hits are being explored. Harnessing high-throughput approaches for engineering of producer cells offers the potential to greatly enhance AAV manufacturing.

Oligonucleotide Therapeutics I

705. In Vivo Immunoprophylaxis in Non-Human Primates Following Administration of Synthetic DNA-Monoclonal Antibodies (dMAbs)

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Monoclonal antibodies (mAbs) are highly effective biologics for the treatment of cancer, autoimmunity, and infectious diseases. Unfortunately, mAb delivery by intravenous (IV) delivery can be time-consuming (>8 hours total infusion and follow-up observation) to ensure slow-administration and reduce infusion-related reactions. *In vivo* delivery of monoclonal antibodies (mAbs) using synthetic nucleic acids is a rapidly advancing field to simplify administration of these potentially life-saving biologics. We recently described engineering and *in vivo* delivery using the synthetic DNA-encoded mAb (dMAb) platform to enable the body's own cells to produce gene-encoded mAbs, achieving biologically relevant levels in systemic circulation. First, we performed novel nucleotide and amino acid engineering mutations within the variable heavy and variable light chain regions to increase *in vivo* expression. This directly led to improved dMAb serum levels in mouse and non-human primate (NHP) models and expression lasting for several weeks. We further evaluated combinations of multiple dMAb, demonstrating potential for combination immunoprophylaxis delivery using this platform. Using these engineering strategies, we designed dMAbs targeting the Zika virus E protein DIII domain. The anti-E protein dMAb candidates demonstrated efficacy in mouse models, including prevention against testicular atrophy. dMAb efficacy was further evaluated in a rhesus macaque NHP challenge. We observed a two-log reduction in Zika virus infection in 4/5 macaques that were administered dMAbs. This is the first demonstration of protection with a gene-encoded mAb in an NHP model. Several delivery device improvements are ongoing to further enhance *in vivo* administration. Based on this data an IND has been opened to investigate the safety and pharmacokinetics of this anti-Zika virus dMAb candidate in twenty-four healthy human participants. This is an important step forward for the field and with potential broader implications on human translation by dramatically reducing the time needed mAb delivery against various diseases.

706. Drug Candidate MTL-CEBPA Sensitises Solid Tumours to Standard of Care Therapies

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C/EBPα is a transcription factor that regulates proliferation and differentiation of hematopoietic progenitors. C/EBPα is down-regulated in MDSC from tumour bearing mice and C/EBPα knock out mice display greater MDSC tumour infiltration, vascularization and growth. MTL-CEBPA is a liposomal formulation of a small activating RNA that up-regulates expression of C/EBPα. In patients with hepatocellular carcinoma MTL-CEBPA was found to be well tolerated and demonstrated pharmacodynamic activity, including immune modulation of peripheral blood, and anti-tumour activity. The combination of MTL-CEBPA with sorafenib showed significantly improved tumour growth inhibition compared to single agents in a diethylnitrosamine induced model of hepatocellular carcinoma in Wistar rats. The combination of MTL-CEBPA with PD-1 mAb showed significantly improved tumour growth inhibition compared to single agents in CT26 syngeneic model of colon cancer in BALB/c mice. Investigators reported that Sorafenib induced complete responses in three of four patients with advanced hepatocellular carcinoma previously treated with MTL-CEBPA. MTL-CEBPA in combination with sorafenib is currently being evaluated in an ongoing Phase 1b study in patients with advanced hepatocellular carcinoma. Our current hypothesis is that MTL-CEBPA sensitises solid tumours to sorafenib and PD-1 mAb by modulating the tumour immune microenvironment.

707. Anti-Inflammatory Activity of MTL-CEBPA, a Small Activating RNA Drug, in LPS-Stimulated Monocytes and Humanized Mice

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Excessive or inappropriate inflammatory responses can cause serious and even fatal diseases. The CCAAT/enhancer-binding protein alpha (CEBPA) gene encodes C/EBPα, a transcription factor that plays a fundamental role controlling maturation of the myeloid lineage and is also expressed during the late phase of inflammatory responses

when signs of inflammation are decreasing. MTL-CEBPA is a small activating RNA targeting for upregulation C/EBPα, currently being evaluated in a Phase 1b in hepatocellular carcinoma. Following dosing, subjects had reduced levels of pro-inflammatory cytokines and we therefore hypothesized that MTL-CEBPA has anti-inflammatory potential. The current study was conducted to determine the effects of C/EBPα saRNA - CEBPA-51 on inflammation in vitro and in vivo following endotoxin challenge. CEBPA-51 led to increased C/EBPα gene and inhibition of pro-inflammatory cytokines in THP-1 monocytes previously stimulated by E. coli-derived lipopolysaccharide (LPS). Treatment with MTL-CEBPA in a LPS-challenged humanized mouse model up-regulated C/EBPα mRNA, increased neutrophils, and attenuated production of several key pro-inflammatory cytokines including TNF-α, IL-6, IL-1β and IFN-γ. Additionally, a Luminex® analysis of mouse serum revealed that MTL-CEBPA reduced pro-inflammatory cytokines and increased anti-inflammatory cytokine (IL-10). Collectively, the data support further investigation of MTL-CEBPA in acute and chronic inflammatory diseases where this mechanism has pathogenic importance.

708. Inhalation of an RNA Aptamer Targeting Extracellular Histones Protects from Acute Lung Injury

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Multiple diverse causes of acute lung injury (ALI) exist, including sepsis, bacterial pneumonia, aspiration of gastric contents, inhalation of toxic substances, and epidemic viruses. When severe, ALI results in significant morbidity and mortality. Early in ALI, the exudative phase is characterized by an increase in alveolar-capillary permeability. The clinical manifestation of this barrier disruption is the development of pulmonary edema, with resultant impairment of lung compliance and hypoxemia. Propagation of injury occurs from the release of histones by damaged cells at the site of injury. Histones normally reside in the nucleus where they partner with DNA, but when released from dying cells or during NETosis, histones cause additional tissue injury. We recently identified using Systemic Evolution of Ligands by Exponential Enrichment (SELEX) technology RNA oligonucleotides (aptamers) that bind with high affinity and specificity to histones implicated in cellular toxicity (histones H3 and H4). The goal of the present study was to determine if pulmonary airway administration of an RNA aptamer could protect against histone-mediated lung injury. Direct exposure of cultured human endothelial cells to calf thymus-derived histones (CTH, 50 μg/ml) for 24 hours caused cell apoptosis (25%) as compared to the vehicle group (10%). Administration of a histone-targeted RNA aptamer (KU7) to cells 30 minutes after CTH exposure reduced cell apoptosis (17%, $p<0.05$ vs CTH). Similarly, CTH exposure caused concentration-dependent death of cultured human epithelial cells, but KU7 given to cells 1 hour after CTH protected from this histone toxicity. *In vivo* CTH administration by oropharyngeal aspiration induced ALI in C57BL/6 mice as evident by an increase in broncheoalveolar lavage fluid (BALF) neutrophils, albumin and cytokines and histologic evidence of alveolar edema, inflammatory infiltrate, and alveolar disruption. We next determined the retention and distribution of the RNA aptamer in lung following oropharyngeal aspiration of fluorescent-labeled KU7 (1.7 nmol in 50 μL PBS). The aptamer was distributed throughout all segments of the lung and remained present in BALF at 24 hours without causing an increase in inflammatory cells or injury. Next, mice received CTH (300 μg in 50 μL PBS) to initiate ALI. Treatment with KU7 (4:1 Molar ratio CTH:KU7) 30 minutes prior to, or 30 minutes after CTH, attenuated BALF albumin. In summary, intratracheal delivery of RNA aptamers protected from histone-mediated lung toxicity and may have therapeutic potential in ALI.

709. mRNA Therapy Improves Metabolic and Behavioral Abnormalities in a Murine Model of Citrin Deficiency

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Citrin deficiency results from loss-of-function mutations in the liver-specific mitochondrial aspartate/glutamate transporter encoded by the SLC25A13 gene. Patients with citrin deficiency present with both hepatic and neurological complications which, can be both severe and life-threatening. Conventional protein replacement therapy (ie. enzyme replacement therapy or ERT) is not an option for these patients or patients with other mitochondrial enzymopathies due to drug delivery hurdles and current gene therapy approaches (e.g. AAV) have been hampered by neutralizing antibodies and genotoxicity. The only curative treatment option for these patients is liver transplantation, which remains high-risk with long-term complications associated with chronic immunosuppression. To develop a new class of therapy for citrin deficiency, codon-optimized mRNA with modified nucleotides encoding human citrin (hCitrin) was encapsulated in lipid nanoparticles (LNPs). We demonstrate the efficacy of hCitrin-mRNA-LNP therapy in human cells in culture and in a murine model of citrin deficiency that resembles the human condition. Of note, intravenous (i.v.) administration of the hCitrin mRNA resulted in significant reduction of 1) hepatic citrulline and blood ammonia levels following oral sucrose challenge and 2) sucrose aversion. In conclusion, mRNA-LNP therapy could have a significant therapeutic impact on the treatment of citrin deficiency and other mitochondrial enzymopathies with limited treatment options.

710. The Delivery of miR-708 Impairs Breast Cancer Metastasis

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Triple-negative breast cancer (TNBC) patients exhibit the worst clinical outcomes due to its aggressive clinical course, higher rate of recurrence, and a conspicuous lack of FDA approved targeted therapies. Here, we show that multilayered nanoparticles (NPs) carrying the metastasis suppressor microRNA, miR-708 (miR708-NP) localize to orthotopic primary tomor site, and efficiently deliver the miR-708 cargo to reduce lung metastasis. Using a SOX2/OCT4 promoter reporter, we identified a population of miR-708^{low} cancer cells with tumor-initiating properties, enhanced metastatic potential and marked sensitivity to the miR-708 treatment. In vivo, miR708-NP directly targeted the SOX2/OCT4-mCherry+ miR-708^{low} tumor cells to impair metastasis. Importantly, the biodistribution, toxicity profile and immunogenic profile were not significantly affected after treatment with the NPs. Together, our preclinical findings provide a mechanism-based anti-metastatic therapeutic approach for TNBC, with a marked potential to generate miR-708 replacement therapy for high-risk TNBC patients in the clinic.

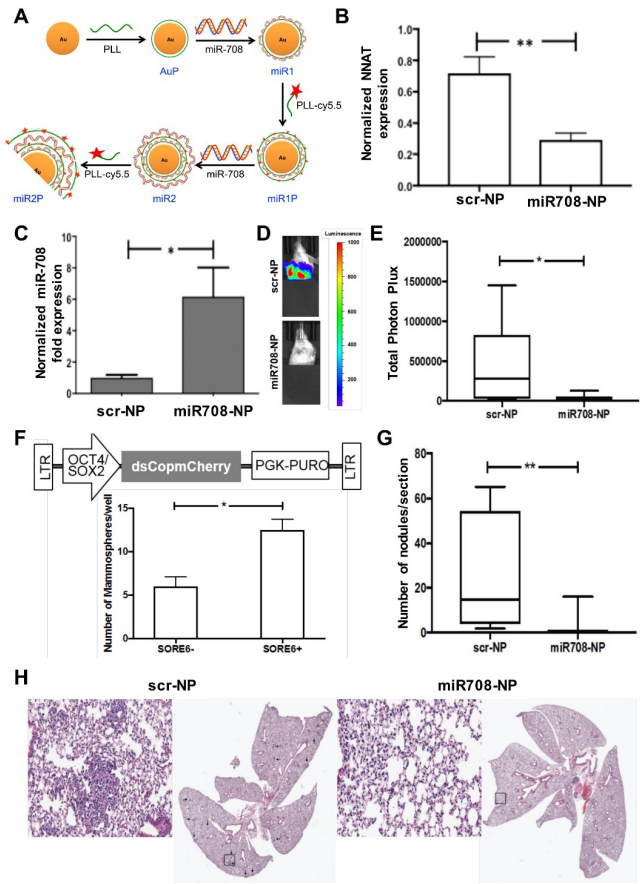

Fig 1: Anti-metastatic effect of miR708-NP. (A) Schematic illustrating the process of preparing multilayered miRNA-coated AuNPs. (B) NNAT expression via qPCR after treatment with NPs: either scrambled (scr-NP) or miRNA-708 (miR708-NP). (C) Primary tumors (n = 5) from the mice in each group (scr-NP or miR708-NP) were lysed, and analyzed for the expression of miR-708. (D, E) The mice were monitored for lung metastasis by BLI, one week after primary tumor resection (n = 9). (F) Reporter construct for SOX2/OCT4 (upper panel); in vitro mammospheres formed by SORE6+ population is significantly higher than SORE6- cells (p = 0.03) (lower panel). (G) 1.5 weeks after resection, mice were sacrificed, and lungs were harvested for H&E analysis. (H) Representative images of lung metastases (black arrows point to metastases in lungs). scr-NP (n=7) and miR708-NP (n=8). *p<0.05, **p<0.01

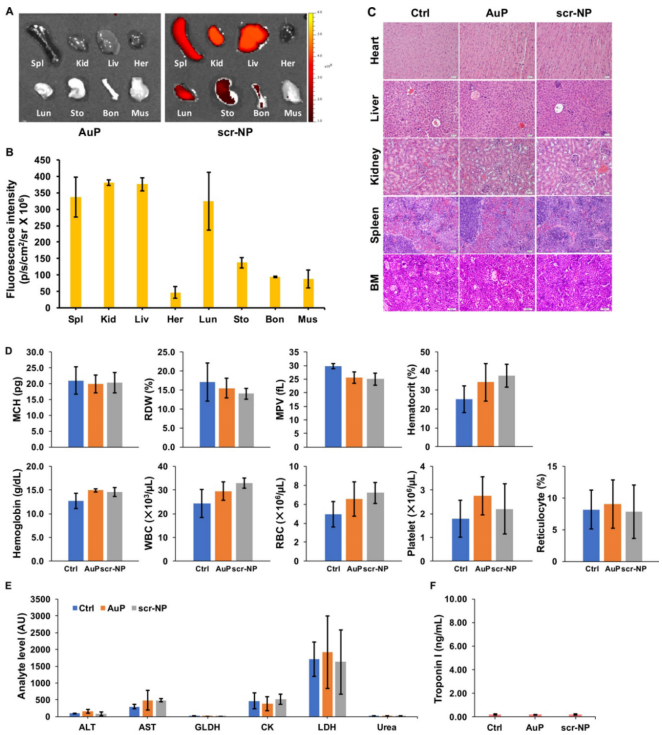

Fig 2: The biodistribution and toxicity profile of NP-mediated delivery system. A-B) The biodistribution study. The fluorescence was measured by optical imaging (Xenogen) one week after treatment of AuP or cy5.5 conjugated scr-NP (n = 3 per group). (Spl = spleen, Kid = kidney, Liv = liver, Her = heart, Lun = lung, Sto = stomach, Bon = bone, Mus = muscle). C) Histopathologic evaluation of the major organs after any treatment. (BE = bone marrow). The scale bar represents 20 μm. D) Whole blood biochemistry indexes. (MCH = mean corpuscular hemoglobin, RDW = red cell distribution width, MPV = mean platelet volume, WBC = white blood cell, RBC = red blood cell). E) Serum analyte activity for liver (ALT, AST, GLDH) or muscle injury (CK, LDH). (ALT = alanine aminotransferase, AST = aspartate aminotransferase, GLDH = glutamate dehydrogenase, CK = creatine kinase, LDH = lactate dehydrogenase). F) The plasma levels of cardiac troponin I for cardiac injury.

711. In Vivo SELEX of an Inhibitory NSCLC-Specific RNA Aptamer (RA16) from PEGylated RNA Library & Synthesized RA16 for Targeting & Inhibition

Hanlu Wang[1,2], Xinxin Ding[1,3], Irvin S. Y. Chen[4], Rihe Liu[1,5], Yongping Jiang[1,2]

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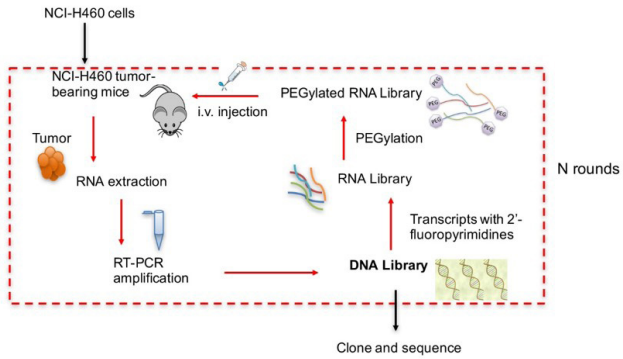
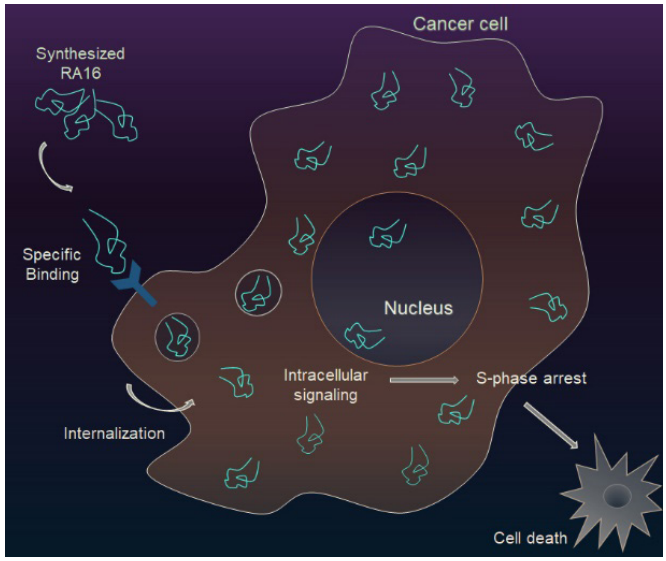
Aptamers are widely used in numerous biochemical, bioanalytical, and biological studies. Most aptamers are developed through an in vitro selection process called SELEX against either purified targets or living cells expressing targets of interest. We report here an in vivo SELEX in mice using a PEGylated RNA library for the identification of a 2'-F RNA aptamer(RA16) that specifically binds to NCI-H460 non-small-cell lung cancer cells with an affinity (K_D) of 9 ± 2 nM. Interestingly, RA16 potently inhibited cancer cell proliferation in a dose-dependent manner with an IC50 of 116.7 nM. When tested in vivo in xenografted mice, RA16 showed gradual migration toward tumor and accumulation at tumor site over time. An in vivo anti-cancer study showed that the average inhibition rate for mouse tumors in the RA16-treated group was 54.26% ± 5.87% on day 16 versus the control group. The aptamer RA16 adducted with epirubicin (RA16-epirubicin) showed significantly higher toxicity against targeted NCI-H460 cells and low toxicity against non-targeted tumor cells. Furthermore, RA16-epirubicin adduct exhibited in vivo anticancer efficacy, with an inhibition rate of 64.38% ± 7.92% when administrated in H460 xenograft mouse model. In addition, we conducted a sequential study of a synthesized RNA aptamer (syn-RA16) and compared it with transcribed RA16 (trans-RA16) in terms of their specific targeting and direct inhibitory activity towards NCI-H460 cells. Similar to trans-RA 16, syn-RA16 was capable of binding NCI-H460 cell with an affinity (K_D) of 24.75 ± 2.28 nM, as well as inhibiting NCI-H460 cell viability in a dose-dependent manner. IC_{50} values were 118.4 nM (n = 4) for syn-RA16 and 105.7 nM (n = 4) for trans-RA16. Importantly, investigations of time course showed gradually internalization of RA16. Aptamer RA16 induced S-phase arrest for NCI-H460 cell growth inhibition. Moreover, in vivo imaging demonstrated the gradual accumulation of both syn-RA16 and trans-RA16 at the tumor site, and qRT-PCR showed the high retention of syn-RA16 in tumor tissues. Furthermore, a truncated fragment of RA16 (S3) was identified and exhibited binding affinity for NCI-H460 cells with a K_D value of 63.20 ± 0.91 nM. In summary, a specific bi-functional RNA aptamer RA16 was selected targeting and inhibiting toward NCI-H460 *in vitro* and *in vivo*. The syn-RA16 and truncated aptamer would greatly facilitate the development of a diagnostic or treatment approach for NSCLC in clinical settings.





Regenerative Medicine II

712. Impaired Therapeutic Efficacy of Bone Marrow Cells from Post-Myocardial Infarction Patients in the TIME and LateTIME Clinical Trials

Xiaoyin Wang[1], Ronak Derakhshandeh[1], Hilda J. Rodriguez[1], Daniel D. Han[1], Dmitry S. Kostyushev[1], Timothy D. Henry[2], Jay H. Traverse[3], Lem Moyé[4], Robert D. Simari[5], Doris A. Taylor[6], Matthew L. Springer[1]

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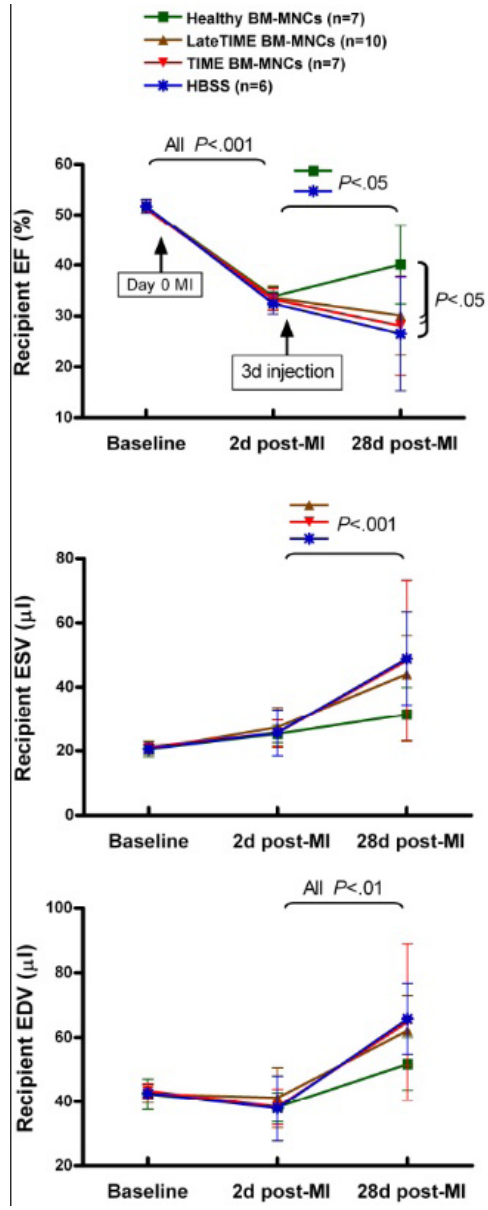
Introduction: Implantation of bone marrow cells (BMCs) into mouse hearts post-myocardial infarction (MI) limits left ventricular (LV) functional decline. However, clinical trials of BMC therapy in patients post-MI have been less successful. While most laboratory experiments use healthy syngeneic BMC donor mice, MI patients receive not pre- but post-infarction autologous cells. We reported that BMCs from post-MI mice are therapeutically impaired, due to MI-induced inflammatory changes in BMC composition. As a result, therapeutic efficacy of the BMCs progressively worsened after MI but recovered as the donor inflammatory response resolved. Hypothesis: The availability of post-MI patient BM mononuclear cells (MNCs) from the TIME and LateTIME clinical trials (harvested at 3 days and 2-3 weeks post-MI, respectively) enabled us to test if human post-MI MNCs undergo a similar period of impaired efficacy. We hypothesized that MNCs from TIME trial patients would be less therapeutic than healthy human donor MNCs when implanted into post-MI mouse hearts, and that the therapeutic properties would be restored in MNCs harvested from LateTIME trial patients. Methods: Groups of SCID mice underwent permanent ligation MI and received intramyocardial injections 3 days later of MNCs pooled from 6 human donors/condition: healthy donors (age 43±6), TIME patients (46±5), and LateTIME patients (47±2); vs. vehicle control. Recipient LV ejection fraction (LVEF) and end-systolic and diastolic volumes (ESV, EDV) were measured by echocardiography. Results: LVEF, ESV, and EDV declined over 28 days in the vehicle group, but improved considerably in the healthy donor group. No therapeutic effect was observed in the TIME or LateTIME groups. Conclusions: Post-MI human BM MNCs lack the therapeutic benefit that healthy human MNCs bestow on post-MI mice, when harvested up to 3 weeks after MI. This may partially explain why BMC therapy clinical trials have been less successful than mouse studies.



713. Targeted Repair of p47-CGD Restores the Ability of iPSC-Derived Macrophages to Kill Bacteria

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Chronic granulomatous disease (CGD) is caused by mutations in the NADPH oxidase and is characterized by defective phagocytes. The NAPDH oxidase is important for the production of reactive oxygen species that further regulate the phagosome milieu, such as the release of granules containing antimicrobial proteins, and the killing of bacteria inside the phagosome, which is defective in CGD patients. Only allogenic hematopoietic stem cell transplantation (HSCT) and retroviral gene therapy are curative treatment options for CGD patients. The first gene therapy trials for CGD were complicated by insertional mutagenesis and transgene silencing of the applied LTR-driven gamma-retroviral vector. More promising results were shown by recent studies with lentiviral SIN vectors and a myeloid-specific promoter. However, targeted gene correction using CRISPR-Cas9 would avoid the risk of vector-mediated insertional mutagenesis and silencing of the therapeutic gene. In our study, we developed a gene editing approach to correct $p47^{phox}$-deficiency, which affects about 25% of CGD patients. Over 90% of these patients carry the mutation c.75_76delGT (ΔGT) in exon 2 of *NCF1* (encodes $p47^{phox}$). This high prevalence most likely originates via gene conversion from one of the two pseudogenes *NCF1B/C*, which are over 99% homologous to *NCF1* and carry the aforementioned ΔGT mutation. For proof-of-principle, we corrected $p47^{phox}$-deficient induced pluripotent stem cells (iPSC) by inserting a minigene donor construct into intron 1 of *NCF1*. We chose this position, which differs by three additional nucleotides from the pseudogenes, to avoid cleavage in the pseudogenes and, thus, potential chromosomal instability due to multiple DNA double-strand breaks. To analyze functionality, genetically corrected clones were differentiated into granulocytes and macrophages. Corrected granulocytes expressed $p47^{phox}$ similar to wild type levels, displayed NADPH oxidase activity and were able to form neutrophil extracellular traps, which enable granulocytes to kill microbes extracellularly. Finally, iPSC-derived macrophages were infected with GFP-labeled *E. coli*. After phagocytosis, corrected macrophages significantly reduced the amount of living bacteria inside their phagosomes as measured by colony-forming units in plated cell lysates compared to uncorrected macrophages. In summary, we demonstrated that the specific insertion of a minigene into intron 1 of *NCF1* corrects $p47^{phox}$-deficiency. Moreover, the pseudogenes remained intact with our strategy, which could be of importance as the pseudogenes might have functional roles. We showed that the corrected iPSC-derived granulocytes and macrophages have all the features necessary to fight infections. In the past, some patients benefited from allogenic granulocyte transfusions. However, due to alloimmunization, this therapy is contraindicative for subsequent HSCT. We propose that autologous iPSC-derived granulocytes and macrophages could be applied therapeutically in the future to combat refractory infections prior to HSCT.

714. Correcting Bleeding Disorders Using Blood Clotting Factors Produced by Shielded Engineered Allogenic Cells

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Background: Current hemophilia therapies require frequent protein infusions yet are unable to address long-term complications due to suboptimal therapy adherence, non-ideal factor kinetics and generation of inhibitors. To overcome these drawbacks in hemophilia, alternative modalities such as gene and cell therapies are being investigated. Allogeneic human cells modified to produce recombinant human hemophilia factors can be shielded from immune rejection by encapsulation. Spheres made with Afibromer™ biomaterials shield engineered human cells from immune rejection while also preventing the fibrotic foreign body response around the implanted spheres, thus enabling a sustained therapeutic effect. Aims: To evaluate whether sustained delivery of blood clotting factors by implantation of engineered human cells producing hFVIII, hFIX or hFVII is dose adjustable, durable, and potentially able to result in better clinical outcomes versus bolus dosing. Methods: Various doses of engineered cell containing spheres encapsulated with Afibromer™ biomaterials were administered intraperitoneally (IP) to murine wild type and knockout disease models. Factor production was evaluated via a combination of protein ELISAs, activity assays (chromogenic) and bleeding assays. Results: Non-virally modified human cells optimized for hFVIII, hFIX and hFVII protein production were placed in Afibromer™ spheres and administered IP to wild type mice. This resulted in therapeutic levels of blood clotting factors. Additional studies in hemophilia A knockout mice resulted in dose-dependent levels of functional hFVIII in plasma, with a corresponding correction of bleeding time and blood loss in a tail bleeding test setting. Spheres producing hFVIII have been tested for long term in vivo viability and protein production (≥6 months). Conclusions: Taken together, these data demonstrate that IP administration of Afibromer™ spheres shielding engineered human cells can result in sustained factor production and efficacious correction of the bleeding phenotype in murine preclinical models. The sustained secretion achieved after a single IP implantation creates a viable alternative to traditional factor delivery or gene therapy with several important advantages. We aim to pursue first clinical studies in patients with Hemophilia A, and to develop additional therapeutics using Shielded Living Therapeutics™ for other serious bleeding disorders. The platform enables itself to deliver a diverse set of therapeutic proteins which is currently under exploration.

715. Engineered B Cells as a Universal Platform for the Treatment of Enzymopathies

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Enzymopathies are a disturbance of enzyme function, including genetic deficiency or a defect in specific enzymes. Current treatment methods are insufficient and rely on hematopoietic stem cell transplant (HSCT) or lifelong enzyme replacement therapy (ERT). ERT can cost hundreds of thousands of dollars per year and HSCTs are highly precarious, with a subset resulting in death from graft versus host disease or infection brought on by prolonged immunosuppression. An alternative approach would be to modify a patients more malleable and accessible cells, such as lymphocytes, to express large quantities of active enzyme and re-infuse these cells into the patient to produce the lacking enzyme. This enzyme can be excreted from engineered cells *in vivo* and taken up by endogenous cells, a process termed cross correction. Recently, there has been a large amount of work on genome engineering of human T cells, typically for cancer immunotherapies. However, the subsets of T cells that are long-lived are metabolically inactive and not ideal for constant protein production. Conversely, B cells can generate large amounts of protective antibodies and continue to do so for years after conversion to long-lived plasma cells. The fact that B cells can become long lived and inherently have the metabolic activity to generate large quantities of protein (i.e. antibody) led us to hypothesize that these cells might be an ideal platform for gene therapy of enzymopathies. To enable the use of engineered B cells for therapy, we recently established the use of CRISPR/Cas9 for gene knock-in (>30%) and knockout (>92%) in human B cells (Sci Rep. 2018 Aug 14;8(1):12144). Now, we are applying these approaches to engineer B cells for the treatment of enzymopathies. Here, we have chosen to utilize our platform for the treatment of Mucopolysaccharidosis type I (MPS I). MPS I is an autosomal recessive lysosomal disease caused by deficiency of alpha-L-iduronidase (IDUA) enzyme resulting in accumulation of glycosaminoglycans (GAGs) storage material and multi-systemic disease. Affected individuals suffer from hepatosplenomegaly, skeletal dysplasias, cardiopulmonary obstruction, and in the severe form (Hurler syndrome) progressive neurologic impairment. In order to engineer B cells capable expressing ultra-physiological levels of IDUA, we developed an approach to simultaneously knockout the endogenous BCR heavy chain and introduce a IDUA overexpression cassette at the heavy chain locus (**Fig. 1**). The design of this cassette is based on standard gene traps, whereby we introduce a vector comprised of a splice acceptor (SA) and polyadenylation (pA) sequence, followed by an IDUA expression cassette near the mu enhancer in the heavy chain locus. By eliminating the cells ability to produce endogenous BCR, and subsequent antibody, it will likely remove a significant metabolic burden, allowing for enhanced production of IDUA enzyme. Moreover, this will avoid any concern associated with transplantation of B cells expressing various endogenous antibodies that could cause adverse events in patients. We have demonstrated that this approach can knockout BCR expression using an EGFP containing cassette. We are currently engineering human B cells with an IDUA encoding vector in this manner and will treat a mouse model of MPS I on a NOD/SCID/Il2rγ background by transplantation of engineered human B cells. Results from these studies will be presented at the ASGCT Annual Meeting.

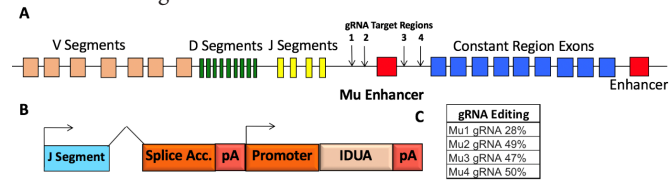

Figure 1. Targeted gene integration at the BCR heavy chain locus. A. Diagram of the BCR heavy chain locus depicting the enhancers, D, J, and constant exons. We have indicated the proposed site of transgene integration as well. **B.** Diagram of cargo design for gene delivery at the BCR heavy chain locus. **C.** Gene editing frequency of gRNAs targeting the Mu enhancer. P2A: ribosomal skip sequence to link cDNAs transcriptionally, pA: polyadenylation sequence, Splice acc: strong splice acceptor element.

716. Induced Neural Stem Cell Therapy to Treat Primary Triple Negative Breast Cancer

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Background: Breast cancer is the deadliest cancer in women in the US. One of the most commonly used strategies for the treatment of these cancers is chemotherapy. However, the systemic toxicity associated with the non-targeted chemotherapy limits the dose and therapeutic effect of chemo drugs. We developed a tumor-homing cell line by transdifferentiating human fibroblasts to induced neural stem cells (hiNSCs) after introducing *Sox2* gene to the fibroblasts with lentivirus. Our previous *in vivo* studies showed that the hiNSCs were capable of migrating to and killing brain tumors when they were equipped with a cytotoxic gene, TNF-related apoptosis-inducing ligand (TRAIL). The goal of the current project is to investigate the therapeutic effect of our targeted hiNSC therapy against primary triple-negative breast cancer. We hypothesized that following systemic administration, our cytotoxic hiNSCs will migrate to tumors and reduce tumor burden. **Method:** *In vitro* co-culture assay was performed to evaluate the tumor killing efficacy of hiNSC-TRAIL against MB231-Br cells with different hiNSC and tumor cell ratio at different time points (24hrs, 48hrs, and 72hrs). House-bred nude mice were inoculated with fluorescence and bioluminescence labeled MB231-Br fat-pad tumor by subcutaneous implantation. After 7 days, hiNSCs labeled with fluorescent and bioluminescent markers were administered by intravenous injection. In order to investigate the migration and persistence of the hiNSCs, the tumors were harvested on day 3, day 7, and day 14 for sectioning. The therapeutic efficacy of the hiNSCs was investigated by evaluating tumor volume by measuring the bioluminescence signal after hiNSC-TRAIL administration. **Result:** The co-culture assay showed that hiNSC-TRAIL was able to reduce the viability of the MB231-Br tumor cells by 82% at Day 3 with 1:1 hiNSC-TRAIL: cancer ratio. After systemic injection, the combination of kinetic imaging and analysis of tissue sections showed hiNSCs in the fat-pad tumor in as little as 3 days. The number of cells that migrated to the tumor peaked at Day 7, and cells were still present in the tumor 2 weeks after injection. Demonstrating therapy, we found the average tumor volume in the animals treated with cytotoxic hiNSCs were reduced by 75% compared to control treated mice 14 days post-treatment. **Conclusion:** These data suggest that hiNSCs are able to migrate to tumor site and reduce tumor burden after

a single dose of systemic cell administration in triple-negative primary breast cancer mouse model. These results provide a solid foundation for further studies to treat metastatic peripheral tumors.

717. Intracranial Pancreatic Islet Transplantation Induces Long-Term Cognitive Improvement in Rats with Sporadic Alzheimer's-Like Disease Dementia

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Introduction: Increasing evidence suggests that Alzheimer's disease (AD) is a type of brain diabetes (type 3 diabetes) associated with brain insulin resistance and deficiency. Intranasal insulin administration has been suggested as a potential approach to overcome brain insulin resistance and improve cognitive functions in patients with AD. As an alternative route for insulin delivery into the brain, we used islet transplantation into the cranial subarachnoid cavity. Pancreatic islets produce and release insulin according to metabolic demand (e.g glucose level). Our recent study showed that a very small number of intracranially grafted islets provide continuous and safe insulin delivery to the brain of rats without alteration of peripheral glucose homeostasis. In addition, islets grafted into the cranial subarachnoid space attenuated cognitive dysfunctions in rats with sporadic AD. In the current study, we investigated a long-term effect of intracranially grafted islets on cognitive functioning in rats with AD. **Methods:** Sporadic AD was induced in inbred Lewis rats by intracerebroventricular administration of subdiabetogenic dose of streptozotocin (icv-STZ). Two months after AD induction, one hundred of syngeneic islets were transplanted into the cranial subarachnoid space. Six months after transplantation, cognitive functions were assessed by Morris water maze test. Islet graft survival was evaluated by immunohistochemical and biochemical methods. **Results:** Spatial learning and memory in transplanted rats were significantly better than in the sham-operated icv-STZ rats. No significant differences in the locomotor activity between transplanted and non-transplanted icv-STZ rats were detected. The grafted islets showed intact morphology, intensive expression of insulin, glucagon and glucose transported 2 (Figure). All transplanted and non-transplanted rats showed intact peripheral glucose homeostasis. **Conclusion:** Intracranial islet transplantation provides an efficient and safe approach for insulin delivery to the brain, leading to a long-term attenuation of AD cognitive dysfunctions.

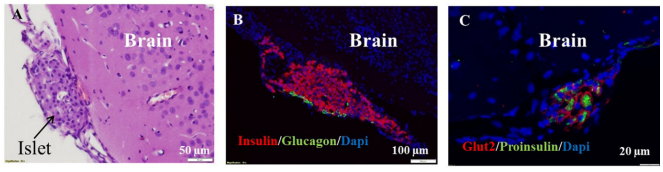

Figure Morphology (A), insulin, glucagon (B) and glucose transporter 2 (C) staining of islets grafted into cranial subarachnoid cavity of icv-STZ rats.

718. Human Mesenchymal Stromal Cells Engineered to Express Collagen VII Can Restore Anchoring Fibrils in Recessive Dystrophic Epidermolysis Bullosa Skin Graft Chimeras

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Recessive dystrophic epidermolysis bullosa (RDEB) is a severe skin blistering disease caused by mutations in *COL7A1*, the gene encoding type VII collagen (C7), the main component of anchoring fibrils (AFs) at the dermal-epidermal junction (DEJ). Loss-of-function mutations in *COL7A1* lead to malformed, reduced or absent AFs, which can compromise the integrity of the DEJ, leading to severe sub-lamina densa blistering and tissue cleavage. Presently there are no curative treatments for RDEB, although early phase I/II clinical *ex vivo* gene therapy trials are investigating retrovirally transduced autologous epidermal grafts and intradermal injections of lentivirally engineered fibroblasts. Alternative systemic investigative approaches include allogenic stem cell transplantation and the infusion of mesenchymal stromal cells (MSCs). We have previously reported that lentiviral (LV) engineered MSCs can substitute C7 secretory function of fibroblasts in the generation of artificial bioengineered skin equivalents and support restoration of the DEJ through AF formation. C7-MSCs have now been investigated for *in vivo* effects and their ability to correct human RDEB grafts on immunodeficient mice. MSCs co-transduced with LV-C7 and a LV-Luciferase-eGFP vector were enriched to 99.5% and the enriched population injected intradermally into grafted RDEB skin. Serial bioluminescence imaging confirmed localized persistence of cells for over a month with minimal loss of signal. Post-mortem the persistence of gene engineered MSCs was further confirmed through detection of eGFP in the dermal compartment. Furthermore, Ki67-positive cells were detected in the dermis, suggesting that injected MSCs had persisted and continued to proliferate in the dermal compartment. Importantly, C7 deposition was seen at the DEJ of the grafts after injection of engineered MSCs, whereas no C7 immunopositivity was detected in PBS injected control grafts. Areas of MSC-injected grafts exhibited significantly increased numbers of sub-lamina densa fibrillary structures that bore the ultrastructural characteristics of well-defined, banded AFs, quantified per 10 microns to be an average of 31 in the treatment group vs 30 in wild type grafts vs 8 in control animals (n=3). In contrast, intravenous delivery of MSCs followed by serial imaging yielded no bioluminesence signals at sites of human grafts, but instead imaging suggested rapid sequestration of injected cells into the lungs. In summary, MSCs engineered to overexpress C7 may support correction of the disease phenotype through AFs reconstitution following localised injection in RDEB. Direct comparisons between engineered MSCs and fibroblasts are warranted for possible therapeutic applications in the treatment of severe blistering disorders.

AAV Vectors III

719. Bat Adeno-Associated Viruses as Gene Therapy Vectors with the Potential to Evade Human Neutralizing Antibodies

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Pre-existing immunity in human populations has been an important concern for safety and efficiency for application of adeno-associated virus (AAV) to clinical gene therapy. Isolation of AAV from species other than human and nonhuman primates plausibly provides the solution to this problem. In this study, four full-length bat AAV capsid genes were isolated in China with their amino acid sequences sharing 61% identity with that AAV2 in average. These capsid genes could package AAV particles in combination with AAV2 *rep* and *ITRs* albeit with a lower efficiency. Bat AAVs could barely infect mouse liver but transduce mouse muscle to some extent after systemic administration into C57B6 mice with a higher muscle/liver ratio than that of primate AAVs. Bat AAV 10HB showed the moderate muscle transduction similar to that of AAV2 during direct intramuscular injection. It was also relatively efficient in resistance of human antibody neutralization to other AAV serotypes after intramuscular injection into the severe combined immunodeficiency (SCID) mice. Evolutionary analysis revealed a number of codons in bat AAV capsid genes subject to positive selection with sites correspondent to V259 and N691 in 10HB capsid localized on surface of AAV2 capsid. Mutagenesis studies corroborated the positive selection in bat AAV capsids to be driven by their tropism evolution in host species. In summary, bat AAV 10HB vector showed the potential for muscular gene therapy especially in existence of human AAV neutralizing antibodies.

720. Efficient Delivery of the Skeletal Muscle Sodium Channel 1 to Cardiomyocytes with Dual AAV Vectors

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Amsterdam UMC, Amsterdam, Netherlands

Cardiac arrhythmias are an important contributor to morbidity and mortality in heart disease, affecting the lives of more than 4 million people in the U.S. alone. The skeletal muscle sodium channel 1 (SkM1) has been identified as a potent gene therapeutic target for both acquired and inherited arrhythmias. When overexpressed in cardiomyocytes, SkM1 can restore impulse propagation in impaired pathways, thereby reducing susceptibility to reentrant arrhythmias. In addition, when co-expressed with the pacemaker channel HCN2, it generates highly efficient biological pacing. Yet, the delivery of SkM1 is challenging because its coding sequence exceeds the AAV capacity limit. Although it has been shown that "forcing" large genes into AAV capsids can achieve transgene expression, this is typically associated with very low efficiency. To overcome this, we developed hybrid dual AAV vectors (Hybrid5 and -3) for the cardiac delivery of SkM1. SkM1 cDNA was split into two fragments where the 5' fragment was packaged into Hybrid5 and the 3' fragment into Hybrid3. Infection of target cells with both hybrid vectors is expected to lead to full-length SkM1 expression after recombination and trans-splicing in the nucleus. As a control, the oversized single AAV vector was also generated. HEK293T cells and neonatal rat ventricular cardiomyocytes were infected with both dual vectors or the oversized single vector, at an MOI of 50,000 per vector. FDA-approved molecules Etoposide and Teniposide were added two hours before infection to improve the transduction efficiency. Cells were harvested 3 days post infection, after which SkM1 mRNA and protein expression were detected using RT-qPCR and Western blot. In both HEK293T cells and cardiomyocytes, transduction with hybrid dual vectors showed significantly higher SkM1 mRNA expression compared to the oversized single vector. Robust SkM1 protein expression was detected in both HEK293T cells and cardiomyocytes after hybrid dual vector transduction in the presence of Etoposide or Teniposide. These results demonstrated the successful delivery and expression of SkM1 in vitro by hybrid dual vectors, which supports the application of SkM1 in antiarrhythmic gene therapy. In vivo validation experiments are currently in progress. After the successful completion of these validations we will proceed with functional testing in ischemic arrhythmias and biological pacing.

721. Dissecting the Positive Selection Events During Evolution of Adeno-Associated Virus Lineages

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Adeno-associated virus (AAV) has been universally used as the vector for human gene therapy. AAV serotypes showed different tissue tropism and immunogenicity, which favored their diverse applications in gene therapy. However, the origin of the AAV serotypes in human and nonhuman primates remains elusive. In this study, AAV capsid genes from human and nonhuman primates were subject to recombination and selection analysis using the cutting-edge bioinformatics tools. Episodic diversifying selection was detected within the AAV lineages after subjecting the unrecombined segments to branch-site test and MEME analysis. Furthermore, partial selected sites from branch-site test and most of them from MEME analysis were distributed on the exterior surface of AAV capsids. Potential functions of these capsid sites involved transduction, antibody recognition or cellular immunity. Two of these sites from AAV2 capsid, T410and E548 were further studied on their evolutionary mechanism. The site 410 was located in a putative human T cell epitope which related it to cellular immunity. The site 548 was in the epitope of mouse A20 antibody which inferred their role in evasion of antibody neutralization. According to the phylogenetic trees, the 410 site was replaced from Q to T and the 548 site was replaced from G to E during the evolution of AAV2 lineage. They were thus reverted to their ancestral condition for evaluation their effects on AAV2 capsid function. A monoclonal antibody C11 was

designed specific to A20 epitope with the G548 reversion. Although the replacement from G to E remarkably reduced the binding of C11 antibody to AAV2, this antibody was non-neutralizing. In comparison, the A20 antibody showed stronger binding of the AAV2-E548 but no difference was observed for the A20 neutralization with them. Significantly, no matter in the pooled human sera (intravenous immunoglobulin, IVIG) or in the human plasma samples individually, the AAV2-E548 showed the stronger affinity but was neutralized to a less extent. Furthermore, the higher selection fitness of AAV2-548E mutant was also observed in their more efficient transduction of mouse liver. All these results strongly inferred the evolutionary adaptation of the E548 replacement in AAV2 capsid within human population. The evolutionary mechanism studies of AAV2 410 site was still on the way. Taken together, positive selection was inferred as an important mechanism for evolution of AAV serotypes, the study of which might promote the comprehension and engineering of their genetic diversity.

722. Abstract Withdrawn

723. Developing a CRISPR Tool to Facilitate Quantification of AAV Transduction in the Mouse CNS

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Engineered recombinant adeno-associated viruses (AAVs) are commonly used vehicle for *in vivo* gene transfer. Several novel AAVs have been developed to achieve wide-scale transduction across multiple cells types in the central nervous system (CNS). Wide scale CNS gene transfer is relevant to gene therapy as well as for disease modeling in mice. The efficiency and tropism of AAV vectors are typically determined by immunohistochemistry-based analysis for co-labeling of an endogenous cell type marker and a fluorescence reporter expressed from the AAV genome. However, immunohistochemistry-based methods can underestimate transduction efficiency if the fluorescence reporter is expressed at nondetectable levels in certain cell population. Here, we describe a CRISPR-based method that enables analysis of AAV transduction in neuronal population in CNS in a more sensitive, quantitative and higher throughput manner. In Cas9 mouse neonates, AAV9, AAV-PHP.B and AAV-PHP.eB were intracerebroventricularly administered at two doses. The AAV vectors express a guide RNA that targets both alleles of the neuronal marker gene NeuN for disruption at high rate (95.7%), and thereby eliminates expression of NeuN in transduced neurons. Simple Western assays of bulk cortex tissues revealed that at 5E+10 vector genomes (vg), AAV9, AAV-PHP.B and AAV-PHP.eB resulted in 42%, 65% and 77% reduction in NeuN protein level respectively. The CRISPR-mediated NeuN reduction corresponds to neuronal transduction efficiency of the AAV vector that delivers the CRISPR guide RNA. At high dose, AAV-PHP.eB resulted in 95%, 91%, 85% and 92% reduction of NeuN in cortex, hippocampus, subcortex and spinal cord respectively, indicating wide-scale transduction of neurons across CNS. We are expanding the capacity of this method to allow analysis of AAV transduction in astrocytes and oligodendrocytes in the mouse CNS.

724. Adeno-Associated Virus Serotype rh74 Prevalence in Muscular Dystrophy Population

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To minimize risk of adverse immune responses, current adeno-associated virus (AAV) gene therapy delivery practices require subjects to be free of antibodies against specific AAV serotypes before treatment. During candidate selection into our Duchenne muscular dystrophy (DMD) and limb girdle muscular dystrophy (LGMD) 2E, 2C, 2D, 2B (NCT03652259, NCT01976091, NCT02710500, NCT03769116) gene therapy studies, study participants were screened for antibodies against AAVrh74 using a validated enzyme-linked immunosorbent assay (ELISA). Subjects with an endpoint antibody titer ratio greater than 2.0 at the serum dilution of 1:400 at screening are currently not permitted to enter our clinical trials. The objective of this study encompassed evaluating the AAVrh74 antibody prevalence in the LGMD and DMD populations. A total of 95 LGMD and DMD subjects were screened for antibodies against AAVrh74. Of the subjects screened as of September 25, 2018, 82.9% did not have antibody titers greater than 2.0 and were eligible for enrollment into our DMD or LGMD gene therapy trials. These findings support the selection of AAVrh74 as a gene therapy vector and indicate promise that AAVrh74-based gene therapy will not induce significant anti-capsid immune responses in the majority of muscular dystrophy patients.

725. Development of Analytical Package for AAV Vectors: Focus on qPCR

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In order to allow AAV vector entry into the clinical trials and subsequently the market, robust analytical methods of the final AAV products as well as in-process AAV must be developed. 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 and, more recently droplet digital PCR (ddPCR) for determining the number of AAV genomes present in AAV preparation. These methods rely on the 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 such PCR approaches extends to estimating the levels of DNA impurities mis-packaged into the particles during production and assembly process. 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. Here, we

describe our panel of qPCR-based AAV titration and residual DNA packaging assays with the aim to create a robust characterisation assay package for AAV vectors. Our earlier work has determined that a large proportion of the mis-incorporated plasmid backbone DNA consists of Kanamycin-gene derived sequences. We have developed a plasmid maintenance system ORT™ (Operator Repressor Titration) in E.coli which does not require antibiotic selection. The use of this plasmid system for triple transfection of HEK293T cells results is equally efficient AAV production, when compared to standard plasmids which are propagated under kanamycin selection. Our work presented here was aimed at (a) using our ORT plasmid system in AAV production and (b) characterisation of the mis-incorporated plasmid DNA packaging with respect to the kanamycin packaging contribution by each of the plasmid through the use of combination of ORT and KAN plasmids. The results identify the contribution of each of the triple-transfected plasmid and suggest an ITR-containing AAV genome dependent mechanism for mis-packaging of the backbone sequences. Further work will address improvement of the AAV upstream production process in order to optimise the DNA usage in transfection and reduce the levels of the kanamycin gene present in AAV capsids. With a thorough understanding of the mechanisms underlying the residual packaging, it would be possible to develop a comprehensive characterisation package for AAV produced for any production platform system.

726. Development of a High Cell Density Perfusion Method for Baculovirus Infected Insect Cells (BIICs) Manufacturing

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Voyager Therapeutics, Cambridge, MA

Recombinant Adeno-associated virus (rAAV) is the vector of choice for a significant portion of ongoing gene therapy clinical trials. The baculovirus/Sf9 system is a cGMP compatible and scalable manufacturing platform established for rAAV production. As with any other biological based production process, there is potential for batch-to-batch variability. Baculovirus inoculum is a critical raw material and can have a strong impact in process yield and/or product quality. Each baculovirus bank does require extensive analytical characterization and production parameters which are often re-optimized for each bank. This adds time and cost for long-term manufacturing. Thus, there are multiple advantages in producing large enough banks of baculovirus inoculum that can enable production of rAAV in large-scale bioreactors with the same lot for multiple annual manufacturing campaigns. This presentation highlights Voyager Therapeutics' efforts in advancing our scalable and robust baculovirus/Sf9 manufacturing platform by development of a novel High Cell Density Perfusion method to manufacture Baculovirus Infected Insect Cells (BIICs) for use as virus inoculum banks. The optimized BIIC production method uses an improved flow system to promote high cell density cultures and to perform a media switch for the cryopreservation of the BIIC cells. Using this improved system, a virus bank is able to be produced at high concentration and at high quantities, allowing the use of consistent virus inoculum for rAAV product development and manufacturing activities. This work demonstrates the strength of the Voyager Therapeutics baculovirus/Sf9 platform, its potential to enable the commercial manufacturing of product candidates, and its promise as a robust supply technology for gene therapy patients.

727. Using Next Generation Sequencing to Assess Accuracy and Quality of Recombinant Adeno Associated Virus Preparations

Karen Guerin, Meghan Rego, Ina Ersing, Leila Haery, Lianna Swanson, Marcella Patrick, Daniela Bourges, Kate Harten, Meron Tasissa, Erin Sanders, Isabelle Mueller, Luke Hanley, Melina Fan
Addgene, Watertown, MA

Recombinant adeno-associated virus (rAAV) are increasingly popular gene delivery tools in research and have been shown to be safe and efficacious in clinical settings. They are available in multiple serotypes, natural or engineered, which provide a great toolbox for targeting of a large number of tissues and cell types. Research-grade rAAVs are relatively easy to produce in a well-equipped lab, and viral vectors cores are available to those labs who are not.Unfortunately there is no standardization of quality control (QC) assays and most research labs only titer the vector preps, typically by qPCR. While it is critical to determine the titer of all preps using a validated protocol, it does not provide any information on the purity of the vectors or the identity or quality of the packaged DNA. It is widely accepted that rAAV preps are heterogeneous due the production methods which requires elements of both viral and cellular origin. In addition, for labs producing and handling multiple vectors at once - like ours - it is important to confirm the identity of the packaged DNA for all vector lots. We use next-generation sequencing (NGS) to extensively and rapidly characterize our viral preps. Here we provide a detailed protocol for next-gen sequencing of rAAV viral genomes extracted from purified rAAV preps. Our data analysis workflow using commercially available software and custom Python scripts (openly available on GitHub) allow us to rapidly and accurately confirm the identity of the viral genome, detect the presence of contaminant(s), confirm the serotype, and detect DNA recombination events at Lox sites. While we focus on the detection of contaminants and recombination events, our Python scripts can be customized to detect other sequences or events, such as reverse packaging of plasmid backbone or DNA from the packaging cell line. We find NGS sequencing of the viral genomes, which we named Viral Genome Sequencing (VGS), to be a powerful way to validate and characterize our research-grade rAAV preparations.

728. Reconfiguration of rAAV Rep-Cap Coding Sequences Significantly Increases Viral Vector Yield and Enables Inducible rAAV Production in HEK293 Cells

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rAAV vectors are demonstrating significant clinical benefit in patients for a range of diseases, however the ability to producelarge quantities of functional rAAV particles in a scalable manufacturing system remains challenging. The majority of currentrAAV production systems exploit

the native Rep-Cap expression configuration found in the viral genome, and adenovirushelper plasmids which contain non-essential regions due to historical cloning strategies. Oxford Genetics has explored the redesignof these two aspects at the DNA level, to increase the yield and quality of rAAV vectors produced. Not only is theresultant system superior to industry standard plasmids in transient transfection in Adherent 293T cells, it is superior insuspension HEK293 cells which allows for scalable production. Furthermore, the development of a tightly controlledinducible system facilitates the creation of stable packaging and producer cell lines.

729. Abstract Withdrawn

730. From Weeks to Minutes - Using nanoDSF to Quickly & Effectively Assess AAV Product Quality

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Differential Scanning Fluorimetry (DSF) was recently described as a tool for discriminating adeno-associated virus (AAV) serotypes based on differences in capsid protein melting temperature profiles, using dyes that fluoresce when bound to hydrophobic surfaces exposed upon thermal denaturation. NanoTemper Technologies has developed nanoDSF instruments that are able to measure changes in intrinsic fluorescence to monitor protein unfolding in a matter of minutes, without the use of exogenous labels or dyes. The Tycho NT.6 is a fast, precise instrument capable of verifying the structural integrity of AAV drug product in a matter of minutes, while the Prometheus NT.48 is a flexible instrument capable of performing detailed characterization of thermal unfolding, chemical denaturation and aggregation. In this study, AAV Drug Product was exposed to a variety of stressed conditions and tested for *in vitro* relative potency. It was found that greater stress exposure resulted in decreased drug potency. Since *in vitro* relative potency methods for AAV can take days or even weeks to perform, nanoDSF was evaluated as a fast and simple tool for measuring changes in AAV structural integrity upon exposure to stress that may be associated with the loss of potency. Upon completion of testing, changes were observed by nanoDSF that were comparable to that of *in vitro* relative potency.

731. ITR qPCR Method Development and Qualification for Quantitation of Adeno-Associated Viral Products

Vinay Kumar Kondeti, Jilin Liu, Danielle McAnally, Harald GP Messer, Mark S. Sherman, Guo-Jie Ye
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Purpose: Real time quantitative PCR (qPCR) is the most common method for quantification of recombinant adeno-associated viral (rAAV) vector genomes. However, development of robust dose-determining assays for rAAV vectors has presented a significant challenge due to the range of dissimilar methods in practice. Indeed, significant inter- and intra-laboratory variations have been documented. Here we report the development and qualification of a universal qPCR assay method that specifically targets the inverted terminal repeat (ITR) sequence of AAV serotype 2 (AAV2) as an archetypal model within the vast majority of rAAV vectors currently in clinical development. We further compared vector genome (vg) titers using ITR qPCR with that obtained using our canonic SV40 qPCR with primers and probes targeted to the SV40 poly A sequence located internally to the vector, as well as that obtained using drop digital PCR (dd PCR) target to either SV40 pA, or ITR sequences. **Method:** qPCR AAV vector samples and the AAV positive control were treated sequentially with DNase and Protein kinase K, followed by heat inactivation of the enzyme. Treated samples and the positive control were subjected to qPCR analysis. The standard was serially diluted six-fold (2E+7 to 2E+2 copies/µL). TaqMan universal master mix along with the forward primer, reverse primer and FAM probe were used. Thermo PCR cycles included UDG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min. The resulting data were analyzed using standards slope and intercept fitting. Identical primer and probe sets were used for dd PCR and qPCR. **Results:** To bridge to our current SV40 qPCR assay method, three different standards were evaluated. These included a linearized plasmid pTR-UF11, a PvuII digested-fragment of pSub-Rep2-Cap2tYF, as well as a 62-bp synthesized duplex ITR amplicon. The 62-bp duplex amplicon standard was found to provide a titer that most closely matched titers derived using SV40 qPCR as well as dd PCR, suggesting that denaturation of the ITR amplicon is easy in comparison to plasmid linearization. We further compared several rAAV preclinical study materials using ITR qPCR, SV40 qPCR and dd PCR (ITR & SV40 specific) and showed agreement across all the qPCR methods. Finally, for selectivity we observed a 100+/-30% spike recovery range in the ITR qPCR assay by spiking known concentrations of dsDNA in various drug diluents, suggesting non-interference by any matrix in drug diluent. LLOQ and LOD for this assay is 200 and 2 vector genomes, respectively, with a precision (Intra-assay, Inter-assay & Inter-analyst precisions) of <5%. **Conclusions:** A highly robust universal ITR based qPCR assay for rAAV vector titration has been developed, with a variation of < 30 % when compared to the titer obtained using SV40 poly A qPCR or dd PCR assays, intra- or inter-laboratories.

732. Chemically Defined, Animal-Origin Free Medium for Sf9 Cells Targeting Enhancement of Virus Production

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R&D, Lonza, Rockville, MD

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 evaluation of Lonza's new chemically defined, animal-origin free cell culture medium which is intended to support cell growth, virus production, and recombinant protein production in suspension culture of insect cells. We share the data on, adaptation of insect *Sf-9* cells to this CD medium, assessment of growth kinetics, as well as production of rAAV (recombinant AAV) vectors in Lonza's CD medium using the insect cells-Baculovirus

expression system (IC-BEVS). The optimized CD medium ensured lot-to-lot consistency, and significantly improved the virus titer production as well as the extracellular localization (thereby potentially improving downstream processing) when compared to other commercial medium.

733. Superior Sensitivity for Noninvasive Tracking of Cell and Gene Therapies Using NISplus 1.0

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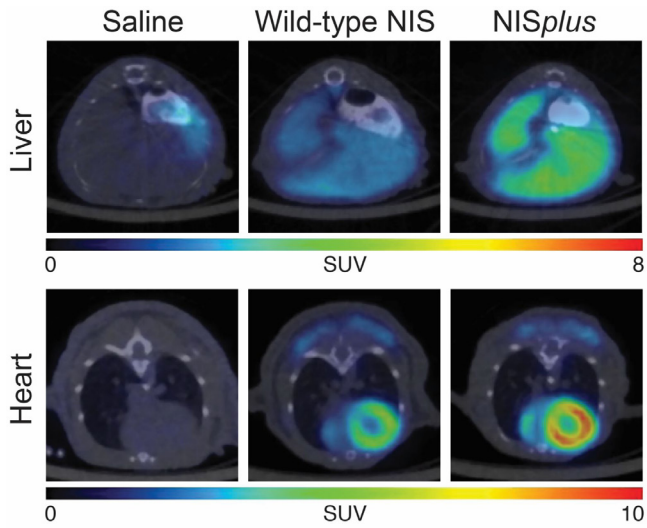
Tracking the in vivo fate of cell and gene therapies represents a major challenge for both pre-clinical and clinical studies. Recent developments in noninvasive imaging using reporter genes have addressed this challenge by facilitating longitudinal in vivo monitoring of therapies. The sodium-iodide symporter (NIS) is a reporter gene that concentrates a variety of clinically approved SPECT and PET radiotracers. NIS has been used to track both therapeutic cells and viruses, producing high-resolution, quantifiable images in living animals and humans. Here, we sought to increase the sensitivity of NIS as an imaging reporter through targeted mutagenesis of the NIS protein. To this end, we first generated predicted 3D structures of the NIS protein using Molecular Dynamic simulations. From these structures, we identified amino acid residues within NIS that were predicted to influence substrate binding and structural integrity. To test our models, we generated NIS proteins containing mutations in one or more of these key residues, and screened the mutants for their ability to concentrate NIS radiotracers. Of a total of 116 different NIS mutants tested, approximately 20% exhibited enhanced uptake of ^{125}I, [^{99m}Tc]-pertechnetate, or [^{18}F]-tetrafluoroborate (TFB) compared to wild-type NIS. The mutant NIS with the greatest increase in [^{18}F]-TFB uptake in vitro (NISplus 1.0; ~30% increase relative to wild-type) was further evaluated in vivo. In order to compare wild-type NIS and NISplus 1.0 signal intensities in vivo, we performed PET imaging on mice treated with AAV9 vectors encoding either wild-type NIS or NISplus 1.0. NIS signal was 2-fold higher in target organs (heart and liver) of mice treated with AAV9-NISplus 1.0 compared to mice treated with AAV9-NIS(WT). Thus, NISplus 1.0 offered dramatically improved sensitivity in vivo compared to wild-type NIS. These findings establish NISplus1.0 as a more sensitive tracker for in vivo biodistribution and cell fate studies, capable of detecting fewer therapeutic cells and lower levels of vector gene expression. NISplus 1.0, therefore, provides a powerful new tool for noninvasive tracking of cell and gene therapies, which is predicted to accelerate the development of these therapies.



734. Validation of AAV Transgene-Specific Quantification Assay Using BioRad Droplet Digital PCR

Scott E. Jenkins, Tomoko Maekawa, Lihini Abeygunawardena, Kendra Hill

Analytical Development, Regenxbio, Rockville, MD

In recent years, the number of clinical trials for gene therapy utilizing adeno-associated virus (AAV) vectors has increased. To support the fast pace of clinical trials and future commercialization, it is essential to have a reliable, precise and accurate AAV quantitation assay at an early stage. Here, we developed an AAV transgene-specific quantification assay using BioRad droplet digital PCR and in-house validation results are presented. During the assay validation, linearity, accuracy, range, limit of quantification, specificity, precision, robustness and sample stability were assessed per ICH guideline Q2(R1). The results provided evidence that the assay is fit for its purpose and can be used for batch release under Current Good Manufacturing Practices (cGMPs).

735. Advantages of Standardization and Large-Scale Manufacturing of Helper 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 of helper plasmids, those that are the same regardless of the specific viral vector produced,

represents an opportunity to significantly reduce timeline, cost, and risk. We have developed processes to quickly produce a set of helper plasmids that consistently generate high-titer viral vectors, are immediately available for research and clinical production, and are free of any royalties or future payments. 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 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. Our data show that fermentation scalability can support large processing events and the production of optimized helper plasmids can enable high-titer lentiviral vector production. Standardization of lentiviral plasmids supports consistency and efficiency at large scale and across multiple programs. Figure 1 shows the scalability of fermentation for a typical plasmid at 30 L and 300 L, with specific yield maintained as scale is increased. Figure 2 shows the performance of lentiviral vectors produced with optimized plasmids, indicating improved performance over other plasmid systems. The availability, cost, freedom to operate, and consistency of these plasmids will help address the growing demands of cell and gene therapy.

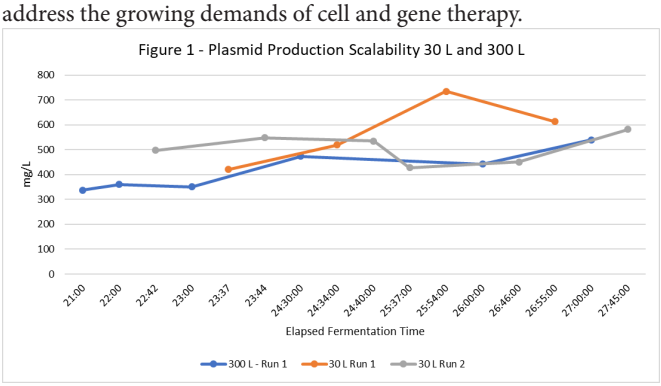
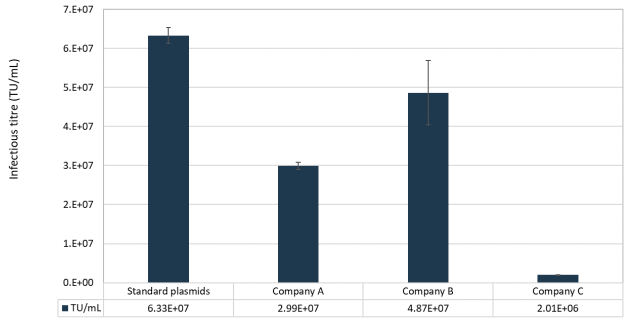


Figure 2 Lentiviral Vector Produced with Standard Plasmid System



736. Tobacco-Smoke Induced Aggravation of Emphysema in SerpinaA1 Null Mouse Model

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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 (A1ATD). Hepatocyte-secreted A1AT is the major circulating serum antiprotease that function to irreversibly inactivate neutrophil elastase, thus protecting elastin in the pulmonary interstitium from degradation. Previous work in our lab used the CRISPR/Cas9 system to successfully disrupt all five copies of the serpinA1 gene, leading to mice with undetectable levels of circulating A1AT and, recapitulating the clinical characteristics of human emphysema. We have shown that these mice develop spontaneous emphysema at 35 weeks of age, making this model highly relevant not only to the preclinical development of therapeutics for A1AT deficiency, but also to tobacco smoke research. In this study we tested the hypothesis that genetically predisposed mice develop accelerated emphysema phenotype when they are exposed to tobacco smoke, that closely mimic emphysema and functional consequences found in human patients over the history of cigarette smoking. Ultimately, we aim to better understand the mechanisms underlying the emphysema disease progression and identify tobacco smoke-related lung parenchyma remodeling and physiopathology. Therefore, to further characterize the mouse model, 10 week old A1AT-KO and WT mice were exposed to the ISO standard puffing profile from 2 or 4 3R4F reference cigarettes using an automated cigarette smoking robot) for 8 weeks. Control (unsmoked) A1AT-KO and WT mice were exposed to room air for the same duration of time. For a comprehensive assessment of the respiratory function, anaesthetized and intubated mice were concurrently subjected to forced oscillation perturbations using the flexiVent FX1 system. In accordance with an anticipated tobacco-smoke induced aggravation of the pulmonary exacerbations, the PV loop of smoked A1AT-KO mice displayed an upward shift typical for emphysema and statistically elevated inspiratory capacity, increased total lung capacity, increased static compliance and decreased tissue elasticity in A1ATD mice, compared to control A1ATD mice as early as at 18 weeks of age. Histopathology studies demonstrate early emphysema presented with typical heterogeneous destruction of the pulmonary parenchyma in smoked A1ATD mice. Namely, the mean alveolar diameter in the lung sections of A1ATD mice exposed to tobacco smoke for 8 weeks was significantly larger than in mice exposed to room air (40.6±5.2 and 30.0±3.1 μm, respectively, p=0.04). No significant changes in alveolar diameters were revealed in A1ATD female mice and in WT mice of both genders. Currently, we are testing if AAV mediated A1AT protein delivery is able to protect lung parenchyma from detrimental effect of tobacco smoke.

737. Characterization of HSV Derived AAV9 Vectors

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[1]Development, Solid Biosciences, Cambridge, MA,[2]PD, Solid Biosciences, Cambridge, MA,[3]Operations, Solid Biosciences, Cambridge, MA

Recombinant adeno-associated viral (rAAV) vectors are excellent tools for the treatment of many genetic and other complex diseases. There are many platforms available to manufacture rAAV such as transient transfection, producer cell line (which may include an adenoviral helper) and herpes simplex virus type 1 (HSV-1) derived vector production in mammalian cells or baculovirus-derived vectors from insect cells. Our AAV9 manufacturing process utilizes HSV-1 which provides both helper functions and serves as the DNA input for rAAV production in suspension human 293 cells. HSV-derived AAV vectors have several advantages regarding their yields, scalability, purity and transduction efficiency. Analytical tools to characterize rAAV products, such as detection and quantification of mammalian cell host or viral helper DNA by PCR, qPCR, next-generation sequencing (NGS) and Southern blots are being developed and/or optimized. Importantly, these tools can detect DNA species and determine their coverage frequency. Development of new assays that enable further characterization of rAAV vectors should be considered as well. In applications that require systemic administration of high doses of vectors, such as Duchenne muscular dystrophy, these analytical tools and assays have the potential to further characterize and improve rAAV products.

738. Development of a Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) Method to Support Process Development of Adeno-Associated Viral Vector Products

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Biogen, Cambridge, MA

Analytical ultracentrifugation (AUC) is one of the most important techniques for biophysical characterization of adeno-associated virus (AAV) based gene therapies in the biopharmaceutical industry. Using sedimentation velocity (SV) AUC, the relative abundance and sedimentation coefficients of distinct viral species can be determined, including lower molecular weight (LMW) species, empty particles, partially filled species, full particles, and higher molecular weight (HMW) species. Historically, the method has proven to be unsuitable for use to support process development due to the high levels of expertise required to perform and low throughput. Through advancements in instrumentation and strategic procedural improvements, we have developed a method that is used to drive decisions in process development.

739. Arming Maternally Expressed Gene 3 RNA against Cancer

Chringma Sherpa, Stuart F. J. LeGrice
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Long non-coding RNAs (lncRNAs) have recently emerged as key players in gene regulation and cancer. Maternally expressed gene 3 lncRNA is by far the best-characterized tumor suppressor lncRNA. In various cancer cell lines and clinical samples, Meg3 is either not expressed or is expressed at low levels, and exogenous expression of Meg3 RNA in such cells can slow growth and induce apoptosis. Targeted restoration or overexpression of Meg3 RNA in affected cells, therefore, offers a promising avenue for cancer treatment. The 1.6 kb length of Meg3 RNA limits delivery options of this RNA for therapeutic targeting of cancer. Here we demonstrate the structure -guided determination of a minimal functional region of Meg3 RNA that retains full functionality in tumor suppression. The shorter half-life and transient expression of Meg3 RNA further limit its utility against cancer. Here we also present our work on the sustained expression of full length and minimal Meg3 RNAs achieved by the delivery of circular forms of these RNAs using an AAV2 vector in U87 glioblastoma cell-line.

740. (r)Evolution of AAV2 Titration ELISA - From Monoclonal to Recombinant

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PROGEN Biotechnik GmbH, Heidelberg, Germany

PROGEN´s AAV2 Titration ELISA is well known to provide reliable and accurate quantification of rAAV2 total capsid titers for many years. It uses the monoclonal antibody A20. This antibody is produced in hybridoma cells with specificity for AAV2 intact particles and is employed for capturing and detecting the capsids in a sandwich assay format. The antibody A20 is not trivial to produce as it shows variable expression levels during production, has the tendency to oligomerize and is very sensitive to storage conditions at low temperatures. Therefore, the production conditions of the A20 antibody need thorough monitoring in order to achieve the required high lot-to-lot consistency. To overcome these obstacles, PROGEN produced a new, recombinant variant of the A20 antibody, called A20R. In contrast to A20 it contains the variable antigen binding region in an IgG1 context instead of the original murine IgG3. The new antibody A20R improves processing, handling and storage conditions during the manufacturing process. Here we demonstrate that A20R shows the same binding specificity to AAV2 compared to A20. Moreover, applying the A20R antibody in the well-established AAV2 ELISA results in the same consistent sensitive and reproducible total capsid titer measurements in comparison to the original A20 antibody.

741. Towards the Development of a Gene Therapeutic Against Age-Related Macular Degeneration: Replacing GFP with Stuffer DNA in a Recombinant Adeno-Associated Virus Vector and Validating its Subsequent Effects

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The wet subtype of age-related macular degeneration (AMD), of which choroidal neovascularization (CNV) is the defining characteristic, is a growing global health problem for which gene therapy may prove to be highly beneficial. We have previously demonstrated that a shRNA delivered via rAAV, which abrogates mTOR activity in a novel manner by simultaneously inhibiting both mTOR complexes, shows promise as a possible AMD therapeutic. Both the target and the use of gene therapy represent a novel treatment modality against this disease. To progress along the path in developing this potential gene therapeutic, we have removed the xenogeneic GFP gene used as a reporter in previous studies from the virus vector and replaced it with a stuffer DNA derived from the 3' UTR of the human UBE3A gene to ensure the optimal size for efficient rAAV assembly. Here, we report that the virus vector (rAAV2-shmTOR-SD) containing the stuffer DNA exhibits anti-angiogenic and anti-apoptotic properties in a laser-induced CNV mouse model at levels comparable to its GFP-containing counterpart, as determined by CD31 immunostaining and a TUNEL assay, respectively. Taken together, these results suggest that it may be possible to develop rAAV2-shmTOR-SD as a human gene therapeutic for the treatment of wet AMD.

742. Novel Process of AAV Purification by Three-Phase Partitioning Combined with Density Gradient Centrifugation Has no Detrimental Effect on AAV Infectivity

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Purification of adeno-associated virus (AAV) has mainly been carried out by column chromatography or density gradient centrifugation methods. Though it is widely used for large scale purification due to its scalability, column chromatography method is expensive and complicated. Different resins/purification processes are required for different AAV serotypes. In addition, empty viral particles, are co-purified with full particles due to their similar binding properties. On the other hand, density gradient centrifugation process using cesium chloride (CsCl) or iodixanol is limited by the volume of each centrifuge tube and thus difficult to scale up. Recently we have reported that three-phase partitioning (TPP) can be used as an economical and scalable upstream purification process for large scale AAV purification and our data have demonstrated that two rounds of TPP can yield 90% purity of bulk AAV vectors at recovery rate exceeding 70%. We have successfully purified AAV2, AAV5, AAV6, and AAVDJ vectors using the same TPP conditions with 90% purity. When TPP was combined with CsCl density gradient centrifugation, large amount of AAV vectors can be purified through conventional ultracentrifuges. In the current study, our goal was to investigate if the TPP process had any negative impact on the AAV infectivity. We purified AAV6 and AAV9-CMV-Luciferase vectors by TPP combined with CsCl density gradient centrifugation or by CsCl density gradient centrifugation alone and performed *in vitro* and *in vivo* studies to compare their infectivities. The data presented here demonstrate that AAV vectors purified by either processes have very similar infectivities, indicating that TPP has no detrimental impact on AAV quality. Since TPP process can remove 90% of the impurities and density gradient centrifugation can separate the empty from the full virus particles, this novel combination process increases the purification capacity of density gradient centrifugation by a factor of 10 and thus provides an economical tool for large scale purification of AAV vectors devoid of empty capsids.

743. Characterization of AAV Percentage of Full Capsids and Comparability across Platforms

Rachael Maureen Ahern, Lauren M. Drouin, Patrick G. Starremans

Analytical Sciences, Voyager Therapeutics, Cambridge, MA

Adeno Associated Virus (AAV) vectors are currently in development for multiple gene therapy applications and can be manufactured using several different methods. An integral quality attribute for AAVs produced by any manufacturing method is the ratio of full capsids (containing the target payload) vs empty capsids. It is thought that the latter, if present in large enough amounts, can interfere with transduction efficiency. An additional quality attribute is the existence of aggregates. AAV exists as a monomer but can also form dimers, trimers or higher order aggregates. Accurate assessment of full vs empty capsids and the quantitation of multimeric AAV aggregates is critical for successful process development and quality control. We performed a comparative evaluation of multiple analytical methods using an in-depth characterization of two AAV2 reference standards produced through an in-house Baculovirus/Sf9 manufacturing platform. Our evaluation showed that the analytical methods which provided the largest throughput also displayed the largest inter and intra assay variability. Likewise, analytical methods which consistently provided high-resolution results would also have long turn-around-times and a low throughput. However, our evaluation did identify one analytical method which, under certain optimized processing conditions, yielded improved resolution over other methods, while also providing lower variability and higher reproducibility. Using this method, multiple intermediate species could be resolved and quantified, with consistently low intra- and inter-assay variability. The optimized analytical method

we have identified provides notable advantages over the other available analytical methods and can effectively support process development and quality control operations in manufacturing.

744. Optimizing the Production of Recombinant AAV Vector Encoding VEGF-B in iCELLis Bioreactors

Lionel Galibert[1], Amira Chafqane[1], Tiina Nieminen[1], Hanna Leinonen[1], Igor Oruetxebarria[1], Pyry Toivanen[1], Vesa Turkki[1], Anniina Valkama[1], Heidi Hynynen[1], Iina Laaksonen[1], Riikka Kärnä[1], Joonas Malinen[1], Sanna Peltola[1], Sonja Kotoneva[1], Eevi Lipponen[1], Seppo Ylä-Herttuala[2], Hanna Lesch[1]

[1]Kuopio Center for Gene and Cell Therapy, Kuopio, Finland,[2]University of Eastern Finland, A.I Virtanen Institute, Kuopio, Finland

Large-scale production of recombinant Adeno-Associated Virus (rAAV) vectors remains a critical step to achieve in the gene therapy field. Previously, we have shown the production and purification of rAAV2 encoding Vascular Endothelial Growth Factor B (VEGF-B) in iCELLis 500/333m^2 fixed-bed bioreactor. Following the large-scale development, we have optimized the plasmids used for rAAV production and compared the impact of newly designed rAAV producer plasmids with the pDG2 reference plasmid. With optimized plasmids, we were able to show an important gain in rAAV2-VEGF-B production level coupled to a reduced level of contaminating intra-capsid DNA following vector production in iCELLis Nano bioreactors. These results take us closer to preclinical and clinical studies where the quality and quantity of the viral vector are of high importance.

745. Optimization and Evaluation of Two Potency Assays for AAV Based Gene Silencing Programs

Timothy P. Boyd, Paul B. Scott, Rachael M. Ahern, Lauren M. Drouin, Patrick G. Starremans

Analytical Sciences, Voyager Therapeutics, Cambridge, MA

Many gene therapies currently under development are designed to reduce the expression of a clinical target through silencing the gene responsible for its production or by promoting the degradation of its encoded messenger RNA. Voyager has multiple rAAV gene therapy programs in development that utilize such a knockdown strategy. One of the critical quality attributes that needs to be monitored during product development is biological potency. According to ICH Q6B specifications, biological potency assays need to be precise, accurate and robust, as well as able to measure the relevant biological activity of the product. Further, according to health authority recommendations, the criteria for what is considered an acceptable potency assay depends on the stage of development of the program and may vary based on readout. In this study, the development and qualification of two relative potency assays based on mRNA silencing are presented. Many of the knockdown assays currently being developed rely on the availability of proprietary commercial kits and reagents. Often, this complicates analytical and supply transparency. To overcome this and obtain benefits of supply chain control, cost effectiveness, and reagent production reliability, commercial kits were mostly excluded from the present assay development efforts. After multiple rounds of optimization for variables such as cell culture conditions, plate layout, multiplicity of infection range, and transduction time, the method was subsequently tested for repeatability, linearity, precision and accuracy. The overall data demonstrate that these methods are robust and suitable for assessing the relative potency of rAAV vectors.

746. The New AAV3 Titration ELISA - Continued Tradition of Reliable AAV Titer Determination

Hueseyin Besir, Iris Queitsch, Katharina Hammer, Caroline Odenwald, Katja Betts, Sven Kuhlendahl

PROGEN Biotechnik GmbH, Heidelberg, Germany

AAV vectors are frequently used by academic and industrial labs for the development of gene therapies. A growing number of serotypes are being used for various diseases. To ensure safety and efficacy of these therapies, a robust and reliable quantification of rAAV titers is indispensable. Meeting this need of the gene therapy community, PROGEN developed a new AAV3 Titration ELISA for reliable determination of total capsid titer. The AAV3 ELISA was developed following the well-established workflow for other PROGEN AAV serotype ELISAs[1,2]. A purified AAV3 gold standard material was generated and quantified by multiple qPCR and electron microscopy experiments in several labs to determine the ratio filled/empty capsid and quantification of viral DNA. Each lot of AAV3 Titration ELISAs is calibrated using this internal gold standard and compared to previous lots in order to ensure minimal lot-to-lot variation. Following the tradition of offering consistent, robust and reliable titer determination for most relevant AAV serotypes, the AAV3 ELISA complements PROGEN's portfolio.

[1] Lock, M. et al. Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. Hum Gene Ther. 10, 1273-85 (2010)

[2] Ayuso, E. et al. Manufacturing and characterization of a recombinant adeno-associated virus type 8 reference standard material. Hum Gene Ther. 11, 977-87 (2014)

747. HEK293-Derived Adeno Associated Virus (AAV) Purification: Comparison of Smalls Scale Laboratory Production Towards Industrial Format Using Monoliths

Kolade Oluwole

Wentworth Institute of Technology, Boston, MA

During recombinant adeno associated virus (rAAV) downstream processing, a large amount of host-cell and product related impurities needs to be removed from the product. Succesful process on laboratory scale, such as Cesium chloride purification, lacks scalability when the process is due to be transferred to larger industrial scale. The aim of the study was to develop robust, fast and effective rAAV virus purification platform, which can be used for several AAV serotypes with various inserts. Lysed harvest and supernatant of rAAV9 were first captured and concentrated on a OH column, followed by intermediate step on

a SO3 column and further polishing using a QA column. Derived purity of industrial scale monolith purification product was compared to laboratory scale purification.

748. Impact of Selected Cell Culture Media and Different HEK293 Production Cell Lines on the Expression of a Variety of Recombinant Adeno Associated Virus Subtypes

Barbara Kraus, Carolin Kahlig, Lucia Micutkova, Gerald Siegwart, Juan Hernandez Bort

Gene Therapy Process Development, Shire, now part of Takeda, Orth / Donau, Austria

Gene therapy is a promising technology to therapeutically address rare diseases caused by genetic disorders. In this context, recombinant adeno associated virus (rAAV) emerged during the last years as the vector of choice for gene therapy. In the present study we investigated the impact of different HEK293 cell lines and several commercially available cell culture media on the upstream yield of a variety of AAV subtypes after transient transfection. All experiments were performed in high throughput micro bioreactors or in small scale laboratory bioreactors. Kinetics of AAV capsid expression monitored over several days post transfection showed different patterns regarding capsid specific ELISA and qPCR. Additionally, we determined to what extend the AAV particles were released into the supernatant or kept in the cytosol of the cells. The outcome of our study was compared against published data.

749. Adeno-Associated Virus-Mediated Expression of Monoclonal Antibodies against Healthcare-Acquired Bacterial Infections

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Adeno-associated virus (AAV)-mediated expression of pathogen specific protective monoclonal antibodies (mAbs) has proven to be an effective method of treatment for many infectious diseases in a range of pre-clinical models. However, the focus of current vectored immunoprophylaxis efforts remain targeted to the treatment of viral and parasitic diseases while overlooking bacterial infections. Healthcare-associated infections (HAIs) that currently affect hospitals in industrialized countries cause significant economic burden on the healthcare system. Therefore, we aim to investigate AAV-mediated gene delivery and subsequent long-term expression of pathogen-specific monoclonal antibodies as a novel strategy to provide protection against, and treatment of, HAIs. Utilizing a novel AAV triple mutant capsid, AAV6.2FF, we can facilitate rapid, robust, and long-term expression of therapeutic antibodies in vivo following intramuscular administration. This was demonstrated previously with high serum concentrations of human IgG (200-525μg/ml) for sustained periods of 22-32 weeks conferring protection against both Ebola virus and Marburg virus challenge using AAV6.2FF-mediated expression of mAbs 100 and MR191 in mice. Re-engineering AAV6.2FF to express hIgG1 mAbs with variable heavy and light chain domains of Actoxumab and Bezlotoxumab, we can produce functional antibodies in vivo against *Clostridium difficile* toxin A and toxin B, respectively. Additionally, AAV6.2FF was re-engineered 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*. Intramuscular injection of $1x10^{11}$vg of AAV6.2FF-Actoxumab and AAV 6.2FF-Bezlotoxumab in separate groups of mice resulted in serum concentrations of over 100μg/ml of human IgG by 14 days post-injection. Intramuscular injection of $5x10^{10}$vg of both AAV6.2FF-Actoxumab and AAV6.2FF-Bezlotoxumab resulted in serum concentrations of human IgG over 250μg/ml by 28 days post-injection. This method of AAV-mediated gene delivery and vectored immunoprophylaxis offers advantages prophylactically or post-infection and shows promise in prolonging the therapeutic effect of recombinant mAb administration.

Gene Targeting & Gene Correction II

750. Meganuclease Suicide System, a Self-Inactivation Method to Reduce Nuclease Expression and Off-Target Activity

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Previous studies have used adeno-associated viral (AAV) vectors to express engineered I-CreI meganucleases, administer them into rhesus macaques, and characterize their ability to edit the PCSK9 gene *in vivo*. First- and second-generation meganucleases (M1PCSK9 and M2PCSK9, respectively) can edit the intended DNA target sequence and reduce PCSK9 protein levels. However, both *in vitro* and *in vivo* studies have shown that these nucleases generate indels (insertions and deletions) in other regions of the genome, suggesting off-target activity. Although the second-generation M2PCSK9 shows improved specificity, it is continuously expressed in hepatocytes following vector transduction, which may induce immune responses and cellular toxicity. We hypothesized that low intracellular levels of a meganuclease are sufficient for on-target genome editing, and that surpassing this threshold increases the chance of off-target editing. To test this hypothesis, we developed a self-inactivation or "suicide" system to restrict expression of the meganuclease. We developed AAV vectors expressing M2PCSK9 by inserting the 22 bp meganuclease target sequence after the promoter. With this design, expressed M2PCSK9 should both edit the PCSK9 gene and cleave the AAV vector genome immediately after the promoter, preventing further transcription of the meganuclease transgene. We constructed alternative vectors by inserting an additional target sequence before the polyA sequence or by inserting a mutant target sequence after the promoter. We also included the PEST sequence in frame with the M2PCSK9 meganuclease, as this sequence should target the transgene protein for degradation by proteasomes. We intravenously administered immunodeficient RAG

knockout mice with an AAV9 vector expressing human PCSK9. Two weeks later, we readministered the mice with the suicide system vectors. All versions of the suicide system reduced the levels of PCSK9 in serum, albeit to different degrees and with different time courses post-vector administration. As expected, M2PCSK9 created indels in both the target sequence in the PSCK9 gene as well as at the target sequence when present in the AAV genome. For some of the suicide system vectors, the on-target editing efficacy was comparable to that obtained with the parental AAV-M2PCSK9 vector with a reduction in protein expression determined by Western blot and a 20-fold reduction in off-target activity at 9 weeks post-vector administration. In conclusion, restricting meganuclease expression through self-inactivation by inserting the target sequence and/or adding a protein degradation signal reduces off-target activity without compromising on-target efficacy. Including this suicide system in gene editing approaches could increase the safety profile of AAV-delivered genome editing nucleases.

751. Nano-S/MARt Vectors: The Next-Generation DNA Vector Platform for Gene and Cell Therapy

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The possibility of modifying cells with an episomally maintained DNA Vector system that lacks viral components and avoids integrative genotoxic effects represents a valuable and exciting alternative to the currently used technologies such as virus and transposons. We have previously demonstrated that we can engineer cells using autosomally replicating S/MAR DNA Vectors and we showed that these vectors are maintained as episomal replicons which provide robust and persistent transgene expression over time and through unlimited cell divisions. In some cell types, such as primary human and murine cells, it is particularly difficult to deliver plasmids that contain a large bacterial backbone comprising an antibiotic selection marker and the pUC replication origin. To overcome the toxicity associated with the bacterial sequences, we have generated a next-generation S/MAR DNA Vector based on the Nanoplasmid vector system developed by Nature Technology Corporation to create a DNA platform suitable for the efficient genetic engineering of cells *in vitro*, ex vivo and in vivo. This novel DNA NanoVector system is characterised by a minimally sized bacterial backbone and an antibiotic-free RNA-Out selection system that allows its manipulation and expansion in an engineered strain of E.coli. The manufacturing of these plasmids generates a high yield of supercoiled DNA by standard DNA preparation without the need for genetic recombination events and/or additional steps of purification as with Minicircle vectors. The presence of the S/MAR region prevents epigenetic silencing, provides epigenetic stability and enhances the transgene expression. We have shown that the S/MAR Nano-Vectors are more efficient with improved efficiency in establishing stable cells when compared to the respective vector with a pUC bacterial backbone. These vectors routinely provide more robust transgene expression than standard plasmid DNA and present increased mitotic stability over hundreds of cell divisions. Here we present the applications of this next generation DNA Vector in experiments that were impossible with other non-integrative technologies.We show their applicability for generating persistently modified Patient-Derived Pancreatic Cancer Cells (PDX), primary mouse embryonic stem cells (mESC), sustained and robust expression of proteins in the liver of mice and the efficient generation of modified T-Cells.To demonstrate that the introduction of a Nano-SMARt vector does not affect the molecular and biochemical behaviour of the cells we have performed microarray studies on cells stably transfected with these NanoVectors and we compared the transcriptome profiles to unmodified parental cells. We have shown that the number of transcripts perturbed by the presence of this novel class of DNA Vectors is minimal which demonstrates that the cells modified with this DNA vector system are essentially isogenic.Due to its easy, quick and efficient production and its efficacy in modifying a wide range of cells without provoking cellular damages or modification in their behaviour, we believe that this novel DNA Vector system provides a unique and innovative approach for generating genetically engineered cells for gene and cell therapy.

752. Engineered AsCas12a Variants with Enhanced Activity and Broadened PAM Compatibility

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CRISPR-Cas12a (Cpf1) is a type V CRISPR effector RNA-guided DNA endonuclease with utility in synthetic biology and genome engineering. As an alternative to the commonly used *Streptococcus pyogenes* Cas9 (SpCas9), Cas12a recognizes TTTV (V = A/G/C) PAM sequences, which permits editing in AT-rich regions of the human genome. However, the extended length of AsCas12a PAM sequence relative to SpCas9 (TTTV vs. NGG) restricts its utility in genome editing. To improve the targeting range and enzymatic activity of Cas12a, we developed a bacterial-based selection assay to select for Cas12a mutants that demonstrate increased cleavage activity and reduced PAM specificity. We first selected Cas12a variants with enhanced activity at non-conical TTTT PAM, since this specific motif is significantly more prevalent throughout the human genome than the other three TTTV motifs. After multiple rounds of selections, we successfully enriched several variants from a Cas12a library with random mutations. We characterized the top enriched variants and revealed their enhanced cleavage activities at both TTTV and TTTT PAM sites when delivered into human cells as ribonucleoprotein (RNP) complexes. Combining mutations from these variants further enhanced cleavage activity, which globally enhanced the total genome editing efficiency of Cas12a over 96 sites found within several unique genomic loci. Early off-target site detection studies with GUIDE-Seq suggest that these mutants retain high on-target specificity of WT Cas12a with ribonucleoprotein (RNP)-based delivery. Overall, we anticipate that this new variant with enhanced activity and broadened PAM compatibility will allow for broader application of the CRISPR-Cas12a system for genome editing.

753. Histone Deacetylase Inhibitors Enhance CRISPR-Cas9-Mediated Genome Editing

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The CRISPR-Cas9 system has become a widely accepted genome-editing tool due to increased adaptability to multiple targets. However with the current state of technology, homology directed repair (HDR) is still inefficient in hematopoietic stem and progenitor CD34+ cells. Histone deacetylase inhibitors such as the FDA approved antiepileptic, valproic acid (VPA), and/or sodium butyrate, can be used in conjunction with the CRISPR-Cas9 system to improve HDR efficiency. At concentrations as high as 50mM, twenty-four hour treatment with VPA had no effect on the growth pattern or viability of K562 myeloid-erythroid leukemic cells or immortalized T lymphocyte Jurkat cells. Similarly sodium butyrate, did not have an effect on hematopoietic cell line growth or viability at concentrations as high as 30mM. As histone deacetylase inhibitors, VPA and sodium butyrate prevent DNA from being wrapped tightly around histones yielding a relaxed state and thus enhancing the accessibility of the genome to the Cas9 endonuclease. A TIDE analysis revealed an increased ability of several guide RNAs specific to the RhD locus to produce a double strand break in K562 cells after pretreatment with either 50μM VPA or 50μM sodium butyrate. Notably, the +39 RhD guide RNA demonstrated a 13-fold enhancement following pretreatment with VPA. Prior to treatment, a 1.7% cutting efficiency was observed with the +39 RhD guide RNA while a 22% cutting efficiency was observed following pretreatment with VPA. Similar enhancements in cutting efficiencies were observed with a second set of guide RNAs designed to target Cas9 to the stop codon of the von Willebrand Factor (vWF) locus. In addition, a T7EI assay confirmed improved guide RNA ability to induce a double strand break following treatment with 50μM VPA. Besides noting an improvement in guide RNA cutting efficiency, we also noted an enhancement in targeting following the addition of a donor DNA template as a result of treatment with a histone deacetylase inhibitor. The donor DNA template contained an exogenous GFP gene. In this manner, targeting could be easily assessed via flow cytometry. K562 cells pretreated with 50μM VPA revealed a significant increase in percent GFP expression as opposed to untreated cells ($p = 0.0005$). This enhancement in gene targeting was observed at two loci (the RhD and vWF locus). Therefore, treatment with VPA or sodium butyrate can be expanded to virtually any experiment utilizing the CRISPR-Cas9 system to enhance cutting and gene targeting efficiency. In the future, histone deacetylase inhibitors can be used as a pretreatment for therapeutically relevant gene editing.

754. The Effects of Manipulating DNA Repair Factors on CRISPR/Cas9-Mediated Gene Editing Outcomes in Human Hematopoietic Stem and Progenitor Cells

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Gene editing of hematopoietic stem cells (HSCs) is a promising strategy for the treatment of monogenic diseases of the blood through site-specific correction of identified causal mutations by targeted nucleases. However, the precise correction of CRISPR/Cas9-induced double stranded brakes (DSBs) via homology-directed repair (HDR) pathway remains less efficient than error-prone non-homologous end joining (NHEJ) in HSCs. Our translational goal is to improve the efficiency of HDR outcomes relative to NHEJ, which will be beneficial for treating diseases such as sickle cell disease. Therefore, it is critical to understand what governs the cellular DNA repair pathway choice, and how it can be manipulated to shift the balance toward HDR from NHEJ. Cellular regulation of repair pathway choice depends on a complex interaction of DNA repair proteins. Previous studies have identified several factors that may influence the repair outcomes in cell lines. However, the effects of these factors have not been evaluated in disease-relevant models, such as primary human HSCs, which are especially challenging to achieve therapeutic levels of HDR in. We tested the effects of manipulating the expression levels of several DNA repair factors that are presumed to be important for pathway choice (such as CtIP, PALB2, RAD52 and 53BP1) on HDR and NHEJ levels in K562 cells and primary human hematopoietic stem and progenitor cells. We tested different methods for DNA repair factor expression (plasmid, lentiviral vector, and mRNA) at various time points, relative to delivery of CRISPR/Cas9 and donor template. We achieved up to 3-fold improvement in HDR levels by overexpressing DNA repair factors in K562 cells. Interestingly, we observed differential effects of DNA repair factor manipulation on gene editing outcomes dependent on the type of donor template used. Ongoing work is currently focused on evaluating the effects of manipulating these factors in primary human HSCs to determine whether HDR can be improved within the long-term repopulating population crucial for successful long-term transplantation.
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755. HK022 Bacteriophage Integrase: An Efficient Tool for Human Genome Manipulation and Therapy

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The HK022 coliphage integrase (Int) is a Site-Specific Recombinase (SSR) that naturally targets the *attB* 21 bp recombination site at the

bacterial chromosome. This site consists an 'overlap' (O) sequence that is flanked by two 7 bp partially inverted (palindromic) Int-binding sites termed B and B' (Fig.1a). We replaced the 'O' with a random 7 bp sequence and demonstrated that it supports Int-mediated site-specific recombination as long as the cognate and larger phage recombination site *attP* features an identical O sequence. This promiscuity prompted us to identify several putative *attB* sites ('*attB*') with random overlaps that flank human deleterious mutations. We harnessed Int to cure the '*attB*'-flanked mutations by Recombinase Mediated Cassette Exchange (RMCE) (Fig. 1b). This unique feature of Int was not demonstrated in any other SSRs. The efficiency of the RMCE on the genomic level was assessed in HEK293 Flp-in cells by fluorescence promoter trap assay (Fig. 1c). In this assay, three plasmids are used. The first is a "docking" plasmid coding 'attB-*1*' downstream to EF1alpha promoter and '*attB-2*' upstream to promoter-less *mCherry* (Fig. 1c, A). The second is an "incoming" vector coding '*attP-1*' upstream to promoter-less *GFP* and '*attP-2*' downstream to *CMV* promoter (Fig. 1c, D), and the third is Int expressing plasmid. First, the "docking" plasmid was inserted by Flp site-specific recombination reaction into the genomic frt-locus (Fig. 1c, B-C). Thereafter, these cells were co-transfected with the "incoming" (Fig. 1c, D) and the Int expressing plasmids. The Int-RMCE product (Fig. 1c, E) is postulated to co-express *GFP* and *mCherry* from the EF1alfa and CMV promoters, respectively. RMCE efficiency was quantified 72 hours post transfection by FACS. We found 1.3% of RMCE efficiency, without any selection enrichment (Fig. 1c, F). We orthogonally assessed the RMCE junctions (Fig. 1c, G-H) and the full exchanged cassette by PCR and DNA sequencing (Fig. 1c, I). To demonstrate our Int-based system functionality for potential therapy of Duchenne and Cystinosis diseases, we assessed the RMCE efficiency in the native genomic location of the '*attB's* located in *DMD* and *CTNS* genes respectively using a GFP trap assay (Figs. 2a and b). FACS analysis showed 0.47% and 1.2% of RMCE efficiencies (Fig.2D). Moreover, the junctions produced by the RMCE (Fig. 2E-F) were confirmed by PCR and DNA sequencing. In conclusion, we developed an efficient human genome editing tool based on HK022 coliphage Int RMCE using *att*-like sites. This system has the prospects to exchange a mutated genomic sequence with the correct one without adding any selection marker or other foreign sequences. Furthermore, it has the capability to swap large transgene cassettes (over 20 kb) compared to the gene-editing endonucleases which is limited to 5 kb. Finally, the Int RMCE system can be made a scalable therapy by integrating cDNA downstream to endogenous promoters.

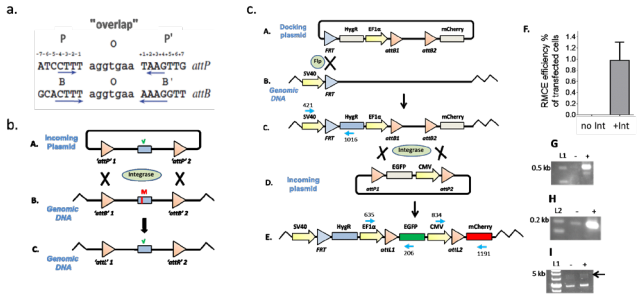

Fig. 1. **a.** Core sequences of the wild type attP and attB. Arrows indicate palindromic sequences. **b.** Scheme of The Int mediated RMCE reaction. **c.** Genomic model of Int-RMCE in HEK293-Flp-in cells. A. Docking plasmid. B. HEK293-Flp-in genomic SV40 promoter-frt cassette. C. Integrated docking plasmid. D. Incoming plasmid. E. Int-catalyzed RMCE product. F. FACS quantitative data. PCR analysis: G. left RMCE junction. H. right RMCE junction. I. full RMCE. L1 and L2 – 1 kb or 100 bp ladders, respectively.

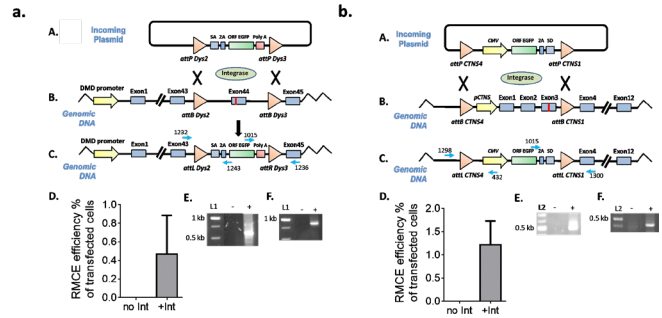

Fig. 2. GFP trap assay of Int-RMCE using '*attB's* in (a) DMD and (b) CTNS genes. A. Incoming plasmid. B. DMD/CTNS locus with '*attB's* that flank a mutated EXON. C. RMCE reaction product. D. FACS quantitative data. PCR analysis: E. left RMCE junction. F. right RMCE junction. L1 and L2 – 1 kb or 100 bp ladders, respectively.

756. Streamlined Genome Engineering: Identifying Successful Homologous Recombination Events Using a Fluorescence-Based Screening Method

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One of the most powerful applications of genome editing is the ability to introduce precise changes at genomic loci of interest by pairing site-specific nuclease activity with repair processes mediated by homologous recombination (HR). Common objectives of this approach include the insertion of long sequences encoding fusion tags or expression cassettes, the introduction of single-base substitutions that mimic single-nucleotide polymorphisms (SNPs) associated with human disease, or the creation of stop codons to generate precise gene knockouts. In the context of most genome editing workflows involving knockin (KI) of insertions or substitutions in cultured cells, there are two different stages when the detection of successful HR events is critically important. The first stage involves optimization of experimental conditions to achieve the highest percentage of error-free recombination events in an edited population before moving forward with the isolation of single-cell clones. The second stage involves identification of cell lines carrying the edit of interest after single-cell isolation and expansion in 96-well plates. To address this need, we developed a simple fluorescence-based method that enables detection of successful HR events independent of their length (from single-nucleotide substitutions to longer insertions) or surrounding targeted genomic loci in edited populations as well as in clones from 96-well plates. The assay consists of PCR amplification of the genomic target site, followed by an enzymatic assay with a dual-color fluorescence-based readout using a standard plate reader. A positive fluorescent signal from the assay is highly correlated with the correct introduction of the desired edit. For scenarios involving KI of longer sequences, the assay allows for the simultaneous detection of seamless insertions at both 5' and 3' ends of the recombinant sequence. For engineering SNPs, the assay enables detection of single-nucleotide substitutions with high sensitivity in both mixed and clonal populations and can be used to positively identify heterozygous clones carrying one copy each of the edited (SNP) and un-edited (WT) alleles. We employed this method in

a variety of experimental contexts, including the creation of isogenic cell lines carrying SNPs in the *FAH* gene related to tyrosinemia, and the introduction of a myc tag fused with the gene *UGT1A9* (related to drug metabolism). In all editing experiments, we performed the assay at both stages identified above: first to detect which sgRNAs generated higher KI efficiencies in the edited cell population, and second to screen for successfully edited clonal cell lines following isolation and expansion of single-cell clones in 96-well plates. During the screening process for successful fusion of the myc tag with *UGT1A9*, we could discern clonal cell lines with either partial or complete insertions due to the assay's ability to interrogate both 5' and 3' ends of the insert. Using previously characterized genomic DNA samples obtained from Coriell Institute, we also demonstrated the capability of the assay to simultaneously detect WT and SNP alleles in the same sample, a common objective for research projects that require engineering cell lines that are heterozygous for a given SNP of interest.

757. Efficient In Vivo Genetic Engineering of Lymphocytes after Systemic AAV Delivery

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Ex-vivo gene therapy using stem cells or T cells transduced by retroviral or lentiviral vectors to treat diseases such as immunodeficiency and cancer have shown remarkable efficacy. However, the entire process of *ex-vivo* therapy is expensive, technically challenging, and not readily scalable to a large patient population in developing countries. On the other hand, direct *in vivo* gene therapy with adeno-associated virus (AAV) vectors has shown to be safe and efficacious in clinical trials for several diseases affecting differentiated tissues such as liver and CNS. However, the ability of AAV to transduce immune cell populations such as T cells and B cells after systemic injection has not been explored. Here by flow cytometric analysis, we show that AAV8 vectors, both as conventional and exosome-associated formulations, can mediate transgene expression in a variety of immune cells after systemic delivery of a clinically relevant dose ($4x10^{13}$ genome copies/kg) into adult mice, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Mean transduction efficiency with a GFP transgene in the aforementioned cell types was 9.9%, 22.7%, and 3.9% at day 12 post injection, respectively. We demonstrate direct evidence of transduction of flow-sorted T cells using detection of AAV genomes and GFP mRNA by qPCR and RT-qPCR, respectively. We also used systemically administered vector to express a human cytokine receptor, IL-2Rα, on the surface of T and B cells, as a practical demonstration of the approach. We are now extending our use of systemically injected AAV vectors to deliver genome editing machinery to modify T cell DNA. Our work will facilitate both basic and applied research involving direct *in vivo* genetic modification of immune cells.

758. Electroporation of Bone Marrow Monocytes and Hematopoietic Stem Cells for Gene Targeting

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Cells in many hematopoietic lineages have great therapeutic potentials. CD34+ hematopoietic stem cells have been studied extensively and gene editing by Cas9 RNP electroporation has been quite easy. However, the viability of gene edited CD34+ cells is impaired and expension of these cells are much slower than non-electroporated CD34+ cells. The other emerging important therapeutic cell type is bone marrow derived monocytes that can differentiate into macrophages and dendritics cells. Macrophages have been implicated in tumor microenvironment regulation and dendritic cells are important in immunotherapy. Electroporation of primary monocytes has been very difficult with low efficiency and low viability. We developed a new method involving several aspects to efficiently electroporate these hematopoietic cells while maintaining their viability. One of the reasons that these primary cells tend to have low viability after electroporation is that they require higher voltage than common cell lines for electroporation and heat generation is a significant problem. We precisely measured the temperature change before and after electroporation and found that pre-cooling the cells for 3 minutes combined with an appropriate pulsing strategy helped control the temperature rise and improved cell viability significantly. The improved cell viability concomitantly allows higher efficiency. In monocytes we achieved breakthrough DNA electroporation efficiency of over 75% while maintaining over 85% cell viability. Our work provided a solution for improving the electroporation of hematopoietic cells and gene targeting with these cells would be made much easier.

759. Targeted Gene Delivery into the Mammalian Inner Ear Using Synthetic Serotypes of Adeno-Associated Virus Vectors

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Targeting specific cell types in the mammalian inner ear is important for treating genetic hearing loss due to the different cell type-specific functions. Adeno-associated virus (AAV) is an efficient *in vivo* gene transfer vector and has demonstrated promise for treating genetic hearing loss. Although more than 100 AAV serotypes have been identified, few studies have investigated whether AAV can be distributed to specific inner ear cell types. Here, we screened three EGFP-AAV reporter constructs (serotypes DJ, DJ8, and PHP.B) in the neonatal mammalian inner ear by injection via the round window membrane to determine the cellular specificity of the AAV vectors. Sensory hair cells, supporting cells, cells in Reissner's membrane, interdental cells, and root cells were successfully transduced. Hair cells in the cochlear sensory epithelial region were the most frequently

transduced cell type by all tested AAV serotypes. The recombinant DJ serotype most effectively transduced a range of cell types at a high rate. Our findings provide a basis for improving treatment of hereditary hearing loss using targeted AAV-mediated gene therapy.

760. Comparison of Three Genome Editing Techniques to Correct the Common W1282X Mutation Responsible for Cystic Fibrosis

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Introduction: Cystic Fibrosis (CF) is a recessive genetic disease that affects multiples organs in patients. The disease is due to different mutations in the Cystic fibrosis transmembrane conductance regulator (CFTR) gene. Patients have to take heavy treatments in order to have their disease stabilized. However, some mutations are not curable with available drugs. This is the case of the mutation W1282X, the sixth most common mutation with a prevalence, in 2018, reported to be 1.2% amongst patients with CF (Cftr2.org). This class I mutation is caused by the change of a G to an A in the 3846 position of the cDNA, and creates a premature TGA stop codon, making cells unable to synthesize a full-length protein, resulting in virtually no CFTR protein available at the cell surface. Whilst many groups have reported the correction of F508del mutation by genome editing technology, there are no published reports on the correction of the W1282X mutation. **Methods & Results:** In this study, we present a comparison of three different techniques to correct the W1282X mutation in HBE W1282X mutant cell lines.We compared Homologous directed repair (HDR) approach using two different CRISPR proteins, SpCas9 and AsCas12a (Cpf1) in a RiboNucleoProtein (RNP)/guideRNA complex format. The donor template was a single strand oligonucleotide of the non-target sequence, containing the corrected mutation flanked by asymmetric homology arms. In both cases, we observed ~20% precise editing by HDR, but we also found a significant level of indels caused by NHEJ repair. Given the high level of NHEJ relative to HDR, and recent observations that double-stranded breaks (DSB) can lead to unwanted chromosomal rearrangements, we have also tested Cas9-adenine base-editing (ABE) to correct this mutation. ABE can convert an A:T to G:C base pair with high efficiency without the formation of a DSB. We have recently confirmed that this enzyme shows high levels of precise editing, with very low levels of indels. However, a current limitation of this technique is that the target residue must lie in a window of 4nt to 7nt region on the same DNA strand as the 5'-NGG-3' protospacer adjacent motif (PAM). There is only one PAM sequence close to the W1282X mutation site, but the targeted A would be outside the base-editing window, and we have been unable to detect ABE using the Cas9-ABE7.10 plasmid. However, the mutation is potentially amenable to editing with a recently described xCas9-ABE, which has less constraints on the PAM sequence (NG, GAA, GAT). **Perspectives:** If it is possible to correct W1282X efficiently with xCas9-ABE, this may be the most suitable for preclinical development. Indeed, a recent study of the editing at off-target sites by Cas9-ABE indicates that it is 20-fold lower than Cas9/gRNA used for HDR. *Aknowledgment: CF Trust and CF foundation for funding this project.*

761. Complete Disruption of ULK1, FAT10 and CtIP Genes by Homology-Independent Multiallelic Knock-In Yielded Distinct Functional Outcomes

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Human cell lines maintained under culture conditions are pivotal models for direct analysis of human gene functions. Since most cultured cells possess diploid or hyperploid genomes, meaning that a single gene is often presented as two or more copies in the genome, knockout or targeted disruption to introduce complete loss-of-function of a selected gene has been technically challenging in these cells. Recently, studies have exploited the NHEJ repair mechanism to knock-in DNA at CRISPR-induced DSBs in both zebrafish and mammalian cells. By targeting constitutively expressed house-keeping gene GAPDH at 3'-UTR using promoterless fluorescence reporters, we directly compared frequencies of knock-in mediated by CRISPR-induced NHEJ and HDR repair mechanisms. We found that knock-in via CRISPR/Cas9-induced NHEJ is superior to the commonly used HDR-based method in all human cell lines examined. Here, exemplified by hyperploid human cell line LO2, we demonstrated that simultaneous knock-in of dual reporters through CRISPR/Cas9-induced homology-independent DNA repair, permits one-step generation of cells carrying complete gene disruption of multiple alleles. Through knocking-in large DNA fragments at a coding exon, we generated stable single-cell clones carrying complete disruption of all four copies of ULK1 gene, lacking all three copies of intact FAT10 gene, or devoid of intact CtIP gene at both alleles. Importantly, we fully confirmed the depletion of ULK1 and FAT10 transcripts as well as corresponding proteins; and in subsequent functional analysis of the ULK1−/− and FAT10−/− cell clones, we observed defect in mitophagy and cytokine-induced cell death, respectively; which are consistent with previous reports. In conclusion, multiallelic gene disruption could be readily introduced through CRISPR/Cas9-induced homology-independent knock-in of dual fluorescence reporters followed by direct tracing and cell isolation. Robust cellular mechanisms exist to spare essential genes from loss-of-function modifications, by generating partial functional transcripts through diverse DNA and RNA processing mechanisms.

762. Novel Transgenic NIS Reporter Mice for Longitudinal Tracking of Pathological Diseases by High-Resolution Imaging

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Introduction: Transgenic rodents expressing beta-Galactosidase, GFP or luciferase reporter genes have greatly facilitated the study of inflammation, fibrosis, and carcinogenesis. Luciferase reporter animal imaging allowed for longitudinal in-life studies of the evolution or amelioration of disease pathology over time and in response to an interrogated therapy. However, since photons are variably attenuated by the tissues through which they pass, luciferase images are 2-dimensional (non-tomographic), poorly quantitative, and do not precisely pinpoint the location of reporting cells. The thyroidal sodium iodide symporter (NIS) mediates the uptake and concentration of iodide (and related radioactive anions) and is the basis for diagnostic thyroid radioimaging. NIS is a superior reporter gene for quantitative expression monitoring in deep-seated tissues because, unlike photons, gamma rays are minimally attenuated by the tissues through which they pass. Here, we describe the creation of transgenic mice that utilize collagen promoter-driven sodium iodide symporter (NIS) reporter gene in conjunction with nuclear imaging as an attractive alternative technology for development of novel disease models. **Methods:** FVB and C57BL/6 transgenic reporter mice expressing human NIS driven by the mouse collagen type-1 alpha-1 (Col1α1) promoter were created. Mice were screened for the pro Col1α1-hNIS genotype by PCR with human NIS specific primers. NIS positive mice were then imaged by SPECT/CT or PET/CT nuclear imaging for detection Col1α1 promoter activity overtime by NIS uptake of radiotracers. **Results:** Both mouse lines had expected physiologic uptake of radioiodine or radioactive anions in tissues that normally express NIS, such as the thyroid and gastic mucosa. Additionally, human NIS PCR positive mice had uptake in tissues that are expected to have high collagen expression, including the joints, tail and spine. In these tissues, we observed that proCol1α1-NIS transgenic mice had higher amounts of radiotracer uptake when compared to non-transgenic mice. Radioiodine uptake detected by SPECT/CT imaging waned as the proCol1α1-NIS transgenic mice aged. Currently, studies are ongoing using the C57BL/6 proCol1α1-NIS mice as a model animal for lung fibrosis. **Conclusions:** For the first time, we describe the creation of proCol1α1-NIS mice that can be used for longitudinal tracking of Col1α1 promoter activity by nuclear imaging. These mice can be used to model a variety of biological processes, including lung fibrosis, carcinogenesis and arthritis. Furthermore, in vivo monitoring using living NIS reporter transgenic rodents will facilitate drug and gene therapy development in many applications.

763. A Systematic Review of the Public Acceptability of Gene Therapy and Gene Editing for Human Applications

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Introduction: Genetic diseases are ideal targets for genetic therapies to correct the function of the abnormal gene. Gene therapy and gene editing technologies are complex and can be difficult for the public to understand possible benefits or side-effects. However, both patient and public support is critical for the successful adoption of a new technology. While both technologies offer hope for a lasting benefit or cure for a range of diseases, the delivery process, and the possibility of permanent changes to the host cell genome, do have potential risks. There are also ethical and philosophical issues that are likely to influence people's perceptions towards gene therapy and gene editing. Given the recent advances in these fields, and their potential for providing substantial clinical benefit, a comprehensive systematic review is essential to assess the beliefs and attitudes towards gene therapy and gene editing for human use, and to highlight factors that influence acceptability for its use. **Methods:** A comprehensive search following PRISMA guidelines was undertaken in April 2018 to identify papers that examined opinions and attitudes regarding the acceptability of gene therapy and gene editing. Overall, 1151 records were retrieved from four databases (Ovid Medline, PsycINFO, Scopus, and Web of Science), and after duplicate removal and independent screening of titles and abstracts (IP, MD), 78 full-text articles were assessed for eligibility. Based on the full-text review 33 were included, with an additional 5 articles identified in a forwards/backwards search (total n = 38 studies). **Results:** Over half of the papers included were published in the last 8 years, reflecting recent advancements in gene therapy and the increasing importance of understanding perceptions. Findings from the qualitative, quantitative, and mixed-methods studies were integrated such that perceptions towards gene therapy/editing were synthesised according to common themes. These were: risks versus benefits of success; treatment specifics (e.g., medical versus other reasons; disease severity and status; somatic versus germline; mode of delivery); ethical or moral issues; the impact of individual differences (e.g., knowledge, age, gender, religion); and changes over time. **Conclusions:** In general, perceptions of gene therapy are positive, particularly for medical reasons or fatal diseases, however these perceptions are also influenced by perceived risk. Somatic gene therapy had higher levels of acceptability than the use of germline transgenesis. Overall, limitations exist in the measurement of perceptions of gene therapy and gene editing. As acceptability of a treatment is essential to consider for future clinical trials, it is important for scientists and clinicians to be clear about the risks and benefits of both of these technologies in the future for improved acceptability. **Acknowledgements:** Supported by the Australian Cystic Fibrosis Research Trust. MD was supported by a Robinson Research Institute Career Development Fellowship.

764. Efficient Gene Editing of NKX2.1 in Human Stem Cells

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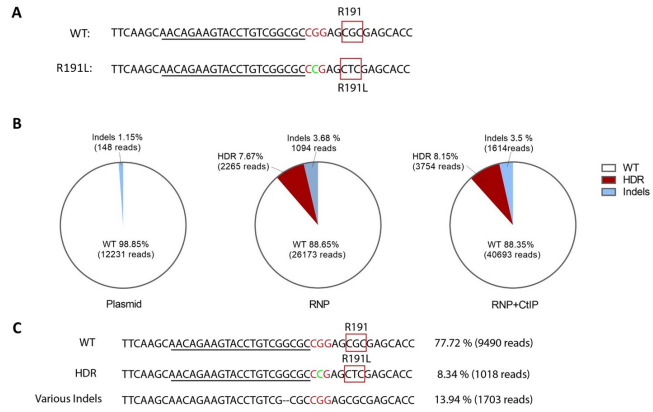
Introduction: Neuroendocrine cell hyperplasia of infancy (NEHI) is a childhood lung disease characterized by hyperplasia of pulmonary neuroendocrine cells within distal bronchioles and alveolar ducts. A heterozygous mutation in the homeodomain of NKX2.1 (c.572 G->T, R191L) has been identified in an extend family with NEHI; however, it is unclear whether this mutation is a cellular intrinsic causative mechanism for the disease. Development of clinically relevant cell lines that carry the NKX2.1 R191L mutation will facilitate the research and drug development for NEHI. **Methods:** Wild-type (WT) human induced pluripotent stem cells (iPSCs) and basement membrane-resident p63+ basal cells (airway stem cell, ASCs) were cultured and used for gene editing. CRISPR/Cas9 guide RNA (gRNA) was designed to target the R191 proximal sequence. Single stranded oligodeoxynucleotide (ssODN) donor template (169 nt) carried 84 nt homology arms on each side, c.572 G->T point change for the R191L mutation, and c.567 G->C point change for a silent mutation at the protospacer adjacent motif (PAM) (Figure 1A). Cas9 was delivered in the format of plasmid DNA (pDNA) or Cas9 and gRNA ribonucleoprotein (RNP) by a tube electroporator (Celetrix LLC, Manassas, VA). 48 hours after electroporation, genomic DNA was extracted, targeted regions PCR amplified, and the PCR products gel purified. The purified PCR products were sent to the DNA Core at Massachusetts General Hospital for deep sequencing. **Results:** We first compared the precise gene mutation (PGM) efficiency when Cas9 was delivered by electroporation in the format of pDNA vs RNP. The Cas9 RNP group led to significantly higher PGM rate (7.67%) than the pDNA group (0%) ($P<0.05$) (Figure 1B). Interestingly, when we co-transfected pDNA of CtIP, a key player in the homology directed repair pathway, in the RNP group, the PGM rate was only slightly increased to 8.15% but not significantly higher than that in the RNP along group ($P>0.05$) (Figure 1B). We proceeded with using tube electroporation to deliver Cas9 RNP and ssODN to the ASCs and achieved 8.34% PGM rate. The remaining alleles consisted of 13.94% of various insertions and deletions (indel), and 77.72% WT (Figure 1C). **Conclusion:** We show that the NKX2.1 R191 locus can be efficiently edited in iPSCs and ASCs by electroporation of Cas9 RNP. This precise gene editing is achieved in one-step without using viral vectors, drug selection, or reporter enrichment. The edited cells represent novel tools for the study of NEHI. Importantly our work demonstrates the feasibility of gene editing therapeutics towards NEHI. Figure 1. Gene editing of NKX2.1. (A) Illustration of the gRNA and the mutation locus. (B) Efficiencies in generating the R191L mutation in human iPSCs when Cas9 was delivered in the format of pDNA (Plasmid), RNP, or RNP along with CtIP (RNP+CtIP). (C) Efficiencies in generating the R191L mutation in human ASCs. Boxed: the R191/R191L locus. Underlined: gRNA sequence. Red colored: PAM sequence. Green colored: silent mutation in PAM. WT: wild-type. HDR: homology directed repair. Indels: insertions or deletions.



765. Generation of ESR1 Y537S Homozygous Knock-In in Polyploid Breast Cancer Cells by Homology-Directed Genome Editing Using Cas9 Ribonucleoprotein and AAV Donor

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Mutations in the estrogen receptor alpha (ERα) 1 gene (ESR1) are frequently detected in ER+ metastatic breast cancer and are considered to confer endocrine resistance in breast cancer patients with advanced disease. To investigate the mechanism of these mutations in driving estrogen-independent activities and reduced sensitivity to ER antagonists, we generated homozygous mutant MCF7 cell lines with all ESR1 alleles containing Y537S knock-in by using Cas9 ribonucleoprotein and an AAV/DJ vector delivering a homology-directed repair template. Among 23 single clones selected, we obtained 6 homozygous Y537S knock-in clones verified by Sanger sequencing and ddPCR analysis of both genomic DNA and mRNA. No off-target effects were observed at 19 CRISPOR/COSMID predicted off-target sites for all 6 clones. Mutant ER protein levels in these clones were significantly lower than ER protein levels in wild-type MCF7 cells and a heterozygous ESR1 Y537S MCF7 cell line previously generated via plasmid transient transfection. However, these clones grew well and ER signaling, measured by ER target gene expression, was constitutively active in hormone-free media. RNAseq analysis indicated the homozygous mutant cell lines had distinct gene expression profiles from those of the wild-type and heterozygous mutant cell lines. Finally, the homozygous mutant cell lines had reduced sensitivity to multiple ER antagonists. In summary, the combination of Cas9 ribonucleoprotein and AAV donor provides a powerful tool for highly efficient and precise homology-directed genome editing, and the establishment of multiple homozygous ESR1 Y537S knock-in mutant cell lines provides a valuable tool for investigating mechanisms of mutant ESR1 mediated signaling and drug response.

766. Rapid Generation of CRISPR/Cas9 Knock-Out Mutants Enabled by ddPCR NHEJ Drop-off Assay

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The widespread adoption of clustered, regularly interspaced, short palindromic repeat (CRISPR) nuclease technology has revolutionized targeted genome editing1. When CRISPR is paired with Cas9 nuclease to target specific DNA loci, specific cleavage and imperfect repair by non-homologous end joining (NHEJ) can lead to disruption of the target gene to create a gene knock-out mutant. CRISPR/Cas9 knock-out mutants provide powerful insights into protein function, and are being used to profile many proteins involved in pathology and infection2. In practice, performing CRISPR/Cas9 mediated NHEJ knock-out editing is a labor and time intensive process, requiring weeks to months of single-cell clonal expansion, surveyor strand assay, and agarose gel electrophoresis. Here we describe a facile, accurate, quantitative ddPCR based assay and method to rapidly calculate NHEJ frequency within 1 day of CRISPR/Cas9 transfection, therefore streamlining the CRISPR/Cas Knock-out generation from a months-long process to under 4 weeks. The ddPCR NHEJ drop-off assay utilizes two fluorescent probes and a pair of flanking primers to detect editing of the targeted DNA sequence, and provide absolute quantitation of NHEJ editing frequency using droplet PCR technology. The ddPCR NHEJ assay is performed on the cell population immediately following CRISPR/Cas9 transfection and can be used to simultaneously optimize transfection conditions and determine the effectiveness of the CRISPR/Cas9 complex targeting efficiency.

Oligonucleotide Therapeutics

767. Splice-Switching Antisense Oligonucleotides for the Treatment of Cystic Fibrosis

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Cystic Fibrosis (CF) is an inherited recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CFTR encodes a chloride channel that, when mutated, results in the buildup of mucus in tissues such as the lung and pancreas interfering with proper organ function. Chronic infections and inflammation in the lung represent a primary cause of morbidity and mortality. CF affects more than 70,000 people worldwide. Approximately 12% of CF-associated CFTR mutations are rare mutations that alter pre-mRNA splicing. The majority of CF therapies in the clinic or in development target only the most abundant CFTR mutations, leaving patients with rare mutations in need of more personalized therapies. Splice-switching antisense oligonucleotides (SSO) have emerged as effective therapeutic molecules that can modify gene expression by modulating pre-mRNA splicing. One of the more common splicing-related mutations in CFTR is the 3849+10kb C>T splice mutation, which creates a de novo 5' splice site and results in the inclusion of a cryptic pseudoexon in CFTR mRNA. This pseudoexon contains a stop codon and results in the production of a truncated CFTR protein. We used an SSO that basepairs to the aberrant splice site created by the 3849+10kb C>T mutation to block splicing to the pseudoexon. Treatment with the SSO blocked cryptic splicing and increased the abundance of full-length CFTR mRNA in patient lymphoblasts homozygous for the splicing mutation as well as in primary bronchial epithelial cells from a homozygous patient and two compound heterozygote patients with the 3849+10kb C>T splice mutation and ΔF508, the most common CF causing mutation. Importantly, SSO treatment significantly improved cAMP activated chloride secretion in differentiated primary patient-derived bronchial epithelial cells measured by an equivalent current (Ieq) assay utilizing a TECC-24, demonstrating that the SSO can restore CFTR function. When analyzed in comparison to current FDA approved CF drugs ivacaftor (VX-770) and ivacaftor/lumacaftor (VX-770 in combination with C18), SSO treatment alone resulted in a greater increase in CFTR function compared to that achieved with either of the other treatments. Together, our results demonstrate the ability of SSOs to correct aberrant splicing in the 3849+10kb C>T CFTR splice mutation as measured by an increase in the abundance of full-length mRNA as well as partial restoration of functional protein activity in patient-derived bronchial epithelial cells.

768. 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 autosomal recessive liver disease caused by mutations in the ABCB4 gene, which encodes the phosphatidylcholine (PC) transporter, MDR3 (multidrug resistance P-glycoprotein 3). PFIC3 is characterized by early onset of cholestasis and variable jaundice which, progresses into cirrhosis and liver failure before adulthood. Standard enzyme replacement therapy (ERT) is not an option for PFIC3 patients due to drug delivery hurdles. Current viral gene therapy-based approaches have also proven unsuccessful due to safety concerns with genotoxicity and the presence of neutralizing antibodies which render the treatments ineffective. The only curative treatment option for PFIC3 patients is liver transplantation, which is high-risk and often presents with significant morbidity. To develop a new class of therapy for PFIC3, we generated a nucleoside modified, codon optimized mRNA encoding human ABCB4 mRNA (hABCB4) encapsulated in lipid nanoparticles (LNPs). We demonstrate the efficacy of hABCB4-mRNA-LNP therapy in human cells in culture and in a mouse model of PFIC3 ($mdr2^{-/-}$)

that resembles the human condition. Of note, systemic administration of the hABCB4-mRNA-LNP in mdr2$^{-/-}$ mice resulted in 1) proper localization of functional hABCB4 protein within the canicular domain of mouse hepatocytes and 2) improved bile PC output in treated mice. Importantly, following repeat dosing of hABCB4-mRNA-LNP in mdr2$^{-/-}$ mice, there was a significant improvement in several clinical and pathophysiological measurements including: progression of liver fibrosis, 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.

769. Development of Synthetic Stem-Loop RNA (Sl-RNA) Fragment Derived from Sendai Virus Genome for Inducing Antitumor Immunities

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In this study, we developed synthetic stem-loop (sl-) RNA fragment which induces strong antitumor immunities. The sl-RNA sequence is derived from Sendai virus DI (defective interfering) particle genome. Previous studies showed that inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) particles called HVJ-envelope (HVJ-E) have multiple-anti-cancer activities. One of the activities is activation of anticancer immunities through inactivation of Treg (regulatory T cell), promotion of NK cells activation and generation of CTL against cancers. Another is the 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-cancer activities are conducted by RNA genome fragments of HVJ-E through 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 (DCs) and the strongest DC maturaion and we found that DI 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. Furthermore, we examined whether a specific structure of the DI RNA genome stimulates the RIG-I/MAVS downstream-related cancer suppressive pathways using HVJ-derived *in vitro* transcribed (IVT) RNAs (this RNA fragment was named IVT-B2). IVT-B2 which is derived from Cantell HVJ DI genome has a special secondary structure with a double-stranded RNA terminus and a single-stranded RNA loop. This IVT-B2 strongly stimulated RIG-I dependent proapoptotic proteins induction in prostate cancer cells. Modified IVT-B2 RNAs which had shorter dsRNA stem lost cancer cell killing activity and proapoptotic gene expressions. *In vivo*, IVT-B2 RNA induced intra-tumoral apoptosis and tumor suppression in PC3 mouse model by both direct tumor-cell killing and NK cell activation. Moreover, sl-RNA has been developed as a third generation of synthetic RNA fragment which originated in Sendai virus genome. Sl-RNA-57 has 25-base double strand stem part and 25-base single strand loop part in the secondary RNA fragment structure. The sl-RNA-57 was injected to B16F10 (mouse melanoma) tumor by pyro-drive jet injector (PJI) three times. The tumor growth was strongly suppressed in sl-RNA-57 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-57 injections. Additionally, macrophage infiltrations and macrophage polarization to M1 (anti-tumorigenic) were observed in the sl-RNA-57 injected B16F10 tumor sections by F4/80 and NOS-2 immunostaining. Originally HVJ-E was recognized as the main antitumor immunity inducing factor. Further investigations revealed that the core of antitumor immunity induction part might be the sl-RNA fragment derived from Sendai virus genome. These findings provide a novel nucleic acid medicine for the cancer treatment.

770. Developing Antisense Oligonucleotides Therapies for Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common inherited muscular dystrophies with an incidence of 1:8,000 to 1:20,000. Studies showed that FSHD is caused by aberrant expression of double homeobox 4 (DUX4) due to epigenetic changes of the D4Z4 macrosatellite repeat region at chromosome 4q35. The aberrant expression of DUX4 causes misregulation of numerous downstream genes and pathways, which in turn lead to the muscle weakness and pathologies. To date, there is no effective treatment for FSHD. Antisense oligonucleotides (AONs) therapy has shown promise for treating an array of disorders and can be used to reduce DUX4 in FSHD. The goal of this study is to develop antisense oligonucleotide strategies to reduce the pathogenic DUX4 mRNA in affected muscles. In this study, we examined three different types of AONs, including 3GA, LNA gapmers and 2'MOE gapmers. The AONs were either delivered by local intramuscular injections (i.m.) or subcutaneous injections (s.c.) to the DUX4-expressing FLExDUX4 FSHD-like mouse model. To localize the AONs, we fluorescently tagged the 3GA and LNA gapmers for injections. Our results showed that the fluorescein-labeled AONs were present in skeletal muscles 24 hours after either one i.m. or one s.c. injection. Three i.m. injections (20ug) of the three types of AONs into the tibialis anterior muscles significantly reduced DUX4 transcripts in the muscles of the FLExDUX4 mice. Systemic delivery,

using six s.c. injections (20mg/kg) of the LNA gapmers and (30mg/kg) of the 3GA significantly reduced the DUX4. In addition to DUX4 reduction, mice received longer treatments showed muscle function improvement measured by grip strength testing. Our findings showed that the AONs targeting DUX4 delivered to skeletal muscles by systemic delivery significantly reduced DUX4 transcripts and was accompanied by recovery of muscle functional deficits in the FLExDUX4 mice. These data support the use of AONs, delivered either locally or systemically, as a viable therapeutic approach to FSHD.

771. Identification of Proteomic Biomarkers Utilizing a Bead-Based X-Aptamer Library and Flow Cytometry Sorting

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Purpose: Effective biomarkers play a key role in clinical applications for patient selection, monitoring response to therapy and improving routine clinical care. Aptamer-based protein biomarker discovery is attractive due to its quick and specific target protein selection. We created a systemic biological approach that combines bead-based modified aptamer libraries with flow cytometry and mass spectrometry to identify proteomics biomarkers. **Materials and Methods:** An ovarian cancer cell line (OVCAR3) was used to model our approach. OVCAR3 cells were treated with or without Epidermal Growth Factor (EGF). Cell lysates were labeled with two different fluorophores respectively, and incubated with a bead-based X-aptamer (XA) library. Bead-XA-protein complexes were sorted by flow cytometer based on two-color fluorescent differential intensity. Proteins were removed by heat, and the XAs were cleaved from beads and amplified by PCR for next generation sequencing (NGS). Identified X-aptamer sequences were synthesized and incubated with cell lysate to pull-down target proteins. The target proteins were identified by mass spectrometry analysis and validated again with cell lysate proteins. **Results:** Four sequences were identified and selected in each group. Specific binding of XA1, our top sequence, was confirmed by incubation with EGF treated OVCAR3 cells. Mass spectrometry analysis demonstrated a significantly changed protein profile in cell lysates treated with EGF (n=3). Based on MASCOT database search, all of the top ranked proteins were related to EGF-induced DNA synthesis, cell proliferation, and differentiation. **Conclusion:** We established a fast and specific approach for proteomics biomarker discovery. The advantages of our approach are combining advanced X-aptamer technology with flow cytometry sorting and mass spectrometry analysis to simultaneously identify high-binding affinity reagents (X-aptamers) and target proteins in a rapid, specific way. This approach can be extended to biomarker studies of other cancers or diseases, and has high impact to clinical diagnosis and therapy.

772. First Human Experience with Novel Triple Effector Gene Therapy for Heart Failure

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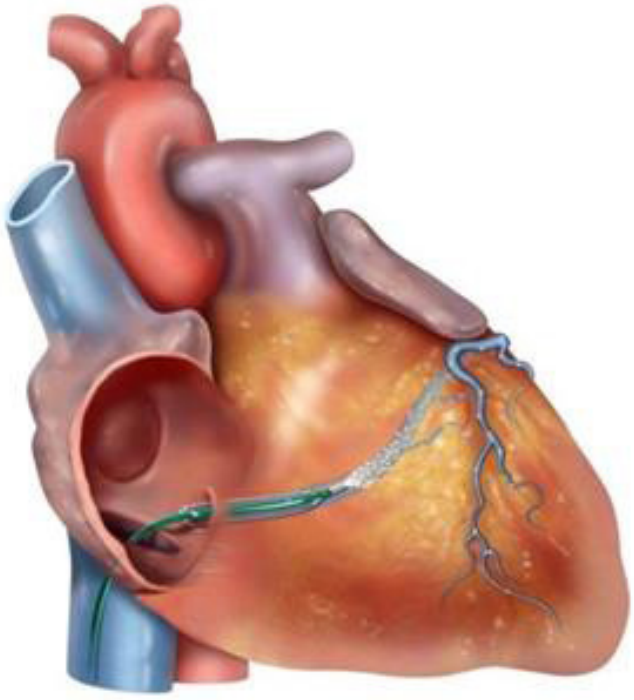
Monogenic therapies have not been successful in clinical translation for treating heart failure. Our goal is to evaluate INXN-4001, a triple effector gene therapy, which impacts angiogenesis, cell homing and contractility in a clinical setting. Patients with durable left ventricular assist devices for the treatment of heart failure were evaluated in two dosing regimens of cGMP INXN-4001. INXN-4001 was delivered with retrograde coronary sinus infusion with balloon occlusion. This FDA approved trial is actively enrolling with primary safety and efficacy signals at 1, 3 and 6 months. In patients dosed to date the efficacy signal is showing an improvement of fractional shortening greater than 40%. Triple effector gene therapy is demonstrating safety thus far with promising efficacy signals. Further follow-up is ongoing to evaluate clinical endpoints.



773. Abstract Withdrawn

774. Gemcitabine-Loaded EGFR Aptamer for Targeted Therapy of Lung Cancer

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Lung cancer is the leading cause of cancer-related death in the world and non-small cell lung cancer (NSCLC) is the most common types of the lung cancer. Epidermal growth factor receptor (EGFR) is an attractive therapeutic target due to its high expression in NSCLC. Gemcitabine (Gem) has shown antitumor activity against many kinds of solid tumors, including NSCLC. Aptamers emerged as promising drug delivery vehicles because of their high binding affinity for the target molecules. The objective of this study was to develop the aptamer-drug conjugates using the EGFR-specific aptamer. EGFR aptamers selected by the SELEX process and gemcitabine was internally incorporated into the EGFR aptamer. *In vitro* binding affinity of EGFR and Gem-loaded EGFR aptamer was evaluated on EGFR-positive A549, H460, H1299 cell lines, as well as GPC3-negative H522 cells using confocal microscopy. To determine whether the Gem-loaded EGFR aptamer was effectively internalized, Z-stack images were acquired after 1 hr incubation of Cy5-conjugated EGFR aptamer at 37°C. The *ex vivo* biodistribution of Cy7-conjugated EGFR aptamer was investigated in NSCLC xenograft nude mice. To confirm the antiproliferative effects of Gem-loaded EGFR aptamer, the MTT cell proliferation assay was carried out in NSCLC cells. In the confocal image of EGFR-positive cells, Gem-loaded EGFR aptamers were selectively bound to the cell surface, while no aptamer binding was observed in H522 cells. Z-stack imaging showed that internalized Gem-loaded EGFR aptamer accumulated in EGFR-positive cells. The *ex vivo* biodistribution data showed that the Gem-loaded EGFR aptamer has higher overall accumulation within the lung compared to scramble EGFR aptamer. A549 cells showed significant reduction in proliferation following treatment with Gem-loaded EGFR aptamer. Our results indicated that Gem-loaded EGFR aptamer was a potential therapeutic agent in EGFR-positive NSCLC therapy.

775. Retrograde Delivery of Novel Triple Effector pDNA Gene Therapy for Heart Failure

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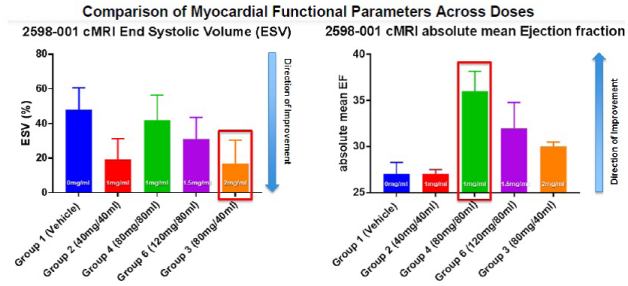
BACKGROUND: Heart failure (HF) is rarely a single gene defect disease. Current monogenic approaches to treat heart failure have failed clinical trials. Our goal was to evaluate a triple effector pDNA Gene Therapy (INXN-4001) in a large animal preclinical model. **METHODS AND RESULTS**: One hundred pigs underwent creation of catheter-based ischemic heart failure. The animals with ejection fractions <40% were then randomized to various treatment doses of INXN-4001 delivered retrograde via the coronary sinus into 8 animals per group. After a single dose, expression of INXN-4001 was found in the heart and kidneys. No other organ had expression. Cardiac MRI was performed at multiple time points. Two of the treatment groups demonstrated the greatest improvement in end systolic volume and ejection fraction respectively. **CONCLUSIONS**: Coronary sinus delivery of INXN-4001 was safe and improved myocardial function in a large animal ischemic heart failure model.



776. Delivery of Non-Coding RNAS by Mesenchymal Stem Cell-Derived Exosomes as an In-Vivo Hit-and-Run Targeted Epigenome Remodeling Approach for Age-Related Disorders and Degenerative Diseases

Roger Bertloltti

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Hot-off-the-press achievements on bioreactor-based large-scale production of extracellular vesicles/exosomes released by mesenchymal stem cells (MSC) and enriched with specific miRNAs/siRNAs, open exciting avenues for in vivo transient epigenetic gene therapy mediated by non-coding RNAs, thereby providing an additional impulse to our targeted epigenome editing approach for age-related disorders and degenerative diseases. Initialy evidenced with promoter-specific siRNAs, transcriptional gene silencing or activation/derepression mediated by non-coding RNAs (promoter/enhancer-specific siRNAs or short sense/antisense RNAs/oligos, epigenome remodeling miRNA cocktails) is a long-lasting epigenetic process that involves DNA methylation/demethylation and post-translational histone modifications as the result of an hit-and-run process driven by hazard-free transitory RNAs/oligos. Importantly enough, the homing ability of MSCs is kept by released exosomes, thereby providing targeted in vivo delivery of therapeutic RNAs/oligos to the sites of injury/inflammation specific to many age-related disorders. A more specific delivery is achievable either by using tissue-specific stem cells or by engineering the relevant exosome-producing cells to express a membrane protein fused to a target-specific peptide as previously done by others for brain neurons. In addition to their homing ability, MSCs are immunoprivileged stem cells, thereby paving the way for efficient off-the-shelf allogeneic exosomes as rejection-free delivery vehicules. Allogeneic MSC-derived exosomes are thus fitted to our age-related focus on NAD+ homeostasis because it is aimed at concurrent rejuvenation of different resident stem cell populations from elderly people, thereby relying on the broad MSC-specific homing ability. A critical decrease in cellular NAD+ content is associated to aging and stands as a major contributor to age-related disorders, in particular through deficient DNA repair mechanisms. NAD+ homeostasis is

therefore a major rejuvenating goal for elderly resident stem cells and we intend to restore it upon epigenetic upregulation/reactivation of the NAMPT and NAPRT genes that encode the rate-limiting enzymes of the NAD+ salvage (conversion of catabolic/dietary nicotinamide) and Preiss-Handler (conversion of dietary nicotinic acid) pathways, respectively. Both genes appear to incur an age-related epigenetic down-regulation linked to CpG island hypermethylation. Based on in-vivo hit-and-run genome-safe exosome delivery of non-coding RNAs/oligos culminating possibly in miRNA gene activation/silencing, our approach is discussed in light of 1) approaches based on NAMPT and NMAT1/T3 transgenes, 2) our and others concurrent epigenome editing approaches based on nuclease-free CRISPR-dCas9 fusion proteins with/without exosome delivery, 3) synergistic dietary complementation, 4) successful allogeneic MSC clinical trials, 5) potential cautious synergistic use of senolytics, 6) potential oncogenic hazard of overexpressed circadian-tuned NAMPT gene and 7) potential choice of exosomes produced by other stem cells such as neural ones for Alzheimer's and other neurodegenerative diseases.

777. Human Cardiac iPSC Evaluation of Novel Triple Effector Gene Therapy for Dilated Cardiomyopathy

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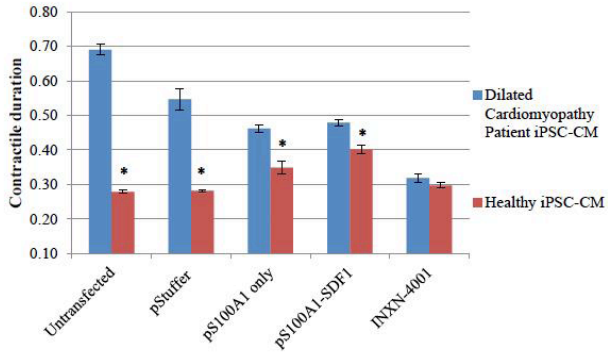
The purpose of this study was to evaluate the functional outcomes of a triple effector pDNA gene therapy candidate (INXN-4001) in comparison with various single and dual effector pDNA constructs, on the contraction properties of iPSC-cardiomyocytes (iPSC-CM) derived from a Healthy Donor and a Dilated Cardiomyopathy (DCM) patient carrying a R173W mutation in the cardiac TNNT2 gene. The effect of these DNA constructs on the electrophysiological properties was also examined to assess potential ion channel toxicity. Baseline contraction properties were measured on a Sony SI8000 Live Cell Imaging System (Sony Biotechnology Inc, San Jose, CA, USA) at 48, 72 and 96-hour time points following pDNA transfection of iPSC-CMs from both healthy control and DCM groups. The transfected cells were evaluated using contraction videos analyzed with Sony S18000C analyzer software, generating data for several contractile parameters, including beating rate, contractile velocity and contraction duration. Conclusion: INXN-4001 demonstrated the most significant improvement, restoring beat rate, contraction duration and contraction rate of DCM iPSC-CM to healthy levels without issues of toxicity.



* indicates statistical significant difference between patient and healthy iPSCs ($p<0.05$)

778. RNA Structurome Analysis of EGFR Pre-mRNA Transcripts using Nanopore Sequencing to Optimize Anti-Sense RNA-Directed Therapeutics

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Pre-mRNA splicing is the process whereby nascent transcripts are transformed into mRNA by removing introns and joining exons together. The pattern in which introns are removed and exons are joined together affects the characteristics of the translated protein. We have developed a gene therapy vector that delivers antisense RNA sequences that bind splicing elements blocking pre-mRNA splicing. Our therapy induces alternative patterns of splicing, reducing the expression of oncogenic proteins. We are using this strategy to target oncogenic epidermal growth factor receptor (EGFR) transcripts which are overexpressed in 60% of glioblastoma multiforme tumors. To optimize efficacy of our current antisense RNAs, we are examining the EGFR RNA structurome to identify regions of transcripts which are not bound by protein nor involved in RNA secondary, tertiary, or quaternary structure. The pre-mRNA structurome affects the splicing pattern. In order to therapeutically modify the pre-mRNA transcript, we are using tools to uncover the RNA structurome of oncogenic transcripts. We have begun experiments to analyze the EGFR pre-mRNA structure using selective 2' hydroxyl acylation and primer extension followed by mutational profiling (SHAPE-MaP). The modified RNA is reverse transcribed, incorporating mismatches at the acylated positions; a comparison of unmodified to modified RNA allows us to determine RNA nucleotides that are involved in secondary structure, part of RNA-binding-protein complexes, or single stranded. Single stranded RNAs and RNAs with minimal structure are considered targetable regions for our anti-sense directed RNA therapy. We hypothesize that the secondary structure of critical splicing elements of the pre-mRNA transcript determines the most effective way to therapeutically alter the splicing of EGFR. GBM cells grown in culture were subjected to the acylation reagents, 1M7, 5-NIA or DMSO (control) in cellular and cell-free conditions. RNA was isolated using Trizol and phenol:chloroform:isoamyl alcohol respectively. The RNA was subjected to DNA degradation followed by reverse transcription with Superscript IV under SHAPE conditions using a gene specific cocktail primer. Reverse transcription under SHAPE conditions

includes the use of manganese chloride as the divalent ion for the RNA-dependent DNA polymerase subunit activity. It was determined that the most effective way to isolate pre-mRNA was to reverse transcribe with a gene specific cocktail primer mix. Primers were designed to target the EGFR transcript ranging from intron 15 to the 3' UTR. The cocktail consisted of multiple sets of primers. cDNA was converted to double stranded DNA and sequenced using the MinION nanopore device to determine the EGFR pre-mRNA structurome.

779. Selection and Characterization of Vimentin-Binding Aptamer Motifs for Treatment of Ovarian Cancer

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The application of aptamers in biomedicine is emerging as an essential technology in the field of cancer research. As small single-stranded DNA or RNA ligands with high specificity for their targets, aptamers provide many advantages over protein-based molecules, such as antibodies, that are currently being used in cancer therapeutics. Vimentin is an intermediate filament protein that has been shown to be overexpressed in endothelial cells of cancerous tissue. High expression levels of vimentin have been associated with increased capacity for migration and invasion of the tumor cells. We have selected and identified thioated aptamers with high specificity for vimentin using human ovarian cancer tissues. The secondary structures of the selected vimentin binding aptamers were analyzed to predict binding motifs for each aptamer. Each binding motif was then synthesized, purified, and then characterized via cell binding assays with cultured ovarian tumor cells. Two vimentin binding motifs with high fidelity binding were selected and further characterized via cell and tissue binding assays. The equilibrium binding constants of these small thioated aptamer constructs were also determined by filter binding assays. Future applications for the vimentin binding aptamer motifs include conjugation of the aptamers to synthetic dyes for the use in interventional radiology, and ultimately more detailed and precise monitoring of tumor progression in ovarian pathology.

780. Minimally Invasive Novel Triple Effector Gene Therapy for Adriamycin Cardiomyopathy

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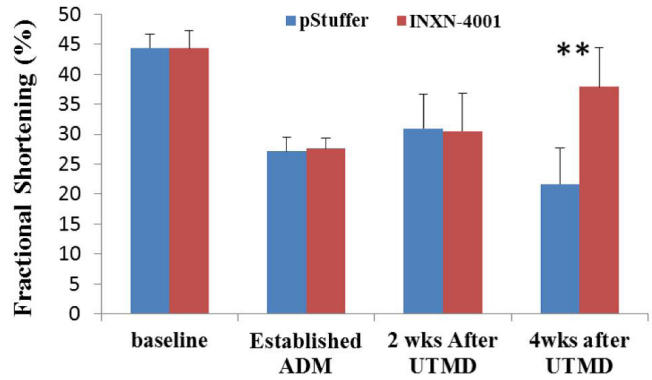
[1]Baylor University Medical Center, Dallas, TX,[2]Intrexon, Germantown, MD,[3]Xogenex LLC, Germantown, MD

Most treatments for Adriamycin (ADM) induced cardiomyopathy have failed as there are multiple underlying mechanisms contributing to heart deterioration. Our goal was to evaluate INXN-4001 (which is a triple effector gene therapy that affects angiogenesis, cell homing and contractility) in a pre-human model using ultrasound targeted microbubble destruction (UTMD). A total of 16 rats with established ADM-induced cardiomyopathy were divided into 2 groups of eight. Each group was treated with one of two DNA plasmids: (1) pStuffer, or (2) INXN-4001. The pStuffer plasmid is a negative control with the same backbone configuration as INXN-4001, in which the open reading frame (ORF) replaced with a non-expressing, similar-sized "stuffer" sequence. Echocardiographic measurements of cardiac structure and function were performed at baseline (before ADM treatments), 3 weeks after ADM treatments, 2 weeks after UTMD-based delivery of the two test plasmids, as well as 4 weeks after UTMD. Echocardiographic measurements performed at 4 weeks post UTMD delivery demonstrated that UTMD-INXN-4001 gene therapy significantly improved fractional shortening index, LV posterior wall diameter, and Interventricular Septal Thickness at Diastole (IVSd) compared to treatment with plasmid control treatments.



781. Transcriptomic Analysis of In Vivo Indoleamine 2,3- Dioxygenase (IDO) Silencing Immune Cells in a Murine Lung Tumor Model

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Indoleamine 2,3-dioxygenase (IDO) is a metabolic enzyme which is involved in tryptophan-degradation and immune regulation. Stimulation of cytokines including interferon-γ and transforming growth factor β and binding of cytotoxic T lymphocyte-associated molecule-4 (CTLA4), IDO expression is induced in dendritc cells and tumor cells. IDO is a key molecule in immune escapes in tumor cells. Furthermore, IDO-expressing dendritic cells can compromise proliferation of T cells and expand regulatory T cells. Therefore, inhibition of IDO is a strategy to revert immune suppression. Our previous studies demonstrated that administration of short hairpin RNA targeting IDO (IDO shRNA) through the skin (via a biolistic device) and muscle (intramuscular injection) significantly delayed tumor growth, induced Th1 immune responses, and activated tumor-specific cytotoxic activity in spleen cells in several tumor models. In addition, IDO shRNA treatment significantly increased the number of tumor-infiltrating $Gr1^+$ neutrophils. When depletion of neutrophils by the anti-Ly6g antibody, IDO shRNA-induced anti-tumor effect was attenuated in a murine lung tumor model. However, detailed regulatory mechanism is not fully understood. Thus, a transcriptomics approach was used to investigate this issue in spleen cells and neutrophils. The spleen cells were collected from the IDO shRNA and scrambled IDO

shRNA-treated murine lung tumor-bearing mice. Besides, neutrophils were also isolated via magnetic beads from spleen cells. The expression profile of mRNA and microRNA were analyzed by RNA sequencing. The potential regulatory network was determined in spleen cells and neutrophils and was further evaluated by experimental evidences. This study characterizes the unknown effect of immune cells after in vivo administration of IDO shRNA.

782. Scalable Manufacture of Mrna Lipid Nanoparticles Using a Novel Microfluidic Mixing Architecture

Jagbir Singh, Gemma Ryan, Ariel Zhang, James Ko, Andre Wild, Euan Ramsay, Lloyd Jeffs, Tim Leaver
Precision Nanosystems, Vancouver, BC, Canada

Precision NanoSystems Inc. (PNI) has developed novel microfluidic-based approaches to prepare a wide range of nanoparticles, including lipid-based nanoparticles for delivery of oligonucleotides (e.g., siRNA), larger polynucleotides (e.g., pDNA and mRNA), PLGA particles, liposomes and emulsions. PNI's NanoAssemblr® technology with the established Staggered Herringbone Mixer (SHM) is widely used to produce homogeneous lipid nanoparticles with defined particle sizes, narrow size distributions (PDI < 0.1) and high nucleic acid encapsulation efficiencies (>90%). PNI has developed a new microfluidic architecture designed for high capacity, homogenous mixing of fluid streams to enable the self-assembly of nanoparticles. This next-generation mixer achieves microfluidic mixing using centrifugal force and allows production rates to be scaled by orders of magnitude with a single mixer, while preserving the precise, time invariant microfluidic mixing properties of the previous generation SHM-based mixer. This new architecture is ideally suited for the reproducible scale-up of mRNA-LNP and other challenging nanomedicine formulations. Here, we show experimentally that Luciferase mRNA-LNP prepared using the SHM mixer at 12 mL/min and the high capacity next-generation mixer at 200 mL/min both gave particles of less than 100 nm, with similar physiochemical properties (i.e., polydispersity, mRNA encapsulation efficiency, particle morphology). These mRNA-LNP also possessed similar biological activity in mice (Luciferase Bioluminescence assay) at doses of 1.0, 0.3 and 0.05 mg/kg, which further demonstrates the parity of the SHM and next-generation mixer architectures. These experimental results demonstrate the equivalence of the next-generation and SHM mixers allowing for the seamless scale-up of mRNA-LNP nanoparticle drugs. The high throughput next-generation mixer can be used for the GMP manufacture 1 - 100 g batches mRNA-LNP to support clinical development programs.

784. Exploring Novel Aptamer-Heparin Combinations to Improve Anticoagulation Therapy in Cardiopulmonary Bypass

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Each year, cardiopulmonary bypass (CPB) is used to facilitate cardiac surgical procedures in more than one million patients worldwide. During these procedures, the coagulation cascade is robustly activated hence potent anticoagulation is required to ensure patient safety and procedural success. Unfractionated heparin (UFH) is almost exclusively used to anticoagulate blood during CPB due to its low production cost, high potency and rapid reversibility. UFH works by binding to and enhancing the action of antithrombin (AT), a natural anticoagulant protein that mainly inhibits the coagulation factors thrombin and activated Factor X (FXa). However, the UFH-AT complex is unable to inhibit clot-bound thrombin and platelet-bound FXa leading to continuous activation of both coagulation and inflammatory proteins, exacerbating risk for CPB-associated thrombosis and systemic inflammatory response syndrome. These factors point to a need to circumvent UFH-associated adverse events in order to successfully execute CPB and reduce CPB-associated morbidity. Our research group has explored the use of RNA aptamers as anticoagulants that bind and inhibit coagulation factors with high affinity. Aptamers are highly attractive as therapeutics due to their target specificity and rapid reversibility with complementary oligonucleotides. However, aptamers alone do not provide potent anticoagulation in the CPB setting. Because our anticoagulant aptamers function by binding exosites on their target proteins and inhibiting protein-protein interactions, we reasoned that they could be combined with direct oral anticoagulants (DOACs) or indirect inhibitors, such as UFH, to reach the potent anticoagulation levels required for CPB. In our preliminary studies, we observed that an aptamer and DOACs targeting FX/Xa could be combined to produce robust anticoagulation with lower thrombin generation and inflammatory protein activation in comparison to UFH alone. Following this, we observed that the FX/Xa aptamer greatly potentiates UFH. This dual-mode approach greatly reduces the UFH dose while recapitulating the level of anticoagulation achieved by standard-of-care therapeutic doses of UFH. Interest grew to understand if a similar thrombin generation and inflammatory profile to the DOAC/aptamer combinations could be observed. We hypothesized that these UFH/aptamer combinations would achieve CPB-sufficient anticoagulation while reducing thrombin generation and activation of inflammatory responses, hence abrogating CPB-associated side effects. Here, I show the additive action of the FX/Xa aptamer with sub-therapeutic doses of UFH in *in vitro* and *ex vivo* models of CPB. The aptamer greatly potentiates UFH in the point-of-care coagulation assays, activated clotting time (ACT) and thromboelastography (TEG) assay. UFH doses ten times lower than the therapeutic dose (5U/mL) in combination with saturating doses (2μM) of the FX/Xa aptamer produce anticoagulation that keeps blood clot-free for periods similar to those by the therapeutic dose of UFH. To account for the thrombogenic shear force caused by continuous circulation in CPB, I treated fresh human blood with UFH/aptamer combinations and let it circulate in an *ex vivo* oxygenator circuit. Although lower concentrations were unable to, 2.0 U/mL UFH combined with 2μM

FX/Xa aptamer was able to keep the *ex vivo* circuit patent for 120 minutes. Current studies are focused on assessing whether these UFH/aptamer combinations are capable of reducing thrombin generation and activation of inflammatory responses similar to results observed with the DOAC/aptamer combinations. Results from this study will contribute to the development of a heparin/aptamer combination that will provide potent anticoagulation, rapid reversibility and limited thrombin generation in CPB.

785. Abstract Withdrawn

786. Therapeutic Polymeric Nanoparticles for Tailored Gene Expression and Improved Wound Healing

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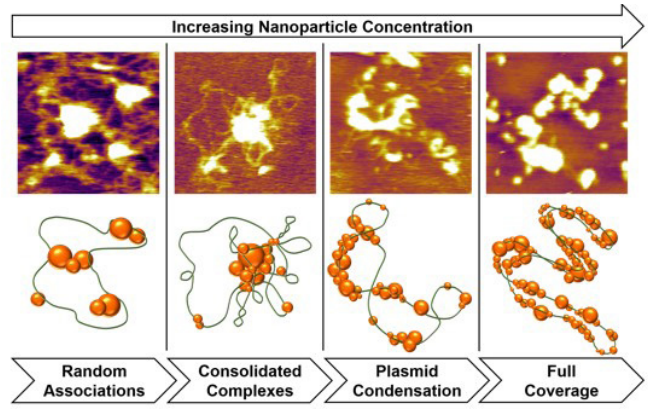
[1]Bioengineering, University of Maryland, College Park, College Park, MD,[2]Surgery, Johns Hopkins School of Medicine, Baltimore, MD,[3]Materials Science and Engineering, University of Maryland, College Park, College Park, MD

Polymeric nanoparticles for the encapsulation of nucleic acids is a popular non-viral gene delivery method; however, this method sets limitations on important characteristics of polymers due to DNA unpacking issues. Additionally, polymers are primarily chosen for their delivery efficiency and biodegradability, and not necessarily for the treatment at hand. In this work, we synthesized therapeutic polymeric nanoparticles using an electrostatic crosslinker. The nanoparticles were created with high molecular weight chitosan and averaged 255 ± 4.8nm in diameter with a 12.11 ± 0.35mV zeta potential. They were used to tangentially adhere to plasmid DNA for gene delivery in the skin. We investigated the in situ effects of nanoparticle concentration on DNA behavior and discovered progressive condensation of plasmids with increasing amounts of nanoparticles in solution. This translated to a unique pattern of gene expression in an in vivo wound healing model. At a low nanoparticle concentration, gene expression was significantly increased for a short-term duration. At a high nanoparticle concentration, expression was prolonged at more moderate levels. Alone, these nanoparticles improved healing in in vivo circular, punch biopsy wound models as animals treated with nanoparticles showed more rapid wound closure compared to an untreated group ($p<0.01$). Using a treatment containing chitosan nanoparticles with a plasmid encoding Hypoxia Inducible Factor-1 in an in vivo ischemic skin flap wound model, there was significant improvement of survival and blood flow to the flap compared to an untreated group ($p<0.001$). This work presents a promising system for gene delivery in the skin for improving wound healing.



Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases II

787. Efficacy and Safety Evaluation of Investigational Liver Gene Transfer for Secretable GAA in the Treatment of Pompe Disease

Sean M. Armour[1], Jayme M. L. Nordin[1], Helena Costa Verdera[2], Daniel M. Cohen[1], Pauline Sellier[2], Marco Crosariol[1], Fanny Collaud[2], Umut Cagin[2], Haley Hanby[1], Francesco Puzzo[2], Virginia Haurigot[1], Giuseppe Ronzitti[2], Pasqualina Colella[2], Xavier M. Anguela[1], Federico Mingozzi[1]

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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 many tissues and results in multi-system pathology. Enzyme replacement therapy (ERT) increases survival, slows disease progression, and is the current standard of care for Pompe disease patients. However, ERT has several significant drawbacks such as limited efficacy, immunogenicity of the recombinant GAA, high cost, and need for frequent lengthy infusions. We have shown that investigational liver-directed adeno-associated viral (AAV) gene therapy expressing a novel secretable GAA transgene results in decreased glycogen accumulation, increased survival, and improvement of cardiac, respiratory, and muscle phenotypes in the *Gaa*$^{-/-}$ mouse model of Pompe disease. We show that secretable GAA outperformed native GAA, even at low doses, as evidenced by enhanced survival and muscle function in Pompe mice. Moreover, secretable GAA vectors demonstrated superior efficacy in restoring *Gaa*$^{-/-}$ mouse muscle strength when compared to the standard of care regimen of 20 mg/kg ERT. In an effort toward clinical translation, we further optimized the expression cassette and selected a highly hepatotropic capsid. A single infusion of the leading clinical candidate, *SPK-3006*, an investigational liver-targeted gene therapy for the treatment of Pompe disease, in non-human primates at three ascending doses demonstrated

dose-dependent expression of GAA in plasma. While pre-clinical efficacy and safety studies are ongoing, no toxicity has been observed in any of the studies to date. These results support moving *SPK-3006* into clinical development.

788. A New Knock-In Mouse Model of mut- MMA: Phenotype and Response to Systemic AAV8 Gene Therapy

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NHGRI, NIH, Bethesda, MD

Methylmalonic acidemia (MMA) is most commonly caused by mutations of the mitochondrial enzyme methylmalonyl-CoA mutase (MUT). In mice, a homozygous exon 3 *Mut* knock-out (*Mut* $^{-/-}$) results in immediate lethality, which has necessitated the construction of tissue specific transgenes that can be bred to knock-out mice to produce viable models of MMA. We have previously developed a transgenic model with a less severe MMA phenotype by creating murine orthologues of mutations that have been defined as less severe (*mut*-) in patients evaluated through our natural history study on MMA, such as p.G717V, a 5'-deoxyadenosylcobalamin K_m MUT mutation. Transgenic mice that ubiquitously express this mutant allele as a stable transgene (*Mut* $^{-/-}$;Tg$^{INS\text{-}CBA\text{-}Mut\ G715V}$) mimic the physiologic and phenotypic manifestations observed in mildly affected MMA patients, but are less severe when compared to other murine models of MMA. This may be attributable to the overexpression of the Mut p.G715V allele from a strong enhancer/promoter (chicken-beta-actin) as a transgene, which can compensate for the reduced activity of the mutant enzyme. We therefore have used genome editing to create an orthologous Mut p.G715V allele, characterized the resulting homozygous mutants (*Mut* $^{p.G715V/p.G715V}$), and assessed the response of the mutant mice to systemic AAV8 gene therapy. *Mut* $^{p.G715V/p.G715V}$ mice were born in Mendelian proportions and exhibited reduced body weight when compared to their wild-type and heterozygous littermates. At 90 days of life, the mutants only achieved 70% of the weight of sex-matched control littermates ($p < 0.0001$). In addition, when maintained on regular mouse chow, the serum metabolites were massively elevated, with plasma methylmalonic acid concentrations ranging between 767-1879 μM, representing a 100-fold or more elevation over age-matched littermates ($p<0.001$). Despite elevated plasma methylmalonic acid levels and growth retardation, *Mut* $^{p.G715V/p.G715V}$ exhibited 100% survival past weaning. Western analysis of mutant liver extracts showed markedly reduced levels of Mut, suggesting that the p.G715V mutant was unstable. *Mut* $^{p.G715V/p.G715V}$ mice also failed to robustly oxidize 1-C-13 propionate compared to age-matched littermates (9.24 ± 2.54% dose oxidized at 25 minutes vs 37.51 ± 11.41% in controls ($p< 0.01$). To test new genomic therapies for MMA, *Mut* $^{p.G715V/p.G715V}$ mice were treated at 40 days with a $2 * 10^{11}$ GC of an AAV8 vector configured to express the human *MUT* gene under the control of the ubiquitous human elongation factor 1-alpha (EF1a) full length promoter, and then were compared to both mutant and control untreated littermates. Within one week, the treated *Mut* $^{p.G715V/p.G715V}$ mice showed a 34.3% average bodyweight increase compared to an average 0.18% increase among sex-matched untreated *Mut* $^{p.G715V/p.G715V}$ mice. In three weeks, they also displayed a decrease in plasma methylmalonic acid levels from 920.2 ± 156.44 μM to 210 ± 12.46 μM. Treated *Mut* $^{p.G715V/p.G715V}$ mice also exhibited a higher 1-C-13 propionate recovery rate at 25 minutes than their untreated age-matched mutant littermates: 25.45 ± 9.76 % vs. 9.24 ± 2.54% ($p<0.01$). Furthermore, the AAV treated *Mut* $^{p.G715V/p.G715V}$ mice regained fertility and appeared more active than the untreated mutants. This new transgene-free mouse model of *Mut* MMA recapitulates the cardinal clinical and biochemical features seen in *mut*- MMA patients. These mice will be useful to explore MMA pathophysiology and to assay the effects of new genomic therapies for MMA, such as AAV gene addition, genome editing, cell therapy, mRNA delivery and microbiome manipulations.

789. AAV9 Intravenous Gene Therapy Enhanced Motor, Cognitive Function and Survival in a Mouse Model of Neuropathic Gaucher Disease

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Gaucher disease (GD) is an autosomal recessive lysosomal storage disorder caused by mutations in *GBA*1 gene, resulting in defective function of acid β-glucosidase (GCase) and subsequent substrate accumulation with progressive deficits in visceral organs and central nervous systems (CNS). Enzyme replacement therapy has been successfully used to correct the visceral disease phenotype. However, there have been no effective treatment options for the CNS manifestations of GD disease and innovative therapies are still needed. Here, we investigate the therapeutic outcome of using human GCase-expressing adeno-associated viral vector serotype 9 (AAV9) to treat both adult (~6 weeks of age) and neonatal (2~3 days of age) mice with neuropathic Gaucher disease (nGD, 4L;C) (GCase^{V394L};SaposinC$^{-/-}$). In adults AAV9 treatment (n=3), intravenous administration of AAV.CB.hGCase.ires.GFP at the dose of 2e13vg led to over 72-fold increase (vs. wild-type levels) of GCase activities in the circulation ~2-week after injection. Using immunofluorescent analysis, we detected widespread gene transfer and transgene expression (GFP signals) in the liver, heart, and kidney with only sporadic signals found in the brain. In addition, no significant improvement was observed in behavior defects with no further extension of lifespan in adult-treated 4L;C mice. The data suggest that AAV9-mediated gene therapy in adult nGD mice is not sufficient to prevent CNS progression with very limited gene transfer in the brain. Conversely, if treated at earlier stage of life, therapeutic vector may be able to mitigate the irreversible organ damage caused by substrate storage and provide a more complete correction of functional defects, especially in the CNS. We intravenously injected the same AAV9 vector at a dose of 5e12vg/mouse into newborn pups (day 2~3, n=5) through superficial temporal vein. AAV treatment prevented body weight loss that we usually observe in untreated 4L;C mice starting at age of day 45. The cerebellar ataxic-like behavior found in 4L;C mice from age of 40 days was normalized after AAV-mediated gene therapy by the hindlimb clasping test. In a repeated open-field

test, the 4L;C mice (50 days and older) displayed significantly less environmental habituation and spent more time exploring the open-field than age-matched WT group, demonstrating short-term memory deficits. However, neonatal AAV treatment completely normalized such behavioral abnormality in 4L;C mice, indicating significant improvement of neurological function. No detectable toxicity was observed in normal controls injected with the same dose of therapeutic vector. Importantly, systemic administration of AAV9.CB.hGCase.ires.GFP in neonatal 4L;C mice resulted in a significant extension of lifespan from 60 days to at least 100 days ($p<0.0001$). These proof-of-concept studies demonstrate the important effects of time window-of-treatment for AAV9-mediated systemic delivery approach on CNS benefits in the treatment of neuropathic Gaucher disease, and support further development to improve AAV-mediated gene delivery and expression in adult-treatment for nGD.

790. Treatment of a Metabolic Liver Disease by In Vivo Genome Base Editing in Adult Mice

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CRISPR/Cas-based genome editing holds great promise for targeting genetic disorders including inborn errors of hepatocyte metabolism. Precise correction of disease-causing mutations in adult tissues *in vivo* however is challenging. It requires repair of Cas9-induced double stranded DNA (dsDNA) breaks by homology-directed mechanisms, which are highly inefficient in non-dividing cells. Here we corrected the disease phenotype of adult phenylalanine hydroxylase *(Pah) enu2* mice, a model for the human autosomal recessive liver disease phenylketonuria (PKU)1 using recently developed CRISPR/Cas-associated base editors. These systems enable conversion of C·G to T·A base pairs and vice versa independent of dsDNA break formation and homology-directed repair (HDR). We engineered and validated an intein-split base editor, which allows splitting the fusion protein into two parts and thereby circumventing the limited cargo capacity of Adeno-associated virus (AAV) vectors. Intravenous injection of AAV-base editor systems resulted in *Pahenu2* gene correction rates that restored physiological blood phenylalanine (L-Phe) levels below 120 μmol/l. We observed mRNA correction rates up to 63%, restoration of phenylalanine hydroxylase (PAH) enzyme activity, and reversion of the light fur phenotype in *Pahenu2* mice. Our findings suggest the feasibility of targeting genetic diseases *in vivo* using AAV-mediated delivery of base editing agents, demonstrating potential for therapeutic application.

791. Treatment of Methylmalonic Acidemia by Targeted Integration of MUT into Albumin Using an Optimized Promoterless AAV Vector

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Methylmalonic acidemia (MMA) is a rare inborn error of metabolism most often caused by mutations in methylmalonyl-CoA mutase (*MUT)*, a mitochondrial-localized enzyme. Patients with MMA suffer from frequent episodes of metabolic instability which accounts for the severe morbidity and early mortality observed in the patients. In addition to dietary protein restriction, liver transplantation is used as a treatment for severe cases and can eliminate metabolic instability. 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, hepatocellular carcinoma was found as a long-term complication in treated, aged mice. To minimize potential vector toxicity and increase the longevity of MUT expression after gene therapy, we have designed a promoterless, AAV vector utilizing homologous recombination to achieve site-specific gene addition of human *MUT* into the mouse (*Alb*) locus, immediately upstream of mouse *Alb* stop codon. This AAV vector, AAVAlb2AMUT2, contains arms of homology flanking a 2A-peptide coding sequence proximal to the *MUT* gene. The endogenous *Alb* promoter drives *MUT* expression after successful integration. We have previously reported that AAV8-Alb-2A-MUT and AAVDJ-Alb-2A-MUT delivered at a dose of 2.5E12 vg/pup or 8.6E11 vg/pup at birth reduced disease-related metabolites, produced durable MUT expression for more than a year following treatment, and was able to rescue the neonatal lethal phenotype exhibited by complete *Mut* knock-out mice ($Mut^{-/-}$). Additionally, we reported that aged treated animals displayed a selective advantage for corrected hepatocytes and showed no evidence of hepatocellular carcinoma. Here, we have endeavored to optimize the AAV-Alb-2A-MUT vector to increase vector integration and improve the phenotype of treated mice. To optimize the vector, we increased the length of the homology arms, changed the AAV ITRs and removed restriction enzyme sequences used for cloning in earlier versions of the vector to create a seamless Alb-2A-MUT junction. Next, we treated a hypomorphic murine model of MMA at birth with this improved AAV-Alb-2A-MUT-2 vector (dose of 2.5E11 vg/pup) and observed significantly reduced levels of methylmalonic acid in treated mice; 1769.5 μM for untreated mice vs. 1313.8 μM for treated mice ($p < 0.05$; n=57 and 18 respectively). Additionally, treated mice displayed a trend of improved weight gain beginning at 3 months compared to untreated mice; on average, +2.5 g for treated mice (n= 22 and 15 respectively). Interestingly, both parameters show improvement with increasing age, suggesting that Alb edited hepatocytes may experience a selective growth advantage after correction. To confirm this hypothesis, we plan to examine liver tissue from treated mice at 1-month, 2-months, 3-months, 4-months, 5-months, and 6-months post-treatment to further characterize the correction time course of expansion of *Alb* edited hepatocytes.

792. Abstract Withdrawn

793. In Vivo glycolate Oxidase Disruption by AAV-Mediated Delivery of Paired S. Aureus Cas9 Nickase to Treat Primary Hiperoxaluria

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Primary hyperoxaluria type I (PH1) is an inborn error of glyoxylate metabolism characterized by oxalate overproduction in the liver and formation of calcium oxalate crystals and stones in the kidneys. The only curative treatment for PH1 is liver transplantation, so there is an urgent need to develop therapeutic alternatives for this disease. The inhibition of glicolate oxidase (GO), an enzyme implicated in oxalate formation in the liver, has been demonstrated to be an efficacious substrate reduction therapy (SRT) for PH1. We have recently shown that AAV-mediated expression of *Staphylococcus aureus* Cas9 (SaCas9) in combination with a unique sgRNA to target *Hao1* gene (coding for GO) leads to the complete elimination of GO expression in the liver and to the amelioration of PH1 phenotype and kidney damage. Although no off-target effects were observed, the expression of an endonuclease capable of generating double-strand breaks (DSB) remains still a concern. In this work, with the aim of increasing the safety of this approach we have used a nickase SaCas9 (nSaCas9) together with two sgRNAs targeting nearby regions of the exon 2 of *Hao1* gene in PH1 mice. While AAV-mediated delivery of nSaCas9 with a single sgRNAs has no effect over GO expression, the administration of 2 sgRNAs located 64bp apart and in PAM-out position was capable of disrupting *Hao1* gene in the liver of PH1 mice. Indels were detected in a high percentage of *Hao1* alleles, which led to a decrease in *Hao1* mRNA and a complete inhibition of the expression GO protein. NGS analysis is being performed to analyze on-target modifications. Finally, functional analysis will be performed in order to verify that the new and safer nickase approach remains therapeutically relevant. In conclusion, we have demonstrated for the first time that an AAV-delivered nickase Cas9 in presence of two sgRNAs generates indels efficiently and decreases expression of the target gene in vivo. The therapeutic efficacy of this approach is under evaluation.

794. Liver-Targeted AAV Gene Therapy Vectors Produced at Clinical Scale Result in High, Continuous Therapeutic Levels of α-GalA Enzyme Activity and Effective Substrate Reduction in a Mouse Model of Fabry Disease

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Fabry disease (FD), an X-linked lysosomal storage disease, is caused by mutations in the *GLA* gene encoding the α-galactosidase A (α-GalA) enzyme. FD is characterized by progressive systemic accumulation of the enzyme's substrates, globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3), in blood and tissues leading to renal, cardiac and/or cerebrovascular disease and culminating in premature demise. The current standard of care is enzyme replacement therapy (ERT). However, ERT requires a lifetime of biweekly infusions and may not clear all substrate from tissues. A more effective and long-lasting treatment would benefit FD patients. An AAV-mediated, liver-targeted gene therapy approach was evaluated in a knock-out mouse model (GLAKO) of Fabry disease that lacks α-GalA activity and accumulates high levels of substrates in plasma and tissues. This strategy employs an episomal AAV (serotype 2/6) vector encoding human *GLA* cDNA (h*GLA*) driven by a liver-specific promoter and manufactured using a clinical scale production method. One-time administration of the AAV-h*GLA* vector leads to hepatic production and secretion of α-GalA into the bloodstream, where the enzyme can be taken up by tissues via mannose 6-phosphate receptor mediated endocytosis. In a 3-month pharmacology and toxicology study, one-time intravenous administration of increasing amounts of AAV-h*GLA* vector to GLAKO mice (age 2-3 months) was well tolerated and resulted in supraphysiological expression of plasma α-GalA (over 300-fold of normal levels) that was maintained for the duration of the study. Dose-dependent increases in tissue α-GalA activity were achieved in liver, heart and kidney. At the end of the study, Gb3 and lyso-Gb3 content was quantified in plasma and tissues via mass spectrometry. GLAKO mice in the high dose group generally had undetectable levels of Gb3 in the plasma, liver, spleen and heart and below 10% of substrate remaining in kidney compared to untreated GLAKO mice. This initial AAV-h*GLA* vector was compared to an improved vector, designated ST-920, containing an additional regulatory element in the non-coding sequence. Both vectors were manufactured using a clinical scale production method and compared in a 28-day study in wild type C57BL/6 mice. ST-920 was well tolerated and produced up to 7-fold higher levels of plasma α-GalA activity than mice administered the same dose of the initial vector. In this study, a single administration of ST-920 was able to achieve plasma α-GalA activity over 1,500-fold of normal levels. The high levels of α-GalA activity seen in these studies, along with the concomitant marked reduction in the accumulated Gb3/lyso-Gb3 in key tissues of the GLAKO mouse model, provide preclinical proof-of-concept for AAV-mediated targeting of hepatocytes to express

therapeutic levels of human α-GalA. The clinical scale manufacturing process developed for these studies will enable rapid production of clinical-grade AAV material.

795. In vivo Genome Editing to Treat Methylmalonic Acidemia (MMA) Caused by Methylmalonyl-CoA Mutase Deficiency (hMUT)

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Methylmalonic acidemia (MMA) is an autosomal recessive metabolic disorder, most commonly caused by mutations in the mitochondrial enzyme methylmalonyl-CoA mutase (*MUT*). Despite advancement in the understanding of disease pathophysiology and resulting improvements to standard of care, patients with *MUT* class MMA continue to exhibit severe morbidity and high rates of mortality. A transgenic murine model of MMA in which Mut is expressed only in the liver demonstrates that restoring enzyme activity in hepatic tissue can rescue knockout mice from neonatal lethality and restore growth to near normal levels. Gene therapy using adeno-associated viral vectors (AAV) targeting *Mut* expression to the liver has demonstrated similar results. However, a long term complication of AAV treatment in mice, including those with MMA, has revealed a significant risk for hepatocellular carcinoma based on timing of therapy, the dose and the promoter/enhancer configuration of the vector. Genome editing using the CRISPR/Cas9 system represents an alternative approach for the *in vivo* therapy of MMA, either at the *Mut* locus, or via the insertion of the corrective gene in a safe harbor. As a first step, we have identified target sites in the *Mut* gene and in the safe harbor locus albumin (*Alb*). *In vitro* screening of the *Staphylococcus aureus* Cas9 (SaCas9) and guide cassettes showed measurable NHEJ activity at the *Mut* and *Alb* loci in Neuro-2A cells. Next, AAV serotype 8 vectors were produced that express the respective gRNAs and SaCas9 under expression of a liver-specific promoter for assessment of *in vivo* activity. Screening of *Staphylococcus aureus* Cas9 and guide RNA cassette showed measurable indel formation (25.5% ± 5.37 n=4) at the *Mut* and (32.5% ± 1.36, n=3) at the *Alb* loci by NGS in an MMA mouse model, when treated with the corresponding guide RNA. A dual vector system including the SaCas9/gRNA and a rescue cassette containing codon-optimized *Mut*, configured to correct at either the *Mut* locus or *Alb,* has been delivered to MMA mice that will be aged prior to sacrifice. This strategy is modular and might be easily applied to other inborn errors of metabolism, particularly those for which correction of diseased hepatocytes results in an easily measurable clinical or metabolic phenotype.

796. Skeletal Muscle-Directed FGF21 Gene Therapy Counteracts Obesity and Type 2 Diabetes

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The prevalence of type 2 diabetes (T2D) and obesity is increasing worldwide. Currently available therapies are not suited for all patients in the heterogeneous obese/T2D population, and there is a need for novel treatments. Fibroblast growth factor 21 (FGF21) is considered a promising therapeutic agent for T2D/obesity. Native FGF21 has, however, poor pharmacokinetic properties, making gene therapy an attractive strategy to achieve sustained circulating levels of this protein. The aim of this study was to evaluate the anti-obesogenic and anti-diabetic effects and the safety of a gene therapy approach based on the overexpression of native FGF21 in skeletal muscle. Treatment of adult mice, fed a high-fat diet for a long time, with AAV-FGF21 vectors resulted in marked increase in circulating FGF21 levels, which was parallel to high expression of vector-derived FGF21 in skeletal muscle. In contrast to HFD-fed null-injected animals, mice treated with AAV-FGF21 showed marked reductions in body weight, adipose tissue hypertrophy and inflammation, hepatic steatosis, inflammation and fibrosis and insulin resistance. Additionally, treatment with AAV-FGF21 vectors reduced the incidence of liver neoplasms associated to long-term-HFD-feeding. Moreover, improvements in healthspan in another cohort of mice treated with AAV-FGF21 vectors when aged 1-year was also observed. Altogether, these results demonstrate that AAV-mediated overexpression of FGF21 in skeletal muscle leads to sustained secretion of therapeutically-relevant levels of this factor to the bloodstream and highlight the therapeutic potential of this approach to expand healthspan as well as to treat T2D and obesity in the future.

797. mRNA Therapy for the Treatment of Glycogen Storage Disease Type 1a (GSD1a)

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Glycogen Storage Disease 1a (GSD1a) is a rare inherited metabolic disorder caused by deficiency of glucose 6-phosphatase (G6Pase). G6Pase is an endoplasmic reticulum (ER) membrane protein required

for maintaining interprandial euglycemia, primarily expressed in liver and kidney. GSD1a patients exhibit life-threatening hypoglycemia, severe metabolic complications, and long-term liver/renal disease. There is no FDA-approved treatment for GSD1a and the current standard-of-care for managing hypoglycemia (uncooked cornstarch diet) has patient compliance issues and fails to prevent long-term complications associated with G6Pase deficiency. Enzyme replacement and gene therapy-based approaches are not an option for patients due to drug-delivery and efficacy/safety considerations. Current viral-based gene therapy approaches to correct G6Pase deficiency, have proven unsuccessful due to neutralizing antibodies and genotoxicity. To develop a new treatment for GSD1a, we encapsulated nucleoside-modified, codon-optimized mRNAs encoding human G6Pase in lipid nanoparticles (*hG6Pase-mRNA-LNPs*). Protein engineering technology was employed to further enhance the expression and enzymatic activity of the resulting G6Pase protein. We demonstrate the therapeutic efficacy of *hG6Pase*-mRNA-LNP therapy in human cells in culture 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 11 days post-dose, along with dose-dependent improvement in serum and hepatic biomarkers. In conclusion, our results strongly suggest that mRNA-LNP therapy could have a significant therapeutic impact on the treatment of GSD1a and potentially to other enzymopathies with limited treatment options.

798. Controlled Gene Expression in the Liver; Design of Constitutive, Inducible and Repressible Synthetic Promoters for use in Gene Medicine and Their In Vivo Validation

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Effective treatment of genetic diseases by gene therapy requires careful consideration of many factors, not least the expression of the therapeutic transgene in the target tissue at the appropriate level and longevity. To ensure the transgene expresses at only the target tissue great efforts have been made to develop tissue specific promoters. Furthermore, there is a growing demand for ever stronger, smaller and more stable promoters that retain this tissue specificity. More recently there has been significant interest in achieving greater control of gene expression through tissue specific inducible and repressible promoters. This is paramount for gene therapy as having control over expression and timing of the gene of interest will increase the number of diseases that can be treated, while increasing efficacy and safety. For example, in gene editing, expression of the editing construct is required for only a short period, enough time to perform targeted editing but preventing off-target effects. It is also desirable that inducible or repressible promoters are single component, i.e., drug activated with no requirement for the co-expression of a trans-activator. This would allow the control elements to be packaged in a single AAV vector, reducing the costs of manufacture and overcoming problems associated with 2 component systems. The need for potent, stable and small tissue specific promoters, whether constitutive, inducible or repressible, has coincided with the development of high-throughput sequencing techniques. These techniques generate a large amount of genomic data which makes it possible to mine the genome for regulatory elements that can used to design synthetic promoters. Using this and in-house data we have developed a promoter design platform, PromPT© that integrates large-scale functional genomics datasets with machine learning algorithms to identify functional regulatory elements. These regulatory elements are then ranked via multiple distinct criteria and subsequently used as component parts in synthetic promoter construction. Here we show that by using PromPT© we can create novel constitutive, inducible and repressible liver selective promoters. *In vitro* and *in vivo* analysis of constitutive promoters developed using our platform have a large dynamic range and show activities many times higher than currently used promoters. In addition, they are highly selective and small therefore fulfilling the criteria required for use in therapeutic constructs. The novel inducible and repressible promoters that we have developed are small, tightly controlled and responsive to chemical stimuli, using either FDA approved or GRAS status chemicals. *In vitro* and *in vivo* analysis of these promoters demonstrated that they were liver specific and had a large dynamic range of >20-fold. The inducible promoters had low levels of background whereas the repressible promoters had high constitutive activity. Both showed high induction/repression and rapid speed of response (9hrs). In summary, we have exemplified a platform for generating novel constitutive, inducible and repressible tissue selective promoters which enable exquisite control of the gene of interest.

799. AAV6-Mediated Choroid Plexus-Targeted Gene Therapy for Alpha-Mannosidosis

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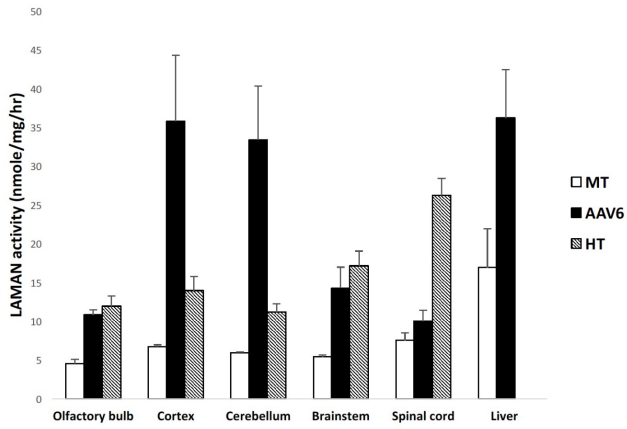
The choroid plexuses (CP) are highly vascularized structures that project into the ventricles of the vertebrate brain. The polarized epithelia of the CP produce cerebrospinal fluid by transporting water and ions into the ventricles from the blood and normally secrete a large number of proteins. Here, we update our work assessing the feasibility of targeting CP epithelia with rAAV gene therapy vectors for treatment of lysosomal storage disease (LSD). Patients with LSDs are unable to metabolize various nutrients, resulting in diminished lifespans and reduced quality of life. There are no ideal or complete 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. Transplantation with native or genetically-modified stem cells has also been carried out for other LSDs in which there is neurological involvement, however this approach requires bone marrow ablation and is associated with significant morbidity and mortality. Furthermore, many LSD patients who are transplanted do not attain normal neurodevelopment, related to inefficient delivery to the CNS. In contrast, rAAV-mediated gene

transfer of missing lysosomal enzymes to CP epithelia would be safer and 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, 2) rAAV transduction of non-dividing cells results in sustained episomal transgene expression, 3) CSF flow extends into the subarachnoid fluid compartment from where proteins (estimated diameter 5nm) can ultimately reach the entire brain via Virchow-Robin spaces, the paravascular, CSF-filled canals surrounding perforating arteries in the brain parenchyma, 4) 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), and 5) 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. Alpha-mannosidosis (AMD) is an autosomal recessive inherited lysosomal storage disorder characterized by cerebellar ataxia, neurocognitive disability, facial and skeletal abnormalities, hearing impairment, and mild immune deficiency. Elevated urinary secretion of mannose-rich oligosaccharides is suggestive of the diagnosis and can be confirmed by genetic testing (*MAN2B1* gene mutation analysis). The onset of symptoms is in early childhood and ataxia, neuromuscular weakness, skeletal deterioration, and neurocognitive decline progress over several decades, rendering most patients wheel-chair dependent and with limited independent living skills. In a reliable mouse model of AMD, we measured LAMAN enzyme levels in brain and peripheral organs after choroid plexus-targeted rAAV6 treatment. Our findings indicate that LAMAN activity increased in brain globally as well as in the liver following 5E+9 vector genomes per mouse to the CSF (Figure). Choroid plexus-targeted rAAV6-LAMAN represents a promising approach to the neurocognitive and systemic aspects of AMD.



800. Gene Therapy and Cell Modeling for MPS VI with Engineered Human IPSC Derived Chondrogenic Cells

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Mucopolysaccaridosis type VI (MPS VI) is a severe metabolic disease caused by deficiency of the lysosomal enzyme N-acetyl galactosamine 4-sulfatase (arylsulfatase B, ARSB). This results in lysosomal accumulation of the glycosaminoglycans (GAGs) dermatan sulfate and chondroitin 4-6 sulfate in various connective tissues and organs, including cartilage. The current available treatment consists of intravenously applied enzyme replacement therapy (ERT) but this treatment is only partlially effective. In particular, the therapeutic efficacy is low in cartilage due to its poor vascularizion and difficulty to be reached via the circulation. Therefore, despite ERT MPS VI patients develop major musculoskeletal problems like contractures of the joints and short stature. This warrants the development of novel therapies to address these symptoms. We have applied the CRISPR/cas9 technology to correct the genetic defect in MPS VI patient-derived iPSCs. Insertion of a copy of the *ARSB* cDNA into the *AAVS1* safe harbour resulted in strong overexpression of the ARSB enzyme (6-9 fold over healthy controls). The iPSCs were differentiated in vitro to chondrogenic cells and these expressed hallmarks of chondrocytes. Immunofluorescent staining of the key chondrogenic transcription factor Sox9 showed expression in >90% of cells after differentiation. RT-qPCR analysis showed elevated expression of chondrogenic factors including Collagen II (10 to 24 fold increase), Sox5 (10 to 20 fold increase) and Sox9 (5 to 10 fold increase), with some variation between patient lines but little difference between isogenic pairs. Furthermore, we showed that chondrocytes derived from MPS VI patients displayed disease-specific hallmarks in vitro and that these were normalized following CRISPR/Cas9 mediated gene correction.The MPS VI disease model will not only be instrumental for understanding the disease mechanisms, but it also provides a tool for testing and developing novel therapeutic approaches toward the cartilage symptoms in MPS VI.

801. A Single Injection of an Optimized AAV Vector into Cerebrospinal Fluid Prevents Neurological Disease in a Murine Model of GM1 Gangliosidosis

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GM1 gangliosidosis is a rare lysosomal storage disease caused by loss-of-function mutations in the gene encoding beta galactosidase (β-gal). The reduced activity of β-gal prevents degradation of GM1 ganglioside, leading to progressive neuronal GM1 storage and cell death, with associated neurological decline. There are no approved treatments for GM1 gangliosidosis. Previous studies in animal models of GM1 gangliosidosis have demonstrated that adeno-associated viral (AAV) vector-mediated gene transfer to the brain can restore β-gal expression and prevent onset of neurological disease. Here, we developed an optimized AAV vector expressing human β-gal, and evaluated the efficacy of a single injection of this vector into the cerebrospinal fluid (CSF) of a murine disease model. AAV vector administration into the CSF increased β-gal activity in the brain, reduced neuronal lysosomal storage lesions, and prevented onset of the gait abnormalities typically observed in this model. These findings demonstrate the potential therapeutic activity of this vector and support subsequent clinical development for the treatment of GM1 gangliosidosis.

802. Comparison of PAH and PAL for Efficacy in the PAH^{enu2} Mice, a Model for Human Phenylketonuria

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Phenylketonuria (PKU) is a genetic deficiency of phenylalanine hydroxylase (PAH) in liver and results in elevated levels of phenylalanine (Phe) in blood and brain and subsequent neurotoxicity. Currently, patients are managed with Phe restricted diet and medical formulas. Compliance among teen and adult patients is poor and patients suffer from neurological and neuropsychological symptoms which affect quality of life. Recently, pegylated phenylalanine ammonia lyase (PEG-PAL) that converts Phe into trans-cinnamic acid rather than the normal PAH-based conversion into tyrosine (Tyr), was approved as a Phe reduction strategy. To better understand the pros and cons of these two therapeutic strategies, we generated recombinant adeno-associated virus 8 (rAAV8) vectors expressing either mouse PAH or *Anabaena variabilis* PAL driven by a liver promoter. The vectors were evaluated in PAH^{enu2} mice, a model for human PKU, and fed with a normal rodent diet. Our results demonstrated that both transgenes efficiently reduced blood and brain Phe levels after single vector administration. However, unlike PAH, PAL overcorrected the blood Phe levels to levels lower than in control mice (heterozygous PAH$^{enu2+/-}$ mice) and did not elevate blood or brain Tyr levels. Both treatments normalized brain tryptophan (Trp) levels as well as neurotransmitter dopamine and serotonin levels (synthesized from Tyr and Trp, respectively) to levels observed in control mice. Furthermore, animal behavior assessed by a nest building assay was normalized by both treatments. Our results support the hypothesis that Phe toxicity and blocking of amino acid transport to brain may account for neurotoxicity rather than reduction in Tyr levels. We also found that intracellular PAL expression resulted in an immune response with accumulation of inflammatory cells in the liver of all PAL treated animals. Analyses of CNS myelination and transcriptional and proteomics profiling are ongoing. In summary, our work demonstrates the importance of Phe reduction as a major therapeutic approach for the treatment of PKU. Safety considerations such as Phe-regulated enzyme activity and immune response to PAL support the restoration of normal PAH-based Phe metabolism in the liver.

803. Gene Correction Efficacy for Pompe Disease, a Lysosomal Storage Disease

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Introduction: For many inherited disorders, genomic editing of endogenous mutations in vivo is a potential treatment, although challenges include safety, precision, delivery and efficacy. This may be exacerbated when a suitable in vitro or animal model does not exist, especially in the case for modeling specific patient mutations. Here we describe a combined computational and experimental model to study the dosing, delivery, efficacy and potency of gene correction to treat the glycogen storage phenotype of Pompe disease. **Results and Discussion**: Using CRISPR-Cas9 genome editing, we demonstrate gene correction of two different mutant *GAA* alleles in an autosomal recessive, patient derived induced pluripotent cell (iPSC) line. Edited cells had normal karyotypes, no detectable off-target mutations or large genetic deletions, and lower amounts of abnormal glycogen storage. Enzymatic cross-correction of the diseased cells from GAA produced within edited cells was robust, and this enzymatic cross-correction was equivalent to standard enzyme replacement therapy (ERT). We further show that correction of both mutant alleles was more effective at rescuing the disease phenotype. Computational tools additionally provide insight into key parameters that control somatic processes at scales larger than cells and small tissue constructs - those involving in vivo genome editor delivery, physiology and tissue crosstalk. Computational modeling for the *in vivo* delivery of these genome editors to the livers of Pompe patients confirmed the efficacy of disease rescue for precise genome editors with six serial doses, as compared to long-term biweekly ERT. Progenitor cell targeting, delivery efficiencies, and suppression of imprecise editing outcomes were identified as key parameters controlling efficacy. This work establishes new quantitative design rules for somatic cell genome editing, and that precise correction

using serial dosing of genome editors to correct multiple mutated alleles may be efficacious in the treatment of polygenic disorders. Overall, both experimental and computational approaches showed the utility of a gene correction approach and highlighted the importance of considering targeting, editing efficiency. New quantitative relationships between editing efficiency and phenotype rescue as well as gene editing precision and delivery are further established in relation to the best available treatment.

804. Modeling Parameters for Optimal AAV Vector Mediated Liver Gene Transfer in a Severe Neonatal Mouse Model of Crigler-Najjar Syndrome

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Recombinant adeno-associated virus (AAV) vectors are well-recognized gene delivery systems for gene replacement therapy of monogenic liver disorders, having excellent benefit-risk ratio as demonstrated in clinical trials for haemophilia and other diseases. 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 due to possible loss of efficacy of the therapy in the long-term. In addition, re-administration of the therapeutic vector later in life is hampered by the production of anti-AAV neutralizing antibodies (NAbs), generated after the first administration. Thus, the identification of key parameters driving the duration of transgene expression following AAV-mediated liver gene transfer in pediatric subjects requires further definition before its translation to the clinic. To this aim, and to achieve safe and long-lasting efficacy of AAV vector-mediated gene transfer in a developing liver, we used neonatal and juvenile Ugt1$^{-/-}$ mice, a well-established animal model for Crigler-Najjar syndrome, closely mimicking the major features of the human disease. An AAV8-hAAT-hUgt1a1 vector was administered to mice at different developmental ages (P4, P11 and P18) and at different doses (from 5.0E11 to 5E12 vg/kg). Animals were followed short-term (1 month) and long-term (7 months) post treatment. No adverse effects were observed in the animals injected at any dose tested. Histological analysis of livers 7 months post-injections showed no major abnormalities in treated mice compared to their controls. Results obtained showed that lower ages showed faster loss of efficacy, a condition that was compensated to some extent by higher doses of vector. Total loss of efficacy was observed in the very early time point (P4) at any dose tested. Moreover, generation of anti-AAV8 neutralizing antibodies was negligible in neonatal mice and significantly increased over time in juvenile animals. As the developmental state of the liver is a critical parameter for the persistence of AAV vector genomes and their expression, the proliferative and pluripotency profile of the liver was assessed at the different ages selected for vector administration (P4, P11 and P18) and compared to the mature liver (P30, P60 and P120). mRNA, protein and immunofluorescence analysis of several markers involved in liver proliferation, pluripotency and maturation (polyipolidization) were measured and correlated with the degree of AAV-mediated gene correction, defining predictive molecular markers of transgene expression persistence. In conclusion, our work establishes the safety and efficacy of gene transfer for Crigler-Najjar syndrome in a relevant animal model of the disease. This work provides guidance for the translation of AAV vector-mediated gene transfer for Crigler-Najjar syndrome to the pediatric patient population.

805. Lentiviral-Vector-Mediated Gene Therapy for Metachromatic Leukodystrophy Decreases Sulfatide Accumulation in the CNS

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Objectives Late-infantile Metachromatic Leukodystrophy (MLD) is an inherited lysosomal storage disease. It is characterized by deficient arylsulfatase A (ARSA) activity resulting in sulfatide accumulation in myelin producing cells. The most common clinical presentation of MLD is late infantile onset between 1-2 yr. with weakness, hypotonia, plateauing and loss of mental development, seizures, loss of vision and hearing and ultimately death within about 5 yr. The pathogenesis of MLD is based on the inability to metabolize the glycolipid sulfatide that is a prevalent lipid in myelin of both the central and peripheral nervous systems. Consequently, in MLD sulfatide accumulates in lysosomes and lysosome-derived vesicles resulting in cellular death and tissue dysfunction. Intravenous enzyme replacement therapies have been unsuccessful as treatment for MLD because of restricted transfer across the BBB. We propose the use of single intracerebral ventricular injection of lentiviral-vector codon-optimized human ARSA to decrease sulfatide accumulation and act as a therapeutic for MLD. MethodsWe designed a lentiviral vector incorporating codon-optimized human ARSA cDNA and green fluorescent protein (GFP) driven by the mammalian elongation factor 1 a (EF-1α) promoter. Stereotaxic injection to the lateral ventricle (2-sided, dual injection) of the mouse was performed in ARSA deficient mouse models. Mice were transduced with ARSA (n=6) or GFP (n=6) vectors. 4-6 weeks post injection mice were sacrificed, and tissues were harvested to assess vector transduction and measure sulfatide concentration. ResultsOur data indicates that intracerebroventricular delivery of a lentiviral vector expressing ARSA (LV-ARSA) transduces the choroid plexus and ependymal cells that line the CSF compartment and expresses ARSA within about 4 days of delivery. The concentration of cerebellar sulfatide which is the principal endpoint is normalized in about 1 month. Conclusion We demonstrate correction of sulfatide accumulation in ARSA-deficient mice through lentiviral vector

806. AAV-Mediated Gene Therapy for Aldehyde Dehydrogenase 2 Deficiency Reduces Acetaldehyde-Related DNA Adduct and Damage of Esophagus

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Aldehyde dehydrogenase 2 (ALDH2) deficiency affects 8% of the world population and 35 to 40% of the East Asian population. ALDH2 is a key enzyme in ethanol metabolism. The most common genetic

variant, the ALDH2*2 allele, results from a glutamic acid-to-lysine substitution at position 487 (E487K). The mutation reduces the oxidizing ability of the enzyme; with ethanol ingestion, the result is systemic accumulation of toxic acetaldehyde. Importantly, ALDH2*2 heterozygotes have a 7 to 12-fold increased risk for development of esophageal cancer, a risk enhanced by smoking and drinking alcohol. In mouse models of ALDH2 deficiency, chronic ethanol consumption induces DNA damage and accumulation of DNA adducts in the esophagus, abnormalities relevant to increased risk for cancer. We hypothesized that a one-time administration of an adeno-associated virus (AAV) serotype rh.10 gene transfer vector expressing the coding sequence of the human ALDH2 (AAVrh.10hALDH2) would correct the ALDH2 deficiency state on a persistent basis, preventing systemic accumulation of acetaldehyde, and esophageal accumulation of DNA adducts and damage caused by chronic ethanol ingestion. To assess this hypothesis, AAVrh.10hALDH2 was administered intravenously at 10^{11} genome copies (gc) to 6-10 wk old ALDH2 knockout homozygous ($ALDH2^{-/-}$) and knockin homozygous ($ALDH2^{E487K+/+}$) male mice (n=10 per group). Four wk after vector administration, mice were given 10% ethanol in the drinking water for 12 wk. Systemic serum acetaldehyde was quantified by liquid chromatography/mass spectrometry (LC/MS). Esophagus DNA adducts were analyzed by measuring N^2-ethyl-deoxyguanosine (N^2-et-dG) by LC/MS, and DNA damage by gamma-H2A histone family member X (gH2AX) immunohistochemistry. After 12 wk chronic ethanol ingestion, compared to non-ethanol drinking littermates, untreated $ALDH2^{-/-}$ and $ALDH2^{E487K+/+}$ mice had decreased body weight and blood hemoglobin levels, performed poorly in a Rotorod test of locomotor activity, had elevated serum acetaldehyde levels and increased esophageal N^2-et-dG adducts and gH2AX positive cells (all $p<0.05$). Strikingly, the AAVrh.10hALDH2-treated $ALDH2^{-/-}$ and $ALDH2^{E487K+/+}$ chronic ethanol-ingesting mice had correction of all of these abnormalities, with increased body weight, normalized hemoglobin levels, enhanced Rotorod performance, reduced serum acetaldehyde levels, and lowered esophageal DNA adduct formation and DNA damage (all $p<0.03$). In summary, the data demonstrate that, in 2 murine models of ALDH2 deficiency, *in vivo* AAV-mediated ALDH2 therapy reduces chronic ethanol ingestion-induced systemic elevation of acetaldehyde levels and prevents abnormalities associated with elevated acetaldehyde levels, including loss of body weight, bone marrow suppression, accumulation of DNA adducts and damage in the esophagus, both precursors of chronic ethanol-induced esophageal cancer. AAVrh.10hALDH2-mediated therapy should be considered as a preventative therapy for the serious systemic consequences of ALDH2 deficiency associated with chronic alcohol exposure.

807. Phenylalanine Hydroxylase (PAH) Liver Distribution and Characterization Following AAV5-hPAH Gene Therapy in Pah^{enu2} Mice

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Phenylketonuria (PKU) is causally linked to the deficiency of phenylalanine hydroxylase (PAH), and characterized by hyperphenylalaninemia, and neurocognitive and neuropsychiatric deficits. PAH metabolizes phenylalanine to tyrosine, which acts as a precursor for downstream metabolites for growth and neurotransmitters. Recent advances in adeno associated virus (AAV) based gene therapy provide an opportunity for sustained amelioration of the PKU phenotype by correction the PAH deficiency. The ENU2 mouse model is a well characterized mouse model of classical PKU, and the effects of AAV5-PAH gene therapy were assessed in this model. Experiments outlined below sought to address the effects of different doses of AAV5-PAH on 1) Bio-distribution of AAV5-PAH vector genomes and transgene derived PAH protein in liver tissue. 2) Effects of AAV5-PAH gene therapy and transgene derived PAH protein on liver safety. AAV5-human PAH (hPAH) vectors were administered to Pah^{enu2} male mice at 2E13 and 2E14 vector genomes per kilogram body weight (vg/kg) via tail vein injection. Mice were euthanized 12 weeks post-vector administration, and perfused with saline prior to liver collection. Liver bio-distribution of AAV5-hPAH vector DNA and hPAH protein was evaluated using in situ hybridization and IHC techniques, respectively. A dose-dependent increase in the number of hepatocytes staining positive for both hPAH DNA and protein was detected. At the 2E14 vg/kg, 52.4 ± 4.6% of hepatocytes stained positive for hPAH DNA and 32.7 ± 11.4% of hepatocytes stained positive for hPAH protein. Overall, there was a greater abundance of both vector DNA and protein in the peri-central regions of the liver lobule when compared to the peri-portal region, which is consistent with what has been observed in mice using AAV5 for gene delivery. To assess liver health following AAV5-hPAH administration, comprehensive analyses consisting of histopathological examination as well as TUNEL and activation of the monocytic cell lineage were performed. To evaluate cell death, the enzymatic assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was used to detect DNA fragmentation. Quantitative analysis of TUNEL positive hepatocytes showed no significant increase in apoptotic cell death in any of the gene therapy-treated groups when compared to the vehicle control group. Immunohistochemistry (IHC) to detect macrophages using the markers IBA1 (total) and CD68 (M1/M2 activated) confirmed a slight increase in the total number of IBA-1 and CD68 positive monocytic cells in the 2E14 vg/kg group. However, there was no significant staining of iNOS (M1 activated), a pro-inflammatory macrophage marker. In addition, such immunohistologic features as hepatocellular necrosis, kupffer cell hyperplasia, and portal triad inflammation or fibrosis were not observed suggesting that liver health is preserved following AAV5-hPAH transduction. In conclusion, there was no hepatic

histopathological evidence of increased cell death or marked signs of inflammation following AAV5-hPAH vector administration at doses up to 2 E14 vg/kg. Bio-distribution of human PAH transgene and protein is dose dependent with the majority of hepatocytes transduced at high dose. These data support that AAV5-mediated gene delivery of hPAH may be a viable option as a potential therapeutic for PKU.

808. CRISPR-Cas9 Generated Pompe Knock-In Murine Model Exhibits Early-Onset Cardiac Hypertrophy and Motor Impairment

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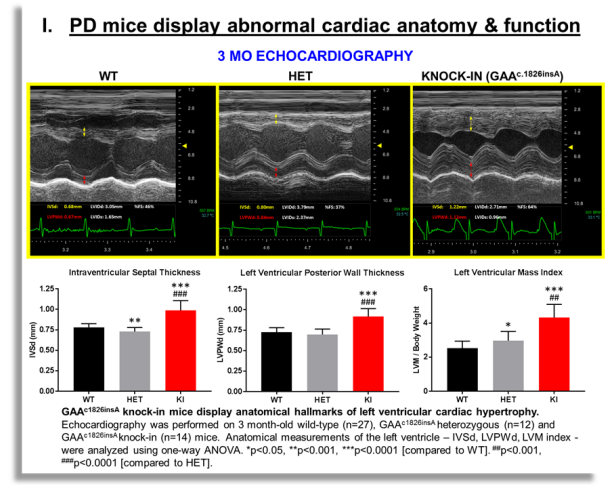
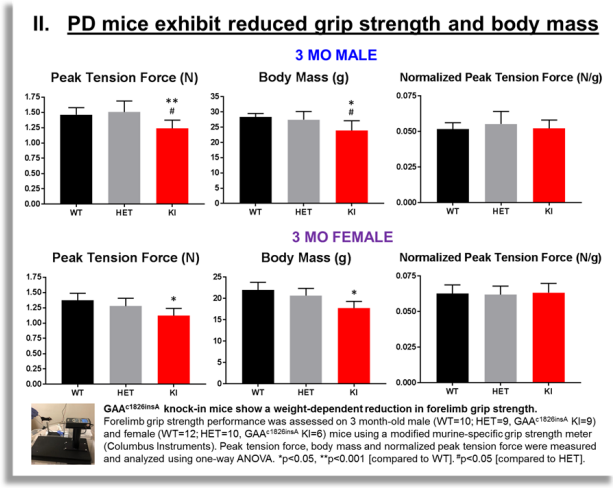
The goal of this study is to generate and characterize a knock-in model of Pompe disease (PD) - a rare, progressive, fatal disorder primarily affecting the cardiac and musculoskeletal systems. While a murine model of PD exists, it bears a Cre/loxP induced exonic insertion of a neomycin cassette and does not completely recapitulate severe human PD - displaying nonfatal hypertrophic cardiomyopathy only late in its natural history. We therefore designed a CRISPR-Cas9 knock-in system targeting the Gaa gene to introduce the known pathogenic CRIM negative Gaa mutation c.1826insA (p.Y609*). Following optimization of our knock-in strategy in cultured murine myoblasts, we successfully generated a Gaa^{c1826insA} mouse model using a dual sgRNA with ssODN donor template approach. Whole genome sequencing and analysis of the Gaa^{c1826insA} murine model establishes that our system is highly specific for the Gaa^{c1826} target locus and does not induce any off-target mutations or genomic rearrangements. Next, we examined GAA mRNA transcript, protein expression and enzymatic activity levels in our PD knock-in mice. Gaa^{c1826insA} mice display significantly reduced levels of GAA expression and enzymatic activity relative to wild-type mice. We performed echocardiography on Gaa^{c1826insA} mice to assess cardiac structure and function. Gaa^{c1826insA} mice exhibit early-onset, progressive cardiac hypertrophy as measured by significant increases in left ventricular wall thickness and mass index by 3 months of age. We also conducted functional tests - grip strength, inverted screen, gait analysis - on Gaa^{c1826insA} mice every 3 months to assess overall motor performance. Gaa^{c1826insA} mice display impaired motor strength and coordination relative to wild-type mice. Altogether, our results demonstrate that the Gaa^{c1826insA} murine model recapitulates human infantile-onset Pompe disease and is better suited for evaluation of therapeutic strategies such as genome correction.





809. The Effects of Sex and Tissue Targeted Gene Therapy on Systemic and Tissue-Specific Damage Due to Propionic Acidemia

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Propionic acidemia occurs due to mutations in the alpha or beta subunit of the mitochondrial enzyme propionyl-CoA carboxylase (PCC). PCC deficiency affects many organs and can lead to potentially fatal cardiomyopathy. We developed a hypomorphic mouse model (Pcca-/-(A138T)) that exhibits many of the symptoms observed in humans including elevations systemic metabolites and neurological and cardiac phenotypes. Systemic therapy with liver biased adenovirus 5 (Ad5) vs. adeno-associated virus 8 (AAV8) carrying PCCA produced marked correction within one week of injection with notable recovery of the abilities of the animals to eat and gain weight. First generation Ad mediated higher correction even when delivering 10 times less PCCA gene than AAV, but this correction was lost over a month due to immune responses. AAV8 correction persisted over 1.5 years in male mice, but PCC expression and phenotypic correction was lost in females. This demonstrated a profound sex bias in AAV vector therapy, at least in mice. Muscle-biased AAV1, liver-biased AAV8, and broadly tropic

AAVrh10 mediated significant biochemical corrections in circulating propionylcarnitine (C3) and methyl citrate (MeCit) with best correction by AAVrh10. AAV1 and AAV8 were made more specific by the use of muscle-specific and hepatocyte-specific promoters. Under these targeted gene therapy conditions, both vectors mediated significant long-term correction of C3 and MeCit, demonstrating that correction of either tissue could blunt systemic metabolite loads. While liver-targeted AAV8- PCCA treatment reduced systemic, it mediated only slight correction of cardiac phenotypes. In contrast, muscle-targeted AAV1-PCCA treatment reduced hypertrophy, lowered BNP levels, and corrected concentric cardiac remodeling to levels not observed in animals receiving liver-specific treatment. These results show that while liver-directed therapy provides systemic benefits, it does little to correct cell autonomous defects in the heart and likely other tissues. In contrast, muscle-targeted therapy blunts systemic metabolites while also better treating cardiac manifestations of the disease.

Musculo-skeletal Diseases

810. AXO-AAV-OPMD: Proof-Of-Concept for a Single-Vector Silence and Replace Gene Therapy for the Treatment of Oculopharyngeal Muscular Dystrophy

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AXO-AAV-OPMD is a gene therapy for patients with OPMD (Oculopharyngeal Muscular Dystrophy) aimed at relieving the dysphagia that is a hallmark symptom of the disease. The desired outcome of this gene therapy is improvement of affected pharyngeal muscle function and dysphagia that is associated with aspiration and impacts the daily life of patients. The disease is caused by a trinucleotide repeat abnormality in the poly A binding protein N1 (PABPN1) gene, resulting in intranuclear inclusion bodies and resultant abnormal muscle function. Efficacy in using one gene therapy vector to silence expression of PABPN1 and a second vector to express an shRNA-insensitive wildtype version of PABPN1 has been demonstrated in an animal model of OPMD. However, utilizing two vectors does not assure balanced knockdown and replacement in a single cell. Therefore, AXO-AAV-OPMD was developed as a single, "silence and replace" therapeutic vector for patients with OPMD. AXO-AAV-OPMD is comprised of a modified AAV serotype 9 (AAV9) capsid that delivers a uniquely engineered recombinant genome encoding a codon-optimized, wildtype PABPN1 protein as well as two short hairpin RNAs (shRNAs) directed against the disease-causing mutant *PABPN1* gene. This study demonstrates the results of AXO-AAV-OPMD administration in the A17 transgenic mouse model, an animal model that recapitulates many of the same phenotypes of the human disease including intranuclear aggregates and a progressive loss of muscle strength. A single administration of AXO-AAV-OPMD into the *tibialis anterior* muscle at doses up to $7.5x10^{11}$ vg/muscle results in robust inhibition of mutant PABPN1 and restores PABPN1 expression to wildtype levels with the shRNA-insensitive codon-optimized protein. AXO-AAV-OPMD also restored muscle strength and muscle weight to wildtype levels and reversed other physiological hallmarks of the disease including intranuclear inclusions and fibrosis.

811. Abstract Withdrawn

812. Abstract Withdrawn

813. All-In-One CRISPRai for Bidirectional Gene Manipulation Facilitating Stem Cell Chondrogenesis and Calvarial Bone Regeneration

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Regeneration of calvarial defect has posed a tremendous challenge in clinical settings. The healing outcome was shown to be augmented when the implanted stem cells were driven towards chondrogenic lineage instead of the conventional osteogenic induction. To facilitate enhancement of chondrogenic while inhibition of adipogenic committment of the stem cell's fate, we developed an all-in-one flatform termed CRISPRai that accommodates three effectors: a catalytically inactive Cas9 (dCas9), a transcriptional activator, and a transcriptional inhibitor along with 2 distinctive single guide RNAs (sgRNAa and sgRNAi) as a selective effector recruitor. Transfection of CHO cells with CRISPRai and a dual reporter confirmed that the dCas9-sgRNAa or sgRNAi complex was able to selectively recruit the activator or the inhibitor resulting in activation of mCherry or inhibition of d2EGFP on the reporter in a orthogonal manner. We further packed CRISPRai, sgRNAa targeting the master regulator of chondrogeneris (Sox9), and sgRNAi for that of adipogenesis (PPAR-γ) into baculovirus vector that allows efficient transduction of rat bone marrow derived stem cells (rBMSC) and verified simultaneous Sox9 activation and PPAR-γ repression. The utilization of the Cre/loxP-based hybrid baculovirus further amplified and extended the activation and inhibition effects. The CRISPRai-engineered rBMSC exhibited stimulated chondrogenesis and repressed adipogenesis in two-dimensional culture and improved formation of engineered cartilage in three-dimensional culture. What's more, implantation of the CRISPRai-engineered rBMSC facilitated calvarial bone healing. This study provides a toolkit to allow multiplexing and bidirectional gene manipulation using a single CRISPR platform.

814. AAV9-U7snRNA Mediated Skipping of DMD Exon 2: Absence of Off-Target Splicing Effects as Demonstrated by RNA-Seq

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Therapeutic exon skipping relies on altering splicing patterns within a target gene in order to establish an open reading frame. We have previously demonstrated that exon skipping of a duplicated *DMD* exon 2 can restore wild-type dystrophin in the Dup2 mouse model, which carries the most common duplication mutation associated with Duchenne muscular dystrophy (DMD). We have developed a viral-based exon-skipping approach utilizing AAV9 to encapsidate four copies of a non-coding U7snRNA with additional sequences targeting the DMD exon 2 splice acceptor (two copies) or donor (2 copies) sites (the scAAV9.U7.ACCA vector). Systemic delivery of this vector results in robust and prolonged skipping of exon 2, leading to two therapeutic transcripts: a wild-type mRNA containing a single exon 2, and a transcript with no exon 2 included (deletion 2) that we have previously shown leads to the translation of a highly functional protein via utilization of an exon 5 internal ribosome entry site. As part of a clinical development program, we sought to determine whether this vector resulted in off-target exon skipping in other genes. Following systemic delivery of clinically relevant doses of vector at day P0, vector- and untreated Dup2 mice (n=3 per group) were sacrificed at day 90 post-injection, and skeletal muscle, liver, and heart were obtained for RNA-Seq analysis using Illumina technology. Sequences were analyzed for differential gene expression, and for detection of local splicing variation (LSV) using MAJIQ software. Splice junction quantification in treated versus untreated skeletal and cardiac muscles showed robust skipping of *Dmd* exon 2, indicating that the virally-expressed modified U7snRNAs were active at their primary *Dmd* exon 2 targets, and confirming the general validity of quantifying treatment-induced alternative splicing from the mapped RNA-seq reads. Only 11 viable candidate off-target LSVs, mapping to 6 genes, were identified, and all were known alternative splicing events annotated in the Vertebrate Alternative Splicing and Transcription Database (VastDB), a large resource of alternative splicing events detected by RNA-seq from a diverse set of vertebrate cells and tissues. Only 1 splice event showed alterations in both skeletal and cardiac tissue, and binding energy prediction suggests that the alterations are indirect effect of treatment on alternative splicing instead of a direct off-target effect due to modified U7 snRNA-target pre-mRNA binding. Gene expression analysis identified only 11 differentially expressed genes in treated versus untreated skeletal muscle. The changes were consistent with a reversal of known dystrophic gene expression signatures, including the downregulation of transcripts consistently identified as observed as upregulated in skeletal muscle from dystrophic mice and humans. These results suggest that for these three tissues, specific transcriptional responses are related to restoration of dystrophin levels due to exon 2 skipping in skeletal muscle and heart, rather than changes in mRNA levels due to off-target U7.ACCA snRNA interactions directly with differentially expressed transcripts.

815. AAV-Mediated Deletion of a Large Mutational Hotspot for Treatment of Duchenne Muscular Dystrophy

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Significance/Background: 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. Gene therapy is an attractive treatment strategy for DMD due to the possibility of targeting the underlying genetic cause. Recombinant adeno-associated viruses (rAAVs) are popular gene delivery vehicles as they are non-pathogenic and capable of efficient and prolonged gene transfer to the muscle. Still, packaging constraints preclude delivery of the full-length dystrophin cDNA. AAV-mediated expression of truncated dystrophin sequences has been utilized to ameliorate the dystrophic phenotype, leading to a condition similar to Becker muscular dystrophy (BMD). However, therapeutic efficacy may be impacted by transgene loss due to muscle turnover as well as a possible immune response to a foreign dystrophin protein. 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 single exons and produce a shorter, yet functional protein. Still, any particular single exon deletion would only apply to a small percentage of patients (<14%), which warrants a more versatile treatment strategy that addresses the heterogeneity of *DMD* mutations. The majority of *DMD* mutations are deletions (~68%) of one or more of its 79 exons that shift the reading frame and terminate expression of the full-length transcript. Deletions mostly occur in two "hotspots" of the gene, which encompass exons 2 through 20 (~1/3 of all deletions) and exons 45 through 55 (~2/3 of deletions). Interestingly, BMD patients with naturally occurring in-frame deletions of the entire 45 to 55 region of the *DMD* gene exhibit delayed disease onset and minimal skeletal muscle pathology. An earlier study conducted in our laboratory showed that the entire 45 to 55 region can be deleted in cultured DMD patient myoblasts following electroporation of plasmid DNA encoding the CRISPR genome editing system. However, the *Streptococcus pyogenes* Cas9 (SpCas9) gene used in this previous work exceeds AAV packaging limitations, precluding future therapeutic application. A smaller Cas9 enzyme derived from *Staphyloccoccus aureus* (SaCas9), which has similar editing efficiency to SpCas9, fits within AAV vector size constraints. Here, we utilize AAV delivery of CRISPR/Cas9 components to mediate excision of the 45-55 region and restoration of dystrophin expression. We have developed new vectors encoding this system, confirmed deletion of exons 45-55 in cultured cells, and an in vivo study to assess editing efficiency and dystrophin expression in a humanized mouse model of DMD (hDMDΔ52/mdx mice) is currently underway. In summary, we have developed a clinically-relevant, CRISPR/Cas9-based therapeutic strategy that has the potential to benefit about half of all DMD patients.

816. Msr Class A1/2 Regulates Fibrosis in Dystrophic Injured Skeletal and Aged Cardiac Muscles

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Duchenne muscular dystrophy (DMD), caused by mutations in the *DMD* gene that encodes dystrophin, shows dystrophic pathology in muscles and with macrophage invasion. M1 macrophages (Mϕ) have a role of inflammation in skeletal muscles, and M2 Mϕ have been shown to have an anti-inflammatory, wound healing and a fibrogenic role in *mdx* mice, a model of DMD. The arginine metabolism by arginase in M2 Mϕ can drive fibrosis in *mdx* muscles (*Genes Dev.* 2008). Recently, we discovered that macrophage scavenger receptor (Msr) is expressed on myotubes in addition to Mϕ, and the uptake of negatively-charged oligonucleotides into myotubes was mediated by Msr class A1 (*Nano Lett.* 2015). In addition, Msr A has been reported to have a role of differentiation from M1 to M2 Mϕ (*Hepatology.* 2015). Here, we extended the previous work to clarify the pathophysiology of Msr A in skeletal and cardiac muscles of newly developed double knock-out (Msr/Dys KO) mice lacking Msr A 1/2 and dystrophin. We found that the fibrotic area in gastrocnemius and diaphragm is significantly less widespread in the Msr/Dys KO vs exon 52 deleted *mdx52* mice, a model of DMD at 12 weeks of age. Furthermore, we have shown that the fibrotic area evaluated by picrosirius red staining in the heart is significantly less extensive in the Msr/Dys KO compared to *mdx52* mice at 60 weeks and *Nox4*, a marker of fibrosis, tended to have lower expression levels in Msr/Dys KO by quantitative PCR. Interestingly, the fibrotic area was significantly reduced in $BaCl_2$ injured tibialis anterior muscles of Msr/Dys KO mice. Using bone-marrow derived macrophages (BMDM), the arginase activity in *mdx52* and Msr/Dys KO were significantly increased compared to WT ones. In M2-like Mϕ that were derived from BMDM using IL-4 and IL-13, the arginase activity in Msr/Dys KO M2-like Mϕ tended to be decreased compared to *mdx52* derived Mϕ. These observations indicate that Msr class A 1/2 deficiency in dystrophic muscle leads to less severe skeletal muscle fibrosis after chemical induction of muscle injury. In addition, the cardiac fibrosis in the aged Msr/Dys KO was significantly decreased compared to *mdx52* mice.

817. nNOS-Based Muscle Disease Gene Therapy Depends on the Dimerization of the nNOS PDZ Domain

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Loss of sarcolemmal nNOS or dislocation of nNOS from the sarcolemma occurs in multiple muscle diseases, such as muscular dystrophies and muscle atrophy. Restoration of nNOS to the sarcolemma has been actively pursued as a promising gene therapy approach to treat muscle diseases. The full-length nNOS protein is composed of the PDZ domain, the oxygenase domain, calmodulin-binding motif and the reductase domain. We and others have previously shown that dystrophin spectrin-like repeats 16 and 17 (R16/17) and syntrophin are cellular components required for sarcolemmal nNOS localization. However, it remains unknown which domain of nNOS is responsible for sarcolemmal localization of nNOS *in vivo*. We hypothesize that sarcolemmal localization of nNOS is mediated by the nNOS PDZ domain. To test this hypothesis, we expressed PDZ-deleted nNOS (ΔPDZ nNOS) to the *tibialis anterior* (TA) muscle of nNOS knockout mice with an adeno-associated viral vector (AAV). Opposite to dominant sarcolemmal localization of the full-length nNOS, ΔPDZ nNOS was mainly in the cytosol of myofibers, suggesting that nNOS PDZ domain may mediate membrane localization of nNOS in muscle. To further confirm this finding, we fused nNOS PDZ domain to a GFP tag and generated another AAV construct to express fusion protein PDZ.GFP in the muscle of nNOS knockout mice. We hypothesize that if the PDZ domain is the membrane-targeting domain of nNOS, the PDZ.GFP should be found at the sarcolemma. Unexpectedly, the PDZ.GFP was largely in the cytosol of myofibers. Since only dimerized nNOS can produce nitric oxide (NO), we next examined whether membrane anchoring of nNOS PDZ domain also needs dimer formation. Since the Fc domain of IgG can induce dimer formation, we fused the Fc domain of mouse IgG2a to the C-terminus of PDZ.GFP and expressed PDZ.GFP.Fc by AAV gene transfer in nNOS knockout mice. As expected, we found the membrane localization of the PDZ.GFP.Fc in the muscle. In summary, our results suggest that nNOS PDZ domain dimerization mediates sarcolemmal localization of nNOS *in vivo*.

818. Implications of Deep Evolutionary History of Locomotion for Muscular Dystrophy Transgene Design

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Genes discovered by positional cloning generally encode low abundance proteins of uncertain function. The incidence of associated

diseases strongly correlates with the size of the genes and/or encoded proteins, complicating gene therapy for some of the most common genetic diseases. For instance, in the case of Duchenne muscular dystrophy, with a global incidence of approximately 1/4000 males, the protein dystrophin is almost three times the size of the largest protein deliverable by AAV (the only current vector system capable of widespread targeting of muscle). We used comparative phylogenomics to gain insight into heretofore unrecognized structural constraints on dystrophin and identified patterns that might contribute to an improved understating of protein function. We reconstructed the deep evolutionary histories of dystrophin and titin to address a seemingly simplistic question with critical implications for DMD gene therapy: which came first, dystrophin or the sarcomere? In large animals, rapid locomotion is invariably powered by sarcomeric myosin, whereas the fastest moving unicellular eukaryotes and earliest branching animal lineages use ciliary dynein as the dominant locomotive power source. Selective pressures driving the evolutionary transition from dynein to myosin must reflect geometric constraints imposed by the organelles in which these motors achieve maximal power density, with sarcomeres but not cilia amenable to three-dimensional scaling. The molecular basis of this pivotal transition is poorly understood. We show that the emergence of sarcomeres correlates with the appearance of an ancestral titin ortholog, whereas dystrophin and its associated complex of membrane-bound glycoproteins arose piecemeal prior to the divergence of earlier branching lineages. We identify invertebrate species that retain the inferred ancestral titin supergene structure, providing a unified view of gene rearrangements that previously obscured gene orthology and the common origin of sarcomeres in animals with radial and bilateral symmetry. 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 dawn of sarcomeres. These findings 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.

819. Improved Osteogenic Commitment of BMP2 mRNA-Transfected MSCs Using NS1 mRNA

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Messenger RNA-loaded matrices for bone regeneration is an interesting alternative to recombinant proteins-infused matrices. Messenger RNA (mRNA) allows in situ production of proteins of interest in their native conformation. Accordingly, successful application of mRNA for bone regeneration should modulate this response to maximize transgene expression and improve osteogenic commitment of mesenchymal stem cells (MSCs). However, in vitro transcribed mRNA (IVT mRNA) triggers an innate immune response during transfection, which results in promotion of its degradation and translation inhibition. RNA viruses express proteins that minimize such responses and allow potent viral RNA translation. Among them, non-structural protein 1 (NS1) from *Influenza A* virus decreases viral RNA intracellular recognition by RNA innate immune sensors. We report that codelivery of bone morphogenetic protein 2 (BMP-2) and NS1 mRNAs improved the expression of BMP-2 for 4-fold in MSCs *in vitro* (Figure 1). This result was due to NS1-mediated reduced IFNs signaling, one of the innate immune RNA response. In addition, the inflammation associated with bone defect results in suboptimal bone formation. We show that NS1 could attenuate pro-inflammatory cytokine-TNFα induced NF-κB activation in vitro. These *in vitro* results demonstrate that NS1 has duo-functions in immunosuppression and anti-inflammation. For *in vivo*, as shown in Figure 2, mRNA, which is firefly luciferase mRNA w or w/o NS1 mRNA, activated collagen matrices (RAM) combined with MSCs were subcutaneously implanted into mice back. In the presence of NS1 mRNA, the luciferase signal is 15 times higher. Finally, preliminary results suggest that implantation of BMP2 and NS1 mRNA activated collagen matrices will accelerate bone regeneration in a bone defect model, e.g. mouse calvarial defect.

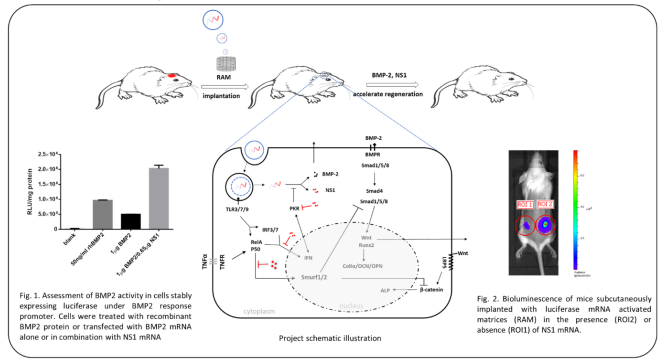

Fig. 1. Assessment of BMP2 activity in cells stably expressing luciferase under BMP2 response promoter. Cells were treated with recombinant BMP2 protein or transfected with BMP2 mRNA alone or in combination with NS1 mRNA

Fig. 2. Bioluminescence of mice subcutaneously implanted with luciferase mRNA activated matrices (RAM) in the presence (ROI2) or absence (ROI1) of NS1 mRNA.

820. A Highly Compact CRISPRa System for In Vivo Gene Activation

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Transcription factors play important roles in regulating gene expression through direct binding to their motifs in genome. Recently, the catalytically dead Cas9 (dCas9) has been engineered to function as transcription factors (CRISPRa) through fusion with transcriptional activator domains, which can be targeted to any genes to drive their expression from their native genomic loci. Despite the attractive potential in manipulating gene expression for therapeutic purpose, the usefulness of the currently developed CRISPRa systems is limited due to their large size, making it highly challenging to package them into adeno-associated viral (AAV) vectors for in vivo applications. In this study, we developed a highly compact CRISPRa system (hc-CRISPRa), allowing us to systemically deliver the CRISPR-based transcription activators into animals using recombinant AAV9. The hc-CRISPRa can efficiently activate the target gene expression similar to the original CRISPRa system. When the hc-CRISPRa with a guide RNA targeting follistatin (*Fst*) promoter was delivered into mouse skeletal muscle (through either intramuscular injection or systemic tail vein injection),

robust elevation of follistatin expression and increased muscle mass were observed. In summary, the hc-CRISPRa system developed in this study could facilitate the bench-to-bed transition of CRISPR-based transcription activators.

821. Comparison of Different CRISPR/Cas9 Strategies for Reframing the DMD Gene in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a devastating muscle wasting disease that typically leads to premature death in the third decade of life. Duchenne is usually caused by out-of-frame mutations in *DMD,* which leads to lack of the protein dystrophin. Becker muscular dystrophy (BMD) is an allelic disease caused by in-frame *DMD* mutations that result in an internally deleted but functional dystrophin protein. BMD progression is generally milder than Duchenne but still has a range of severity from mild to severe BMD. This difference in disease progression is likely caused by the folding and stability of the internally deleted dystrophin protein. It has been hypothesized that retaining proper repeat phasing may partially dictate the functionality of the protein. Some especially mild BMD patients, such as those with an exon 45-55 deletion, have repeat phasing that is predicted to be maintained and can be asymptomatic into their seventh decade. Thus, a therapeutic strategy for Duchenne is to restore the *DMD* reading frame, converting a DMD mutation into a mild BMD mutation. One way in which the *DMD* gene could be reframed is by employing the CRISPR/Cas gene editing system. It is currently unclear whether single vs double cut or various sized deletion strategies have different efficiencies or off target effects. Here we compare different CRISPR/Cas9 strategies to reframe the *DMD* gene through either a single cut splice site disruption, a single exon deletion, or various sizes of multi-exon deletions from 96-708kb. We assess the efficiency of deletion *in vitro* and *in vivo* using our humanized dystrophic hDMD del45 mdx mouse model. This work offers a comprehensive comparison of different CRISPR approaches for reframing the *DMD* gene for Duchenne muscular dystrophy.

822. Identification of Novel Muscle Specific Promoters for AAV Gene Expression in Skeletal and Cardiac Muscles

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Successful expression of an AAV transgene in a specific tissue relies on both the capsid tropism and the specificity of the promoter that drives the packaged expression cassette. We sought to engineer novel promoters for AAV-based gene transfer specifically in muscle tissue. Given that AAV has a limited packaging capacity, such promoters should not only drive strong muscle-specific expression but should also be as short as possible. A bioinformatics approach was employed to generate promoter sequences with predicted muscle specificity. These were first screened *in vitro* for specific expression in mouse myoblasts (C2C12), rat cardiac cells (H9C2) and human myoblasts (hSkM), with lack of off-target expression in HEK293 cells. Candidates with the highest expression in comparison to a known muscle-specific promoter were further characterized *in vivo* by both electroporation into tibialis anterior and systemic injection in mice, resulting in several novel and potent muscle-specific promoters that can be used in AAV gene transfer to muscle. These findings are particularly relevant for treatment of diseases like Duchenne muscle dystrophy.

823. DUX4 Knockdown with LNA and 2'-MOE Gapmers: Developing a Treatment for Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disorder with a global prevalence of 1:8000 to 1:22000. It is characterized by progressive muscle wasting, occurring first in the upper body, followed by the lower extremities. FSHD is currently incurable. FSHD is caused by the aberrant expression of *DUX4* in muscle, a gene typically silenced in healthy myofibers. *DUX4* codes for a transcription factor, and is known to activate pathways involved in muscle atrophy and apoptosis. We aim to use antisense oligonucleotide gapmers specifically targeting *DUX4* to reduce its expression *in vitro* and *in vivo*, and potentially remove its harmful effects on muscle. Upon hybridizing to *DUX4*, gapmers allow for RNase H1 recruitment to the site, resulting in target degradation. We used two different chemistries, locked nucleic acid (LNA) and 2'-*O*-methoxyethyl (2'-MOE), with 7 and 3 gapmers designed for each, respectively. Significant *DUX4* knockdown at nearly 100% was achieved when LNA and 2'-MOE gapmers were transfected at 100 nM into immortalized FSHD patient-derived myofibers. This corresponded with significant knockdown of *DUX4* downstream targets *ZSCAN4*, *TRIM43*, and *MBD3L2*. Gapmer activity was dose-dependent, with significant *DUX4* knockdown still achievable at a 10 nM dose for both chemistries. Observable improvements in muscle fusion and reductions in apoptosis were found upon treatment with LNA gapmers. Furthermore, RNA sequencing revealed that treatment with an LNA gapmer *in vitro* decreased the expression of ~86% of genes that were significantly upregulated in FSHD. For *in vivo* testing, two successful LNA gapmers, 1 and 4, were injected thrice intramuscularly every other day into the tibialis anterior muscles of *FLExDUX4* mice, a mouse model of FSHD. Significant *DUX4* expression knockdown was observed compared to saline-injected controls. We therefore conclude that our gapmers are highly effective at knocking down *DUX4* expression *in vitro*, with our LNA gapmers capable of both potential functional improvement *in vitro* and *DUX4* knockdown *in vivo*. Our study should facilitate the identification of a drug candidate for FSHD therapy, providing the potential for developing a cure for this disabling genetic disorder in the future.

824. Systemic Treatment with Dental Pulp-Derived Stem Cells for Muscle Dysfunction in Dystrophic Muscle

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Background: Duchenne muscular dystrophy (DMD) is an incurable genetic disease with early mortality that exhibits skeletal muscle weakness with chronic inflammation. Dental pulp stem cells (DPSCs) have the potential of becoming therapeutics because of their immunosuppressive properties and multipotency. In the present study, we designed and examined strategies for safe and effective DPSCs transplantation using DMD models to develop a novel approach for functional recovery of the skeletal muscles. **Methods:** DPSCs were intravenously injected into *mdx* mice and canine X-linked muscular dystrophy in Japan ($CXMD_J$) 4-8 times while acute phase. To assess the safety and effects of therapeutic interventions, comprehensive analysis was preformed to observe blood exams, growth, voluntary physical activity, grip strength and muscle histopathology. **Results:** There were no serious adverse events in the DPSCs-treated animals, supported by voluntary physical activity of *mdx* mice and improved growing of $CXMD_J$ during development. Significant decreased grip strength in *mdx* mice was recovered in DPSCs-treated mice group to a normal level. In the case of high-dose transplantation, the effect was maintained until the mice were 1 year old. DPSCs-treated *mdx* mice could run faster and for a longer distance compared with untreated mice in dose dependent manner. Improved histological appearances in DPSCs-treated DMD muscle were demonstrated by attenuated pathological inflammation and reduced centrally nucleated fibers, which are induced by degeneration and regeneration of myofibers. DPSCs-treated $CXMD_J$ also maintained locomotor function, suggesting that specific pathophysiologic features of the dystrophic muscle were restored for long-term.**Discussion:** We suggest that the systemic injection of DPSCs ameliorated the progressive phenotype in *mdx* mice and $CXMD_J$. The therapeutic effects might be associated with the production of paracrine or endocrine factors that regulate inflammation, but a high dose of DPSCs and repeated treatments were required for the prevention of the DMD phenotype. Together, even if further study is needed for clinical usage, this type of stem cell treatment could be promising for DMD therapy.

825. Polyrotaxane Nanocarriers Can Deliver CRISPR/Cas9 Plasmid to Muscle Cells to Successfully Edit the DMD Gene

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Gene editing has shown promise in experimental models of Duchenne muscular dystrophy (DMD), a progressive muscle wasting disorder. However, previous studies have mainly relied on viral delivery systems, which are likely to be limited to single dosing and thus may not be therapeutically practical for gene editing systems like CRISPR/Cas9. Therefore, there is a need to develop safe and efficacious non-viral approaches that allow for systemic delivery and repeat dosing of CRISPR/Cas9 therapies to skeletal muscle. Nanoparticles offer significant advantages over viral delivery methods because they can be modified, package various payloads, and protect the cargo. Here, we develop polyrotaxane (PRX) nanoparticles designed to deliver plasmid DNA to muscle by utilizing our novel multi-arm PRX design, which has shown improved pharmacokinetics in vivo. PRXs are composed of positively charged alpha-cyclodextrin rings threaded onto a polyethylene glycol (PEG) backbone, which self-assembles with negatively charged nucleic acid. We tested iterations of PRX nanoparticles on primary humanized dystrophic muscle cells obtained from hDMD de145 mdx mice, which contain a mutated human DMD gene to target with our CRISPR/Cas9 platform. We demonstrate nanoparticle optimization through iterative modification and show that addition of a redox-responsive linker enhances plasmid release and that peptide conjugation leads to quicker uptake and improved transfection efficiency in vitro. Finally, in vitro delivery of PRXs complexed with our CRISPR/Cas9 platform demonstrates effective deletion of DMD exons 45-55, which is a therapeutic platform that has potential to restore the reading frame for half of all patients with DMD. In summary, this work represents the first PRX platform that has been optimized and designed for delivery of large plasmid DNA, including CRISPR/Cas9, to dystrophic muscle cells.

826. Mouse Models of Nemaline Myopathy Display Structural and Functional Abnormalities of Mitochondria

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Nemaline myopathy (NM) is a genetically and clinically heterogenous neuromuscular disorder that can cause death or lifelong disability. Studies of weakness in NM have focused primarily on issues of skeletal muscle structure and contractility, as essentially all of the genes associated with NM are related to the sarcomeric thin filament. The genetic variability does not entirely explain the significant phenotypic heterogeneity observed in NM patients and mouse models thus suggesting the presence of additional factors in determining disease phenotype. We performed proteomic assessments of the *Acta1* H40Y and *Neb* cKO mouse models of NM to identify additional biological processes of interest in NM, and our results implicate distinct elements of mitochondrial dysfunction in each model. *Acta1* H40Y mouse model has abnormalities in signaling molecules associated with mitochondrial fission and follow-up testing has identified mild structural and biochemical abnormalities of mitochondria in *Acta1* H40Y tissue. In contrast, the *Neb* cKO mouse shows extensive upregulation of proteins related to the mitochondrial electron transport chain (ETC), with increased activity of several ETC complexes on biochemical testing. The *Acta1* D286G has yet to be studied using proteomics, but biochemical testing suggests another distinct pattern of mitochondrial abnormality that relates to decreased ETC complex III activity. While additional classification of functional and structural mitochondrial abnormalities in *Acta1* D286G, *Acta1* H40Y, and *Neb* cKO mice are in progress, this is novel finding provides strong evidence that the disease process in NM exerts significant effects on mitochondrial function. These studies form a foundation for future work on the impact of mitochondrial function on contractile dysfunction in NM and a potential option for future therapeutics.

827. Characterization of the Cytokine Responses in Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by muscle degeneration and weakness due to mutations in the dystrophin gene, leading to an absence of dystrophin protein. There is no cure for DMD, and current treatments include the use of steroids to slow muscle degeneration and immunosuppressants to reduce muscle damage. While DMD is a degenerative muscle disease with immune components, previous research has primarily focused on the immunological findings in the blood rather than in the muscle tissue itself. In the context of clinical trials, where new forms of dystrophin are produced, it is important to distinguish immune events in the muscle related to the disease from those induced by experimental treatments. This study is designed to characterize cytokine and immune responses in tissue in two strains of mdx mice at early and late-stage disease. Our current work utilizes both the C57BL/10ScSn *mdx* mice, the most widely used animal model for DMD research, and the DBA/2-*mdx* mice, which are thought to better represent human disease. Endpoints including cytokine production in blood, spleen, and muscle and immunohistochemistry for immunological markers are in progress. Thus far, there is no difference in interferon-γ production by ELISPOT at 4 weeks old, and we are currently evaluating other Th1, Th2, and Th17 responses at 4 weeks and 6 months of age. Immunophenotyping of tissue at 4 weeks reveals equivalently increased numbers of CD4+ T cells in both mdx strains, minimal CD8+ T cells, and deposition of IgG in necrotic myofibers. Further evaluations at 6 months of life are in progress. Ongoing studies to further characterize the immune response include RT-q PCR to determine expression of immunoglobulin transcripts within the muscle tissue. Overall, our work will improve understanding of the immune events occurring within the tissue in mdx mice. Extension of similar studies into human tissue will be very useful in understanding immunological responses to novel treatment strategies.

828. Development of a Quantitative Evaluation Method for Cognitive Impairment in MDX Mouse

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[Background]Duchenne muscular dystrophy (DMD) is a congenital disease causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. Dystrophin and components of the dystrophin-associated glycoprotein complex (DGC) are located at the plasma membrane and play an important role for muscular stability. While in the central nervous system (CNS), these proteins are located at the postsynaptic membrane of GABAergic synapses and contribute to cognitive function. The loss of dystrophin and DGC lead to muscle inflammation, degeneration and cognitive dysfunction. Supplementation of dystrophin using rAAV-microdystrophin is sufficient to improve pathogenesis of animal models of DMD. Meanwhile, it is unclear whether cognitive dysfunction is improved or not. To assess the cognitive function and to evaluate the microdystrophin therapy in the brain, we designed a quantitative evaluation method using a combination of behavioral test and recoding of spontaneous locomotor activity. **[Methods]**We injected 1e13 v.g. / kg of rAAV9-CMV-microdystrophin via retro orbital vein to 3-day-old male mdx mouse. At 8-week-old, we performed the behavioral test

and recording of spontaneous locomotor activity. [**Results**]*Mdx*mouse treated intravenously with rAAV9-microdystrophin showed a significantly reduced freezing ratio in the behavioral test compared with the non-treated mdx mice. The data from the movement sensor and the activity wheel indicated that the mdx mouse treated with rAAV9-microdystrophin appeared to have a better response although this did not reach statistical significance. [Conclusion]Our results demonstrate that rAAV9-microdystrophin to mdx mouse improved cognitive dysfunction. This evaluation method would be a useful approach to investigate the cognitive function in mice. These findings also support the value of microdystrophin therapy for mdx mouse in the CNS complications.

829. Proteomic Profiling in Nemaline Myopathy to Identify Molecular Phenotypes

Rebecca A. Slick[1,2,3], Jennifer A. Tinklenberg[1,2,3], Mark Vanden Avond[1], Margaret Beatka[1], Hui Meng[1], Emily M. Siebers[1], Aron Geurts[2], Henk Granzier[4], Edna Hardeman[5], Liwen Zhang[6], Federica Montanaro[7], Kristin Nowak[8], Michael W. Lawlor[1,2]

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Nemaline myopathy (NM) is a rare muscle disorder that can cause death and lifelong disability. It is genetically and clinically heterogenous but is considered one disease entity based on the presence of nemaline body aggregates within the diagnostic muscle biopsy. Though nemaline bodies are diagnostically useful, their presence does not correlate well with disease severity or prognosis. The typical sub-classification system for NM patients is based on causative gene, although this is also a poor indicator for patient prognosis. We hypothesize that currently undetermined biological processes play a role in the muscle weakness of NM patients and that these can be revealed by examining disease signatures of NM mouse models. Proteomic analysis was performed on the *Neb* cKO and *Acta1* H40Y mouse models of NM in comparison to their wild-type (WT) counterparts, to identify protein expression profiles at early and late stages of disease. The top canonical pathways affected include calcium signaling, the protein ubiquitination pathway, eIF2 signaling, and the regulation of eIF4 and p70s6k signaling, as well as, processes relating to neuromuscular transmission and metabolism in the *Neb* cKO and *Acta1* H40Y mouse models respectively. Mitochondrial dysfunction was present in both models, but interestingly, different elements of mitochondrial biology were affected in each model. Additionally, a subset of overexpressed proteins was identified in each model that constituted potential immunohistochemical biomarkers that could be used in the classification of disease models and patients. Current work on the project includes 1) further evaluation of pathways that are potentially relevant to disease progression and treatment and 2) immunohistochemical evaluation of potential disease biomarkers in muscle tissue from NM models and patients. Increasing the knowledge of biological processes contributing to the heterogeneity of NM will enhance the ability to design scientifically sound treatment studies, while also improving prediction of patient prognosis to positively impact the quality of clinical care.

830. Allele-Specific RNAi and RNA Base Editing for Glycine Substitution Mutations in Collagen VI-Related Dystrophy

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Collagen VI-related dystrophies (COL6-RD) are a group of congenital neuromuscular disorders of muscle and connective tissue. They are caused by mutations in any of the three *COL6* genes; *COL6A1*, *COL6A2* and *COL6A3* encode the collagen α1, α2 and α3 (VI) chains, respectively. These three chains undergo hierarchical assembly to form the extracellular matrix protein collagen VI which is primarily secreted by muscle interstitial fibroblasts. In dominant-negative mutations causing COL6-RD, the mutant chains are translated and participate in the hierarchical assembly process, eventually leading to a dysfunctional collagen VI microfibrillar network. Haploinsufficiency for dominant-negative mutations is clinically asymptomatic, so allele-specific knockdown of the mutant allele is a rational and previously validated therapeutic approach for this class of mutations. Triple helical glycine substitutions are a recurrent sub-type of dominant-negative mutations, causing COL6-RD by disrupting the Gly-X-Y repeat motifs characteristic of collagens. A specific such mutation we are currently targeting is the recurrent G293R substitution caused by c.877G>A in the *COL6A1* gene. To employ an allele-specific siRNA-based approach to knockdown this mutation, we designed and scored 19-nt long siRNAs based on rules described by Elbashir, Tuschl et al. (2001, Genes and Development). We tested the potency and specificity of the top scoring siRNAs against eGFP-fusion reporter constructs expressing the WT (c.877G) or the mutant G293R (c.877A) allele in HEK293T cells. We identified a single siRNA that, albeit highly potent, was non-discriminatory between the wild-type and mutant G293R alleles. To improve specificity, we introduced a single mismatch in the siRNA design at varying positions from the mutation and identified sequences that were highly specific to the G293R allele while sparing the wild-type allele over a wide range of concentrations. Additionally, *in vitro* treatment of these siRNAs in patient dermal fibroblasts dramatically improved the 'spotty' appearance characteristic of deposited mutant collagen VI microfibrillar matrix and reduced the intracellular retention of collagen VI, as determined by IF and western blot analyses. As an alternative to siRNA-based therapeutics and to consider additional therapeutic avenues for effective *in vivo* delivery, we are currently validating Cas13b-mediated RNA interference (RNAi) of the G293R transcript. We have designed six guide RNAs which are position matched to the siRNAs and of varying lengths (19nt, 24nt and 30nt) and varying position of the mutation, to assess whether these guide RNA sequences can effectively target the G293R in an allele-specific fashion. Following successful RNAi through both aforementioned approaches, we plan to utilize RNA base-editing to

deaminate the adenosine c.877A to inosine, which is interpreted as guanosine, and restore the mutant *COL6A1* transcript to a wild-type state. Such an approach to treat missense, single-base substitution mutations in COL6-RD has not been previously demonstrated and, if effective, could be the preferred therapeutic approach for glycine substitution mutations of COL6-RD, as it could restore *COL6A1* expression from both alleles.

831. CRISPR-Induced Deletion for the Correction of the Human Dystrophin Gene Using the Cas9 from S. Aureus

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Duchenne muscular dystrophy is a severe genetic disease, affecting 1/5000 newborn boys, characterized by muscle wasting and weakening that lead to cardio-respiratory failure and to death in the twenties. This X-linked disease is related to a mutation (mostly deletion of one or several exons) in the DMD gene (coding for dystrophin) that is responsible of a shift in the reading frame thus creating a premature stop codon that abrogates dystrophin protein synthesis. We report that by using a combination of sgRNAs recognized by the SaCas9 (*S. aureus),* we can create a large genomic deletion that reframes the dystrophin gene in myoblasts from DMD patients and allows expression of dystrophin in myotubes. Our approach differs from others as in addition to restoring the expression of the dystrophin protein, we also aim to restore the correct structure of the spectrin-like repeat (SLR) in the dystrophin central rod-domain as observed in patients with Becker muscular dystrophy, who do not have a severe phenotype, because their internally deleted dystrophin protein is functioning normally. Following the screening of sgRNAs targeting the exons 46 to 58 of the DMD gene, we identified pairs of sgRNAs able to form hybrid exons 46-51, 46-53, 47-52, 49-52, 49-53, and 47-58 in 293T cells. In these hybrid exons, the nucleotide sequences was exactly as expected in 54% to 90% of the amplicons. Interestingly, two pairs of sgRNAs generated large genomic deletions connecting the exon 47 to the exon 58 that might correct up to 40% of the DMD mutations. In addition, the resulting amino-acid sequences of these two hybrid exons were modeled by iTasser. These models indicated that the spectrin-like repeat structures (SLR) of these internally deleted dystrophin proteins were perfect. These pairs of sgRNAs were thus tested in myoblasts of 4 different DMD patients and we detected the formation of the hybrid exons. Deep sequencing indicated that 40% to 89% of these hybrid exons contained the exact expected nucleotide sequences. Moreover, after formation of myotubes by the myoblasts containing a hybrid exon, the truncated dystrophin protein was detected. Subsequent experiments were performed by intravenous injection of two AAV9 (each at 1.10^{14} vg/kg) coding respectively for the SaCas9 protein and a pair of sgRNAs in the del52hDMD/mdx dystrophic mouse model. Six weeks later, we observed the formation of the hybrid exons 47-58 in the heart, the diaphragm and several other skeletal muscles. Moreover, the dystrophin protein was detected in these edited tissues. Finally, this work supports the feasibility of creating a large genomic deletion permitting the production of an internally-truncated dystrophin protein in which all SLRs are correctly phased. Such feature is important to improve dystrophin protein function since abnormal SLR's phasing results in a more severe phenotype in Becker patients.

Cancer-Immunotherapy, Cancer Vaccines II

832. Rewiring T-Cell Signaling Responses to Extracellular Soluble Cues with Chimeric Antigen Receptors

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Soluble extracellular factors are a commonly used medium of communication between a cell and its environment. Using the chimeric antigen receptor (CAR), we establish a general framework for engineering T cells to sense soluble ligands and specifically demonstrate its utility in sensing transforming growth factor beta (TGF-β), an immunosuppressive factor commonly upregulated at solid tumors. Empiric data and biochemical modeling together demonstrate that CAR signaling in response to soluble ligands relies on ligand-mediated CAR dimerization. Ligand exposure additionally triggers the TGF-β CAR to form ZAP70-rich microclusters in an actin-dependent manner. Further characterization of TGF-β CAR-T cells shows Th1-cytokine production and T-cell expansion after exposure to the otherwise immunosuppressive TGF-β. TGF-β CAR-T cells also protect surrounding T cells from TGF-β-induced functional defects and differentiation into the regulatory phenotype. Finally, we demonstrate a method by which the TGF-β CAR signaling strength can be rationally tuned. Principles learned from our results serve as an initial step toward engineering cell-based therapies with designed interactions with soluble, extracellular ligands.

833. GMP Manufacturing of Allogeneic Sleeping Beauty (SB) Transposon-Engineered CD19 Chimeric Antigen Receptor (CAR) Cytokine Induced Killer (CIK) Cells

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Adoptive transfer of patient-derived T cells engineered to express a chimeric antigen receptor (CAR) by viral vectors has achieved complete remission and durable response in highly refractory populations. Unmodified allogeneic Cytokine Induced Killer (CIK) cells (CD3+CD56+ T cells) have clearly demonstrated a high profile of safety in ALL patients. Here, we demonstrate the feasibility and reproducibility of a good manufacturing practices (GMP)-compliant culture of allogeneic CIK cells modified by non-viral Sleeping Beauty (SB) transposon to obtain CD19CAR T cells. PBMCs were electro-transferred with the SB GMP-grade CD19.CAR/pTMNDU3 plasmid and pCMV-SB11 plasmid (kindly provided by L. Cooper, Houston). CIK cells were then generated according to the method enclosed in the filed patent EP20140192371. The manufacturing process were performed in a academic cell factory authorized by Agenzia Italiana del Farmaco (AIFA). We manufactured ten batches by seeding a median of $103.16x10^6$ allogeneic PBMCs derived from 50 ml of PB. After 20-28 days of culture (median 22) we harvested a median of $3.6x10^9$ nucleated cells (range 1.40 - $15.75x10^9$). At the end of expansion, cell viability was 97.24% (range 91.99%-98.96%), manufactured cells were mostly CD3+ lymphocytes (mean 98.73% ±SD 1.55%). Of these, 46.17%±17.92% were CAR+ and 43.89%±10.13% were CD56+, while median fold increase was 176.6 (37.0-1350) and had a median vector copy number (VCN) of 3.5 VCN/cells. In all the ten batches, CARCIK-CD19 cells demonstrated potent and specific *in vitro* cytotoxicity towards the CD19+ REH target cell line (mean 80.68%, range 61.89%-97.72%). Cell products appear to be highly polyclonal and no signs of genotoxicity by transposon insertions could be observed by integration site (IS) analysis performed using Sonication Linker Mediated (SLiM)-PCR. All the batches were released after about 10 days after the end of production. The quality requirements for batch release were met in all 10 productions. Overall, these results demonstrate that clinical-grade SB transduction of allogeneic CIK cells with CD19 CAR is feasible and allows efficient expansion of highly potent CARCIK-CD19 cells starting from easily available small amounts of PB, with important implications for non-viral technology. A clinical trial investigating allogeneic CARCIK-CD19 in r/r pediatric and adult ALL post HSCT is currently ongoing (NCT03389035).

834. CTV-1-Activated CAR-NK (MBIO-109): A Potent "Off-The-Shelf" Tumoricidal Therapeutic

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Primary human natural killer (NK) cells are known to detect and eradicate malignant cells. NK cells are the first lymphocytes to reconstitute after allogeneic hematopoietic stem cell transplantation and play an important role in mediating the graft-versus-tumor response. There are, however, limitations to using NK cells for immunotherapy including tumor immune escape mechanisms, as well as inhibition of the NK cells by inhibitory molecules expressed on the tumor cell (including inhibitory HLA types). To overcome these limitations, we have combined two very potent approaches: First, we prime the NK cells with a CTV-1 lysate that results in enhanced cytotoxicity against tumor cell lines. CTV-1 is unique in its priming capabilities in that the stimulation event is separated from the lytic event allowing the NK cell to be stimulated in vitro, but only lyse upon target cell encounter - thereby avoiding NK cell exhaustion and alleviating the need for patient IL-2 injections. CTV-1 primed NK cells retain their primed state even after cryopreservation, and CTV-1 priming capabilities are retained even as a lysate. Also, CTV-1 primed NK cells lyse a wide range of NK-resistant tumor cells irrespective of the degree of HLA matching. Second, we lentivirally transduce the NK cells with a CAR (chimeric antigen receptor) molecule to improve target recognition and further enhance activation.Using NK cells sourced from healthy donor peripheral blood, we show here that NK cell priming is very efficient, and that CAR-transduction rate is enhanced. We also show that we can effectively produce off-the-shelf, proliferative, tumoricidal CAR-NK cells and that these cells can be utilized as a novel anti-cancer therapeutic.

835. Targeting Adenosinergic Immunometabolic Suppression Using Natural Killer Cells Engineered with NK-Specific Signaling Constructs for Immunotherapy of Solid Tumors

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Natural killer (NK) cells are powerful effectors of innate immunity, and utilizing genetically engineered NK cells as immunotherapies has had promising clinical responses in the treatment of various cancers.

However, more progress is needed for the clinical treatment of solid tumors. The activation of cancer-associated ectoenzymes CD39 and CD73 catalyzes the phosphorylation of ATP to AMP to produce extracellular adenosine (ADO), which is a highly immunosuppressive mechanism contributing to the pathogenesis of solid tumors. We have previously established that tumor microenvironment ADO results in impaired metabolic and anti-tumor functions of cytokine-primed NK cells. Specifically, peripheral blood-derived NK cells stimulated with IL-2 (200 IU/ml or 400 IU/ml), IL-15 (100 ng/ml), or a combination of IL-12 (30 ng/ml) and IL-15 (100 ng/ml) showed suppressed anti-tumor immunity due to ADO. This was observed through the downregulation of activating receptor expression (including NKG2D), cytotoxicity inhibition, impairment of metabolic activity via both glycolysis and oxidative phosphorylation, and alteration in gene expression signatures that match altered IFNy signaling. Priming the NK cells with cytokines partially diminished the immunosuppression induced by ADO. The greatest response to ADO was the combination of IL-12 with IL-15, which yielded a much higher increase in IFNy expression with ADO compared to either IL-2 or IL-15 alone. A significant upregulation of the IFNG gene was also observed, which matched enhanced IFN-γ expression. To target ADO-producing CD73 on cancer cells, we have postulated that NK cells redirected against CD73 using NK-specific signaling components derived from FCyRIIIa (CD16) might provide enhanced cytolysis against CD73+ solid tumors. These signaling elements have not been used in traditional CAR architectures, but are actively involved in mediating NK cell cytotoxicity natively. To that end, we engineered NK cells with this type of construct, which was synthesized by fusing CD73 ScFv with intracellular and transmembrane regions of FCyRIIIa or the T-cell receptor zeta chain signaling domain. The novel genetic construct was transcribed into mRNA and electroporated or retrovirally transduced into NK cells. Engineered NK cells expressing the CD73.FCγRIIa construct were tested for transfection efficiency, stability, and effector function against $CD73^{+}$ solid tumors. The innovative genetic construct that fuses CD73 ScFv with FCyRIIIa derived transmembrane and intracellular signaling components and T-cell receptor zeta chain signaling domains provides a novel, alternative signaling source for the construction of tumor-responsive NK cells. In summary, the microenvironment of solid tumors is highly immunosuppressive via the activity of CD73 expressed on cancer cells, which generates adenosine, a metabolite we have shown to impair NK cells' anti-tumor immunity. To hinder CD73+ solid tumor growth, redirecting NK cell function using an NK-specific anti-CD73 targeting construct is being pursued pre-clinically as a novel adoptive immunotherapy with NK cells.

836. Engineering Antigen Presenting Cell (APC) Cancer Therapies Using the SQZ Platform

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Availability of antigen on the surface of antigen presenting cells (APCs), crucial to the activity of potent cancer vaccines, is often hindered by inefficient presentation of cancer antigens on major histocompatibility complex class I (MHC-I). In this work, we use the microfluidics-based Cell Squeeze® platform to temporarily disrupt the cell membrane and deliver antigen directly into the cytosol of diverse cell types. This approach allows for the rapid and efficient loading of antigen into both professional and unconventional APCs for subsequent priming of CD8+ T cell responses in murine and human systems. In murine models, we demonstrate that the Cell Squeeze® platform efficiently delivers protein and peptide antigens to diverse cell populations (including both conventional and non-conventional APCs). Importantly, we demonstrate that even unconventional APCs are able to present immunogenic epitopes on MHC-I. When transferred in vivo, diverse types of antigen-loaded APCs (termed SQZ-APCs) drive the expansion and activity of antigen-specific CD8+ T cells. When used to treat mice implanted with the TC-1 tumor model for human papillomavirus 16 (HPV16) associated cancer, HPV16 synthetic long peptide (SLP) E7-loaded APCs exhibit strong anti-tumor effects. These responses correlate with a significant increase in antigen-specific CD8+ tumor infiltrating T lymphocytes. Compared to a traditional subcutaneously administered peptide vaccine, SQZ-APCs elicit a greater intra-tumoral CD8+ T cell response and drive improved tumor growth inhibition. Moreover, combination of the SQZ-APC vaccine with chemotherapy dramatically abrogates tumor growth and does so to a greater extent than either therapy alone. In addition, we show that primary human cells can be efficiently loaded with SLPs containing immunogenic epitopes derived from cytomegalovirus. These SQZ-APCs efficiently activate both antigen-specific expanded CD8+ T cell lines as well as unperturbed patient-derived CD8+ cells. We demonstrate that diverse populations of human SQZ-APCs retain APC activity following cryopreservation, enabling flexibility in the generation of clinically relevant material. Finally, this process has been scaled to engineer greater than a billion APCs per minute, allowing for the rapid and efficient manufacturing of APCs for clinical translation. Through the direct cytosolic delivery of antigens, the Cell Squeeze® therapy platform enables engineering of diverse cell populations as potent APCs to be used in cancer therapy.

837. Impact of RetroNectin-Mediated T-Cell Activation on Expansion and Phenotype of Chimeric Antigen Receptor T Cells

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Introduction: Adoptive immunotherapy with chimeric antigen receptor T (CAR-T) cells for hematological malignancies has shown promising results. Enhanced *in vivo* expansion, long-term persistence of CAR-T cells, and efficient tumor eradication through these cells may be depend on the proportion of naïve (T_N) or less-differentiated T cells in the CAR-T cell product. RetroNectin, recombinant human fibronectin fragment, is a chimeric peptide that consists of three functional domains: a cell-binding (C-domain), a heparin-binding (H-domain), and a CS-1 site. RetroNectin is well established as an adjuvant for enhancement of retroviral-mediated gene transduction by aiding the co-localization of target cells (e.g. hematopoietic stem cells

and T cells) and virions. Furthermore, in conjunction with interleukin (IL)-2 and anti-CD3 antibody, RetroNectin has been shown to further increase the expansion of T_N cells from peripheral blood mononuclear cells (PBMCs), and to improve the expansion and posttransplant persistence of genetically modified T cells. However, its property to expand CAR-T_N cells in combination with alternate cytokines such as IL-7 or IL-15 is less known. In order to find an effective way to expansion of CAR-T_N cells, we investigated the impact of using RetroNectin on proliferation, cytotoxic activity, and phenotype of CAR-T cells. **Methods:** CAR-T cells were generated upon transduction of peripheral blood mononuclear cells (PBMCs) from healthy donors with a CAR-CD28-CD3zeta retroviral vector under different stimulating culture conditions: anti-CD3 antibody/RetroNectin or anti-CD3/anti-CD28 antibodies adding either IL-2, IL-7, IL-15, or IL-7/IL-15. Phenotype and function were tested using flow cytometry and CTL assays. **Results:** RetroNectin-mediated activation resulted in an expansion of CAR-T_N cells ($CAR^+CD45RA^+CCR7^+$), compared to anti-CD28 antibody-mediated activation. Especially, IL-7 preferentially induced differentiation into both $CD4^+$ and $CD8^+$ CAR-T_N cells, but IL-7 stimulation resulted in lower proliferation and cytotoxic activity. IL-15 and IL-7/IL-15 induced $CD4^+$ rather than $CD8^+$CAR-T_N cells. **Summary:** The results showed a marked expansion of CAR-T_N cells with the use of RetroNectin. However, activation with RetroNectin in combination use of IL-7 should be performed with caution due to lower proliferation and cytotoxic activity of CAR-T cells. This effect is probably attributed to the RetroNectin-mediated intracellular signaling of T cells. Further studies are also needed in order to clarify enhanced *in vivo* expansion, long-term persistence of CAR-T cells, and efficient tumor eradication.

838. T Cells Armed with Novel Anti-CD30/Anti-CD3 Bispecific Antibodies for Immunotherapy of CD30+ Malignancies

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Introduction: Chimeric antigen receptors (CARs) have delivered striking clinical successes for patients with leukemia and lymphoma, though responses are frequently accompanied by life-threatening toxicities such as cytokine release syndrome. Furthermore, gene-modified autologous T cell products can be expensive and complex to produce, thus limiting their accessibility. Bispecific antibodies (biAbs) can similarly redirect T cells to kill tumor-associated antigen expressing cells, and may be more straightforward to produce. CD30 is a promising immunotherapeutic target due to its expression on a number of malignancies including Hodgkin's lymphoma, anaplastic large cell lymphoma, and adult and pediatric AML. Expression of CD30 on normal tissues is limited, with low expression on activated T and B cells. Several approaches targeting CD30 are currently being evaluated in preclinical and clinical settings, including an antibody-drug conjugate and CAR-T therapies. The aim of the present study is to develop novel CD30/CD3 biAbs and assess their ability to mediate elimination of $CD30^+$ tumor cells *in vitro* and *in vivo* when loaded onto human pan-T cells. **Methods**: We purified protein, inoculated mice, and generated 15 anti human CD30 hybridoma cell lines. Five hybridomas were selected for further analyses. All candidates were shown to bind specifically to CD30 by flow cytometry and ELISAs. Candidates were also characterized by epitope mapping, along with both DNA and protein sequencing. Antibody affinities to CD30 were measured by surface plasmon resonance. Two candidate antibodies that bind different epitopes of CD30, 8D10 and 10C2, were selected for development of biAbs. Purified antibody was heterconjugated with anti-huCD3 antibody (OKT3) to produce anti-CD3 x 8D10 (bi8D10) and anti-CD3 x 10C2 (bi10C2). Human CD3+ T cells were activated with CD3/CD28 beads and expanded in IL-2. Next, they were incubated with biAbs for one hour prior to evaluation. BiAb-armed T cells were assessed by flow cytometry to measure binding and conjugation ability. *In vitro* cytotoxicity of biAb-armed T cells against target cells (CD30+: SU DHL-1, RPMI6666, Raji LV CD30; CD30-: Raji, OCIAML2) and T cells ($CD30^{low}$) was assessed by ^{51}Cr release. IL-2 and IFN- γ production by biAb-armed T cells co-cultured with these cell lines was measured by ELISA. **Results**: Bi8D10 and bi10C2 bind to both tumor cells and T cells. Bi8D10 T cells, but not bi10C2 T cells, show efficient conjugation with CD30+ tumor cells. Bi8D10 T cells effectively kill all CD30+ cell lines tested. Bi10C2-T cells are less effective, however, at lysing RPMI6666 and Raji LV CD30 cells. When co-cultured for 24 hours with CD30+ tumor cells, bi8D-T cells and bi10C-T cells robustly produce IL-2 and IFN- γ. Neither bi8D10 T cells nor bi10C2 T cells kill or produce cytokine in response to $CD30^-$ or $CD30^{low}$ cells. **Conclusions**: Bi8D10 T cells are more effective than bi10C2-T cells at conjugating T and tumor cells, and lysing a variety of CD30+ cell lines. Importantly, our CD30 biAbs do not kill $CD30^{low}$ T cells, allowing for lysis of tumor cells in the absence of fratricide. *In vivo* studies of bi8D10 T cells are underway. Our previous studies demonstrate that while CAR-T-Rapa cells have cytotoxic abilities equivalent to CAR-T cells, they produce significantly less proinflammatory cytokines, such as IFN- γ, GM-CSF, and TNF- α. We are investigating this alternative T cell population with our biAbs. Cytokine production and cytotoxicity studies are underway to determine whether biAb-armed T-Rapa cells have comparable killing to biAb-armed T cells, while maintaining a low cytokine secretion profile.

839. Generation of Selectable, Multi-Edited Allogeneic CD3+ T Cells

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The engineering of allogeneic "off the shelf" T-cell therapeutics would be facilitated if multiple gene edits could be created by a single manufacturing step. Towards this goal, we are developing a strategy for multi-editing of T cells at the TCR alpha (TCRA) locus and a second locus, (locus "B") to generate non-alloreactive, engineered T cells that can be inducibly expanded both in vitro and in vivo. This approach employs a two component cytokine receptor, termed a chemically induced signaling complex (CISC) to generate an IL2-like trophic/growth signal in the presence of rapamycin. The proposed strategy involves simultaneous integration of one CISC component at the TCRA locus and a second component at the "locus B". Using Cas9/guide

RNA ribonucleoproteins (RNPs), both TCRA and a "locus B" were successfully knocked down at >90% efficiency. Targeted integration of the CISC components at the TCRA and "B" loci was accomplished using AAV donor templates with homology arms directed at each locus. Combined targeted integration rates of ~15% were achieved over multiple experiments in cells from normal donors, indicating that approximately half the single edited cells are amenable to multi-editing and would be expandable via rapamycin-mediated CISC activation.

840. Precision Genome Editing: Identification of Highly Efficient and Specific Guides for CRISPR-Cas9 Gene Editing in Human T Cell Loci

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Gene editing is a promising technology with applications in a variety of fields, including human therapeutics. However, one of the challenges presented by gene editing is the potential for unwanted off-target edits. In order to successfully deploy genome editing for various biotechnology and clinical applications, it is critical to design strategies that maximize on-target activity while minimizing off-target editing. Here, we present a target selection workflow that enabled the identification of Cas9 guides incorporating DNA residues that exhibited both high on-target activity and significant specificity in human primary T cells. In brief, our workflow used a combination of computational, sequencing and functional assays to recover guides that enabled substantial on-target editing. Subsequently, guides were analyzed for off-target activity using the SITE-Seq™ assay, a comprehensive and unbiased off-target analysis method, thus allowing us to discard guides exhibiting low specificity. For example, high specificity guides were identified that showed >90% disruption of the TCR alpha constant (TRAC) locus in human primary T cells. Finally, analysis of indels generated after Cas9-mediated double-strand breaks indicated that reproducible and non-random DNA repair outcomes occurred, providing confidence in top quality target selection. Together, these target selection tools enabled identification of highly efficient and specific reagents for human therapeutic gene editing.

841. CRISPR/Cas9 Gene Editing to Produce Multiple Allogeneic CAR-T Cell Candidates Showing Consistently High Potency, Durability, Lack of Alloreactivity, and Ability to Overcome Immune Suppression

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The CRISPR/Cas9 system enables the highly efficient editing of genomes in multiple cell types. The proliferative nature of T cells makes them particularly amendable to CRISPR/Cas9 gene editing for both gene knock-out by non-homologous end joining (NHEJ) and site-specific genetic knock-in by homology-directed repair (HDR). Here we show the consistent production of potent gene-edited allogeneic CAR-T cells targeting multiple tumor antigen targets. Gene knock-out via NHEJ is coupled with HDR to knock-in the CAR construct. The resulting CAR-T cells exhibit the following properties: (1) highly efficient deletion of the T cell receptor (TCR) to enable allogeneic administration, as supported by lack of graft versus host disease (GvHD) when administered to NSG mice; (2) specific and potent activity against antigen-expressing tumor cells; (3) durability and persistence, as exhibited by multiple tumor cell re-challenges without exhaustion; and (4) resistance to PD-L1-induced immune suppression. These attributes may give the CRISPR/Cas9 gene-edited allogeneic CAR-T cells described here the potential to provide clinical benefit in both hematological and solid tumors.

842. High-Efficiency CAR-T Cell Manufacturing by Improved Electroporation

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CAR-T cells are currently manufactured for clinical use by infection of human T cells with viral vectors containing the CAR gene. The current viral CAR-T manufacturing process is lengthy and costly and electroporation has emerged as a promising alternative. However, clinical use of electroporation technology in CAR-T has been difficult and several clinical trials have met significant problems due to the low transfection efficiency and/or high cell mortality. Our novel understanding of the electroporation mechanism revealed that the current widely-used electroporation methods have significant mistakes in the physical design as well as electroporation buffer design. The first problem is the electroporation sample container design. It is well known that electrochemical reaction generates gas bubbles that are harmful to the cells and there was no good solution to the problem. Here we used a novel pressurization approach to largely eliminate the effect. Combined with other improvements including electroporation

buffer design and post-electroporation cell culture strategy, we have been able to achieve over 80% plasmid transfection efficiency in unstimulated T cells and over 90% plasmid transfection efficiency in stimulated T cells. The viability in survived cells is over 95% measured by live/dead staining and the true survival rate measured by survived cell number is over 66%. The new electroporation method can achieve over 90% in gene editing and the method is also widely applicable in electroporation of NK cells, DC cells and monocytes. Our method can potentially eliminate the need for expensive cell expansion and virus production altogether, therefore cutting the huge economic burden of CAR-T therapy.

843. Change of Tumor-Infiltrating Lymphocytes after Neoadjuvant Chemotherapy & Their Clinical Significance in Breast Cancer

Mieson Lee[1], Bo-Im Kim[2], Won Seon Bang[2], Gyungyub Gong[1], Hee Jin Lee[1]

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The level of tumor-infiltrating lymphocyte (TIL) is a predictive factor for responsiveness of neoadjuvant chemotherapy (NAC) in breast cancer. However, changes of TILs after NAC & their clinical significance have not been clearly defined. We performed analysis of a series of consecutive breast cancer patients treated with NAC. We histologically evaluated & compared the level of TILs in paired pre-chemotherapy biopsies & post-chemotherapy operation specimens without pathologic complete response (pCR) upon NAC. We also correlated changes of TILs with response to NAC & patients' survival outcome. Average level of TILs was significantly lower in post-chemotherapy operation specimens than pre-chemotherapy biopsies in HR+ (biopsies, 6.9 ± 11.8; operations, 3.3 ± 11.2; P<0.001) & TNBC (biopsies, 18.4 ± 17.2; operations, 14.9 ± 25.9; P=0.038). The level of TILs was increased after NAC in 3.9%, 11.3%, 19.8%, and 17.6% of HR+, HR+/HER2+, HER2+, & TNBC, respectively. In contrast, the level of TILs was decreased after NAC in 16.3%, 17.5%, 31.9%, & 40.7% of HR+, HR+/HER2+, HER2+, & TNBC, respectively. The level of TILs in post-chemotherapy operation specimens was significantly correlated with increased residual cancer burden (RCB) class and decreased Miller-Payne grade only in HER2+ breast cancer (P=0.046 and P=0.023, respectively). Increase of TILs in post-chemotherapy operation specimens compared to pre-chemotherapy biopsies showed significantly positive correlation with RCB class (P=0.048) in HR+/HER2+ breast cancer. In survival analysis, patients with increase of TILs in post-chemotherapy operation specimens showed significantly better disease-free survival than those with no change or decrease of TILs in TNBC (P=0.042, hazard ratio=0.290, 95% confidence interval=0.088 ~ 0.958). However, TILs in pre-chemotherapy biopsies or post-chemotherapy operation specimens were not associated with survival outcome in breast cancer patients without pCR. Breast cancer subtypes showed different pattern of TIL changes after NAC. Increase in the level of TILs in post-chemotherapy operation specimens compared to pre-chemotherapy biopsies was an independently better prognostic factor in TNBC.

844. Minicircle DNA as Starting Material for Development of ATMPs

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Minicircle DNA is currently gaining increasing importance for clinical research applications in gene therapy and genetic vaccination. For direct gene transfer into humans, *GMP Grade* DNA is mandatory whereas for GMP production of e.g. mRNA or viral vectors (Lenti, AAV etc.), in many cases *High Quality Grade* (HQ) DNA is accepted as a starting material. If the therapeutically active substance is a genetically modified cell, e.g. for an advanced therapy medicinal product (ATMP), the situation is currently controversially discussed, here the DNA may either serve as a starting material or be part of the drug substance. Based on results clearly showing the advantages of *minicircle* DNA in direct comparison to the corresponding plasmid, *minicircle* DNA shows a great benefit on the one hand avoiding the presence of unnecessary bacterial backbone sequences, on the other hand due to higher transfection efficiencies. Hence, these constructs provide a striking benefit especially for production of AAV vectors and CAR-T cells and both are currently the most promising tools moving forward to clinical applications. Consequently, in close collaboration with the leading experts in the fields of AAV and CAR-T cell-based therapies, we are developing a process for the production of *High Quality Grade minicircle* DNA, meeting the requirements of most regulating agencies. ***High Quality Grade*** *minicircle* DNA is produced in a dedicated facility. Starting from a characterized Research Cell Bank (RCB), the manufacturing process passes through different well-documented production steps. The HQ fermentation facility is completely separated from purification (chromatography) to ensure that the sensitive DNA downstream processing is not affected by any living contamination. The proprietary special purification procedure results in pure, supercoiled (ccc) *minicircle* monomers, meeting the regulatory requirements to have a defined, homogenous product, proven by a number of quality controls for the cell bank and to the *minicircle* DNA product.

845. Development and Validation of In-House CAR-T Cell Manufacturing

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Treatment of patients with autologous T cells expressing chimeric antigen receptor (CAR-T) is a promising adoptive cell therapy already FDA approved for the treatment of hematologic malignancies. Manufacturing of approved CAR-T products is limited primarily to commercial production facilities and remains a limiting factor for wider implementation of the technology. We have developed methods for in-house manufacturing of CAR-T products in order to reduce cost, decrease turnaround time and increase flexibility to manufacture newly developed products. Manufacture of CAR-T products for a clinical trials falls under the scope of an Investigational New Drug (IND) application to the FDA. We describe here our experience developing

the processes required for submission of an IND to treat patients with novel CAR-T products. We built on the T Cell Transduction (TCT) process on Miltenyi CliniMACS Prodigy to generate methods for manufacturing GMP grade CAR-T cells to treat indicated tumors. The TCT process is completed entirely on the instrument to reduce the opportunity for introduction of human error or contamination. Steps include purification of T cells from a leukapheresis product followed by activation and introduction of the CAR vector. Importantly, the development of release testing to demonstrate purity, potency and safety must be performed. Final product is characterized to meet standards required by the FDA for GMP grade CAR-T products and will include sterility and functional tests to ensure safe and efficient delivery of product to the patient. As the scope of CAR-T therapy expands in the clinic, the bottleneck persists with the limited manufacturing capacity of commercial facilities and obstacles to carry out clinical trials. The result is patients waiting for a lifesaving treatment until there is a slot open to manufacture their product. Our goal is to eliminate the need for external manufacturing by making products in-house, resulting in a cheaper and more flexible process for generating CAR-T products and a shorter time from target discovery to patient delivery.

846. Novel Humanized EGFR Variant III-Specific Chimeric Antigen Receptor T-Cells for Glioblastoma

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YooYoung Pharmaceutical Co., Seoul, Korea, Republic of

Glioblastoma is the most challenging brain tumor. There is currently no effective treatment. Chimeric antigen receptors (CARs) redirected T-cells to specific tumor antigens hold promise in the treatment of glioblastoma, and have the potential to improve both survival and quality of life. We exploited the variant III mutation of the epidermal growth factor receptor (EGFRvIII). EGFRvIII is a tumor-specific mutation observed in 30% of glioblastoma, and expression of EGFRvIII is linked to poor long-term survival. We generated *novel* fourth-generation EGFRvIII-specific CAR-T cells, and tested in vitro for its ability to direct EGFRvIII-specific CAR-T cells to specifically lyse, proliferate, and secrete cytokines in response to EGFRvIII-bearing targets. These data provide evidence for an effective therapy of EGFRvIII-positive glioblastoma.

847. Walk-Away Automation of Cell Immunostaining Assays by Centrifuge-Less DA-Cell™ Washer and Plate for Superior Data and Faster Workflow

Namyong Kim, Melvin Lye

Marketing, Curiox Biosystems, San Carlos, CA

We describe the DA-Cell™ system, a novel wall-less plate and laminar-flow cell washer that enables the automated washing of suspension cells and retains more than 95% cells at a fraction of the time and with higher viability of cells. The wall-less DropArray (DA) plate consists of an array of 96 hydrophilic spots separated and surrounded by a hydrophobic surface, which functions as a virtual wall. In a typical immunostaining assay, a 50 µl drop, containing cells and antibody mix, is dispensed on each spot of the DA plate. During incubation, cells settle on the surface of the spots. The plate then undergoes a laminar-flow washing process in DA-Cell washer by repeated cycles of aspiration and dispensing of buffer through two sets of nozzles. The controlled buffer flow minimizes turbulence and cell loss. The cell washing process only requires 3-4 minutes by eliminating the need of a centrifuge, which also reduces stress on the cells and possible cross-contamination of antibodies on cell membranes, leading to better segregation of cell populations in flow cytometry. Additional incubation time of 10-20 minutes improves cell retention to more than 99%. Most importantly, cell incubation and washing on a DA-Cell system minimizes operator variability as mixing and washing steps are mechanically controlled and mostly automated, significantly improving reproducibility and consistency of flow cytometry analysis. We demonstrate the integration of DA-Cell washer into a benchtop fully-automated liquid handling and scheduling robotic work station for sample preparation, 'DA-Cell Auto'. The automation of cell washing with DA-Cell eliminates the challenges of integrating centrifuge to robotic workstations, while reducing the number of commands by 6 times and decreasing the chances for errors and malfunction. Time take for two rounds of centrifugation is also reduced from 30minutes to 7minutes. Moreover, the small footprint of DA-Cell Auto is approximately half that of conventional centrifuge-based workstation, enabling easy adoption In the laboratory. Here, we show a series of immunostaining assays comparing the DA-Cell system with a conventional centrifugation. Based on the staining index, absolute cell counts and reproducibility data, we demonstrate that the DA-Cell system produces superior results while simplifying and expediting cell preparation for flow cytometry analysis.

848. Centrifuge-Less Red Blood Cell Lysis and Immunostaining of Whole Blood for Flow Cytometry Using DA-Cell Washer and Plate

Namyong Kim, Chyan Ying Ke

Marketing, Curiox Biosystems, San Carlos, CA

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 lysis and leukocyte immunostaining on a centrifuge-less platform DA-Cell™, using a novel wall-less plate and laminar flow washer. The DropArray (DA) 24-well plate consists of an array of hydrophilic spots surrounded by hydrophobic surface, which functions as a virtual wall that separates each spot. Each well is capable of staining and lysing 100uL whole blood. During lysis, WBC settle to the surface of the spot, allowing the spent lysis buffer to be removed by a gentle laminar-flow washing process in the DA-Cell washer, eliminating centrifugation that stresses cells and disrupts antibody binding. We demonstrate similar or higher retention of CD45+ lymphocytes with lysis in DA-Cell plate compared to conventional tube centrifuge.

In this presentation, we will also compare the staining index and resolution of cell cluster by flow cytometry. In summary, DA-Cell system provides gentle, fast and convenient blood lysis, while improving data quality with superior antibody staining.

849. The Development of an Integrated Platform for IDH1.R132H Mutant Specific TCR Discovery in a Glioma Vaccine Trial

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Immunotherapy, including the adoptive transfer of T-cells genetically engineered to recognize tumor antigens, has revolutionized cancer medicine. Central to an effective and specific cancer immunotherapy is the discovery of tumor-specific targets. However, interpatient heterogeneity requires highly personalized, scalable, cost- and time efficient approaches for the identification and safe deployment of T-cell receptors (TCRs) recognising such targets. Despite technological advances in single-cell sequencing, bioinformatics and molecular biology, a fully integrated procedure for identification, characterization and delivery of tumor-specific TCRs is still lacking. One example of such a target is the recurrent driver mutation IDH1.R132H found in >70% of low grade gliomas, which confers a neomorphic enzymatic function upon the gene encoding for isocitrate dehydrogenase 1 (IDH1). Recently we have conducted a multicentre, first-in-man trial in which IDH1.R132H^{+ve} glioma patients were vaccinated with a IDH1R132H-peptide. Each patient in the NOA-16 trial (n=33) was HLA typed and subject to extensive immune monitoring of their vaccination response, including functional characterisation using ELISPOT assays and TCR beta repertoire profiling using Adaptive's ImmunoSEQ® platform. In total we generated ~250 samples encompassing over 15 million TCR beta clonotypes. To identify IDH1.R132H specific TCRs within these samples we combined extensive bioinformatic repertoire analyses with experimental expansion of antigen specific TCRs using the MANAFEST platform and 10x Genomics' Single Cell Immune Profiling. Here we 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. This pipeline has allowed for the identification and functional validation of IDH1.R132H specific TCRs as a first step towards implementing adoptive T cell therapies in glioma patients, and serves as a model for TCR discovery in other cancer modalities.

Hematologic & Immunologic Diseases II

850. Efficient and Sustained FOXP3 Locus Editing in Hematopoietic Stem Cells as a Therapeutic Approach for IPEX Syndrome

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Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a rare monogenic primary immunodeficiency, characterized by multi-organ autoimmunity. IPEX syndrome is caused by defective regulatory T (Treg) cell generation and function due to mutations in transcription factor FOXP3. The dysregulation of Treg cells is the main pathogenic event leading to early disease onset and fatality typically within the first few years of life. Supportive therapies combined with pharmacological immunosuppression are currently used for controlling the symptoms of the disease. Allogeneic hematopoietic stem cell transplantation leads to best outcomes provided a matched donor is available. Gene therapy by transduction of CD4+ T cells with lentiviral vectors constitutively expressing FOXP3 can convert effector T cells into Treg cells, although long term survival of transduced cells in vivo is still under investigation. Replacement of the defective FOXP3 gene with a functional cDNA in hematopoietic stem cells is likely to provide an effective and sustained long-term cure as this will allow locus specific expression of FOXP3 regardless of the downstream mutation. To this end, we tested multiple guide RNAs for their ability to cleave at the FOXP3 locus and achieved up to 80% cleavage in mobilized CD34+ peripheral blood stem cells (PBSC) from multiple GM-CSF-mobilized donors with the top performing guide RNA. We then utilized the CRISPR/Cas9 (delivered as RNPs) to introduce a double strand break and provided donor templates with homology arms flanking a GFP expression cassette via an AAV6 donor vector to achieve homology directed repair (HDR). HDR rates ranging from 40-60% were achieved in PBSC from multiple healthy donors. Next, we assessed the long-term engraftment of edited (GFP-expressing) PBSC in NOD.Cg-KitW-41J Tyr+Prkdcscid Il2rgtm1Wjl recipient mice. Experimental mice were analyzed 12-16 weeks post-transfer of edited PBSC for engraftment of hCD45+ cells in the bone marrow. Up to 80% of the cells in the bone marrow were hCD45+ with ~5% of those cells expressing GFP, indicating the ability of edited cells to engraft long term. All recipients harbored edited cells in the myeloid, granulocyte, and B cell populations and these lineages were present at ratios equivalent to recipients of mock-edited human CD34+ cells, suggesting that the differentiation capacity is not compromised by editing the FOXP3 locus. Finally, we edited the FOXP3 locus with AAV6 donors designed to introduce a FOXP3 cDNA to enable expression from the endogenous promoter upon targeted integration. Using this approach, as determined by droplet digital PCR, we achieved efficient locus editing with rates comparable to our GFP donor constructs. Overall, these findings demonstrate efficient editing of the FOXP3 locus in human hematopoietic stem cells and sustained

engraftment of FOXP3 edited cells at levels predicted to provide clinical benefit. Taken together, these finding support pursuit of this approach as a potential long-term therapy for IPEX patients.

851. Gene Therapy of Patients with Leukocyte Adhesion Deficiency Type I (LAD-I): Preclinical Studies and Clinical Trial Design

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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, required for normal leukocyte trafficking and extravasation to infection sites. The characteristic clinical feature of LAD-I is the increased number of infections, which cannot be properly resolved by phagocytic cells. During recent years we aimed at developing a gene therapy approach for LAD-I based on the gene correction of autologous hematopoietic stem cells (HSCs) with a lentiviral vector (LV) in which the expression of hCD18 is driven by a chimeric promoter with higher gene expression activity in mature myeloid cells. The infusion of gene-modified HSCs corrected the characteristic phenotype of hypomorphic and knock-out LAD-I mouse models, including leukocytosis and defective migration of leukocytes upon appropriated stimulus, with a concomitant absence of hematotoxic or genotoxic effects. Based on these preclinical studies, the therapeutic Chim.hCD18-LV obtained Orphan Drug designation in 2016, both in Europe (EU/3/16/1753) and in the USA (DRU-2016-5430). Further studies were conducted with a GMP-produced Chim.hCD18-LV to optimize the transduction of human $CD34^+$ cells using a combination of specific transduction enhancers. The addition of these molecules consistently increased the transduction efficiency of $CD34^+$ cells, without detrimental effects on their clonogenicity or repopulating ability in NSG mice. Finally, three validation runs using optimized transduction conditions have been performed demonstrating robust and reproducible transduction efficiencies (2-4 copies of the therapeutic vector per cell) and maintained clonogenicity and repopulation ability of either fresh or cryopreserved $CD34^+$ cells. A Phase I Clinical Trial for LAD-I has been authorized in Spain that will allow us to initiate the treatment of pediatric patients with severe LAD-I. The clinical trial will use G-CSF/plerixafor-mobilized $CD34^+$ cells that will be transduced with the Chim-hCD18-LV and then cryopreserved until verification of the specifications of the medicinal product. Cells will be then thawed and infused in patients subsequent to myeloablative conditioning.

852. Correction of SCID-X1 T-Cell Deficiency by a Gene Therapy Approach Based on Baboon Pseudotyped LVs

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T-cells represent valuable tools for treatment of cancers, infectious or inherited diseases but they are mainly short-lived *in vivo*. Therefore, gene transfer into long-lived persisting naïve T-cells or T-cell progenitors could represent a main improvement for T-cell therapies. We have demonstrated that baboon envelope glycoprotein pseudotyped lentiviral vectors (BaEV-LVs) outperformed by far other LV-pseudotypes for transduction of adult naïve or memory T-cells and thymocytes. Moreover, BaEV-LVs transduced efficiently T-cell progenitors generated from hematopoietic stem and progenitor cells (HSPCs) exposed to the Notch Delta-like ligand-4 (DL-4). In NOD/SCIDγ$C^{-/-}$ mice, these transduced cells efficiently seed the thymus to produce mature T cells in a faster kinetic as compared to HSPCs counterpart. Here we evaluated the ability of γC-encoding BaEV-LVs to correct HSPCs from a patient with X-linked severe combined immunodeficiency (SCID-X1). Patient's HSPCs cells were cultured in the conventional gene therapy conditions (GT) or in combination with the DL-4-based T-cell progenitor differentiation technique (DL4). The viability and the transduction efficiency was similar in both condition demonstrating that BaEV-LVs has no toxic effect on Patient's HSPCs. As expected, a higher number of T-cell progenitors was observed in the DL-4 culture as compared to the conventional culture. We then evaluated the efficiency of gene corrected cells to differentiate into T-cells after co-cultured on OP9-hDL1 stromal cell line. Double positive $CD4^+CD8^+$ cells and $CD3^+$ T cells were present as early as week 2 in the transduced DL-4 condition and only at week 3 in the transduced GT condition. Higher numbers of T cells were observed in the DL4 condition as compared to the GT in the earlier weeks. Our results demonstrate that BaEV-LVs can drive correction of γC deficiency in both conventional GT and DL-4-culture conditions but T-cell differentiation was faster and more efficient under DL-4-conditions. Collectively, these data indicate that BaEV-LVs are valuable tools to induce genetic modifications of naïve T-cells or gene-corrected T-cell progenitors from SCID patients. Ultimately, the co-injection of LV-corrected T-cell progenitors and HSCs represent an interesting strategy to accelerate T-cell reconstitution in immunodeficient patients.

853. Lentiviral CYBB Gene Therapy Restores Oxidase Activity and Relieves Disease Symptoms in an X-CGD Infant

Lung-Ji Chang[1,2,3,4,5], Yuan Sun[4], Shih-Ting Tsao[2], Juan Xiao[4], Rui Zhang[2], Siqian Gui[2], Jie Zheng[5], Jian-Xing He[5], Huyong Zheng[5]

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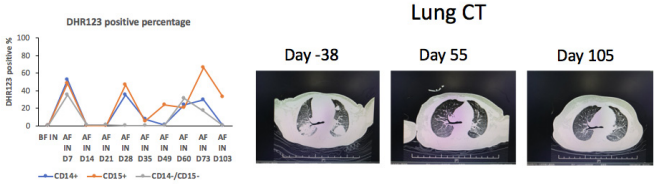
X-linked chronic granulomatous disease (X-CGD) is a genetic disease caused by a mutated CYBB gene (gp91phox) affecting the NADPH oxidase activities. The dysfunction of NADPH oxidase results in the lack of reactive oxygen species (ROS) in activated phagocytes including neutrophils, monocytes and macrophages, leading to death from recurrent infections. There is no cure for the disease except for allogeneic hematopoietic stem cell transplantation (allo-HSCT). Gene therapy has become one of the most promising treatment for X-CGD since most patients carry a single mutated gene. We have used a lentiviral EF1a-CYBB gene (TYF-CYBB) to modify CD34+ cells of X-CGD patients (NCI registry NCT03645486). The one-month-old male infant has a CYBB mutation carrier mother and genetically normal father. He had severe pulmonary infection, which was controlled by anti-fungal and anti-bacterial medication at the time of the recruitment. The patient underwent G-CSF mobilization and bone marrow aspiration at 37 days and 4 days before stem cell collection. CD34+ cells were selected and subjected to two rounds of lentiviral transduction and used for transplantation. The patient received busulfan and fludarabine conditioning prior to infusion with 2.04x10^6/kg TYF-CYBB-modified CD34+ cells. Peripheral blood was monitored weekly for the oxidase activity by DHR123 assay and the TYF-CYBB gene copy number was determined by qPCR. We observed two peaked oxidase activities during the first 103 days after infusion. The first peak appeared at day 28 after infusion with 47% DHR123 positive cells in peripheral blood. The second peak was at day 73 with 66% oxidase positive cells, compared to 0% DHR123 positive cells before infusion. LV-CYBB gene copy number in peripheral blood cells reached 3.75% and 2.41% at day 21 and day 35, respectively. CT scan of the lung showed complete resolution from infections and resolved granulomatous complications. The general physiological condition of the patient has improved without further medication, and we did not see any adverse effects from myeloablative conditioning or the infusion of the TYF-CYBB modified CD34+ cells. Our study showed that the lentiviral TYF-CYBB gene therapy can successfully resolve CGD associated symptoms in the patient in two months. Continued follow-up is needed to evaluate the long term efficacy of this gene therapy regimen.



854. In Vivo Expansion of CMV-Specific Anti-HIV CAR T Cells Following CMV Superinfection

Nathan Michel Johnson[1], Chengxiang Wu[1], Shan Yu[1], Hui Li[2], Gautam Sahu[3], Preston Marx[1], Dorothee von Laer[4], Gail Skowron[5], George Shaw[2], Richard P. Junghans[6], Amitinder Kaur[1], Stephen E. Braun[1]

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Human Immunodeficiency Virus-1 (HIV-1) has killed over 35 million and infects 1.8 million new people each year. Antiretroviral therapy (ART), although effective in controlling plasma viremia and transmission, does not purge the latent or persistent reservoirs necessary to eliminate infection, and must be maintained for life. It is thus imperative to discover therapeutics that provide both lifetime suppression of viral loads and depletion of viral reservoirs. Recently, studies have demonstrated control of viral replication and decreasing viral reservoirs in 50% of rhesus vaccinated with a CMV vaccine vector. They propose that continuous immunosurveillance of SIV by T effector memory (T_{EM}) cells is maintained by the persistent CMV vectors. Utilizing CAR T cells, this strategy could be extended to control viral replication in HIV$^+$ patients. To mimic immunosurveillance elicited by the CMV vector, we stimulated rhesus PBMCs with rhCMV peptide pools (IE1, IE2, and pp65) to expand CMV-specific T cells. In contrast to CD3/CD28 bead stimulation, which typically gives rise to T central memory cells (T_{CM}) differentiated from naïve cells, response to CMV restimulation induces expansion of T_{EM} cells. Utilizing this approach, we genetically modified CMV-specific T cells with retroviral vectors expressing CD4-CAR, converting them into HIV-specific effector cells. These vectors utilize a bicistronic design, expressing the CD4 extracellular domain with intracellular T cell signaling domains, as well as an maC46 domain to protect against viral entry. The genetically modified T cells target a critical step in the viral life cycle independent of MHC presentation, targeting heterogeneous viruses while avoiding the potential for viral escape. We hypothesize that continuous stimulation of CD4-CAR T cells through their CMV-specific TCR will not only maintain activated T_{EM} CTL capable of targeting HIV infected cells, but also circumvent the potential for T cell exhaustion that occurs when relying solely on *ex vivo* stimulation to generate therapeutic levels of T cells. Using a cohort of 4 macaques chronically infected with SHIV, we infused CMV-specific CD4-CAR/maC46 T cells. Upon CAR infusion, ART was terminated, allowing viral rebound and antigen to reappear for *in vivo* expansion of CAR T cells. We find CMV-specific CAR T cells exist *in vivo* primarily as T_{EM}, comprising on average 1.5% (SD ± 0.9) of CD8$^+$ T_{EM} in peripheral blood 5 months after infusion. To further boost these CAR T cells *in vivo*, we superinfected animals with rhCMV at 6 months post infusion. Three animals showed an increase in the circulating CD8$^+$ T_{EM} CD4-CAR population to an average of 2.7% (SD ± 0.6) (Fig1), while the fourth animal did not respond. Despite this expansion of CAR T cells, PVL remained unchanged. These studies suggest that viral specific CAR T cells are capable of proliferating in response to endogenous viral specific TCR stimulation, and that boosting via the viral specific TCR is a viable strategy for enhancing CAR T cell frequency *in vivo*.

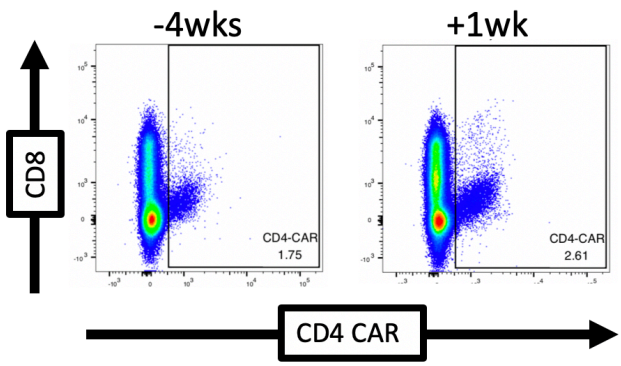

Fig1. CAR T cells pre and post CMV Superinfection Representative flow of T_{EM} ($CD28^{-}$,$CD95^{+}$) at 5 months (-4wks CMV Superinfection) and 6 months (+1wk CMV Superinfection) following CAR infusion from a responding animal.

855. Gene Editing for Treatment of X-linked-Agammaglobulinemia: Comparison of Three Alternative Nuclease Platforms in Promoting Homology-Directed-Repair

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The immunodeficiency, X-linked agammaglobulinemia (XLA), is caused by mutations in the gene encoding Bruton's Tyrosine Kinase (BTK). BTK is required for B-cell development and activation following BCR-engagement and is also required for optimal signaling function in myeloid cell populations. Patients lacking functional BTK lack mature B cells and fail to produce antibodies leading to recurrent and life-threatening bacterial infections. Available treatments are limited to intravenous immunoglobulin therapy and judicious use of antibiotic therapy. BTK is an ideal target for gene editing in hematopoietic stem cells (HSC) as BTK-expressing B cells exhibit a marked selective advantage in vivo with as few as 1% normal HSC capable of rescuing B cell development in murine models, suggesting that a similar proportion of repaired HSC may rescue the B cell compartment in XLA patients. Thus, introduction of an intact BTK cDNA under the control of the endogenous promoter and enhancer in HSC is anticipated to rescue the immunologic and functional deficits and provide a potentially curative therapy. In the current study, we demonstrate efficient gene editing of the BTK locus in human mobilized, CD34+ peripheral blood stem cells (PBSC). To achieve this goal, we generated AAV donor templates designed to drive homology directed repair (HDR)-based, targeted integration of an expression cassette into the first coding exon of BTK gene. In parallel, we developed and directly compared three alternative designer nuclease platforms targeting BTK: transcription activator-like effector nucleases (TALENs), megaTALs (fusions of a homing endonuclease and single TALE effector domain) and CRISPR/Cas9. MegaTALs specific for the BTK were iteratively engineered utilizing yeast-surface display binding/cleavage and selection strategy. Top-performing nucleases were subsequently tested in vivo. TALEN and CRISPR/Cas9 nucleases were developed using established methods and tested in parallel. Next, utilizing co-delivery of each top-performing designer nuclease and its respective, cleavage resistant, AAV6 donor construct, we determined the rates of HDR using all three platforms in PBSC. To simplify the analysis, the AAV donor templates were designed to knock-in a promoter driven GFP cassette into the first coding exon and were flanked by 1 kb of homology arms. Cell viabilities post editing were comparable across the three platforms at ~70% while average HDR rates were 18%, 13% and 11% for megaTAL, RNPs and TALENs respectively (n=4 CD34+ donors). Minimal GFP expression was seen in samples treated with AAV alone. We also assessed the ratio of HDR vs non-homologous end joining (NHEJ) in PBSCs edited using these alternative nucleases and AAV donors by ICE (Inference of CRISPR edits) analysis (for NHEJ) and droplet digital PCR (for HDR). The HDR/NHEJ ratio was 2-fold higher in megaTAL treated cells compared to the other platforms. Importantly, edited HSCs retained the potential to give rise to multiple lineages in colony forming unit assays. While our data demonstrate efficient editing with all candidate platforms, our findings suggest that the megaTAL platform may provide greater efficiency based upon higher overall HDR and a more favorable HDR/NHEJ ratio. Together these data support the continued investigation of megaTALs and HDR based gene editing as a potential therapeutic strategy for the correction of XLA.

856. Cure of X-SCID Mice after Ex Vivo Non-Viral Genome-Editing Therapy of Hematopoietic Stem Cells

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X-linked severe combined immunodeficiency (X-SCID) is a congenital disorder of hematopoietic stem cells (HSCs) caused by genetic defect of the interleukin-2 receptor gamma-chain (*IL2RG*) gene on the X chromosome. X-SCID is a major type among all SCIDs. Disruption of *IL2RG* causes under development of T cells and NK cells and makes patient extremely vulnerable to infection. Although a cure for X-SCID can be brought by allogeneic HSC transplantation, more than half of the subjects cannot find their matched donors and die within 2 years of birth. Although, gene therapy of HSCs has been attempted as an alternative treatment, the therapy may cause insertional leukemogenesis, due to vectors random integration into genome. Therefore, repairing the mutated genome of patients' HSCs with genome-editing technology such as CRISPR/Cas9 without viral delivery methods is a hot topic. In this study, we aimed to develop a targeted genome-editing therapy, which is "universal" to work with

most of the mutations on exons and uses "natural promoter" using a non-viral delivery method. Our strategy of *ex vivo* gene-editing therapy of X-SCID is 1) generation of double strand break in intron1 of *Il2rg* by electroporation of Cas9 gRNA to HSPCs 2) insertion of *Il2rg* exon 2-8 cDNA+splice acceptor (knock-in vector) which allows corrected *Il2rg* expression under the natural promoter and works regardless of mutations 3) transplantation of the *ex vivo* genome-edited HSPCs. We constructed a knock-in vector to function regardless of mutations of *Il2rg* and allow the corrected *Il2rg* to express under the "natural promoter"; that is to say, we inserted *Il2rg* exon 2-8 cDNA fragment with splice acceptor into *Il2rg* intron 1 so that the endogenous exon 1 and inserted exon 2-8 express under the natural promoter. We electroporated both Cas9-RNP and knock-in vector to SCID mice-derived (CD45.2) bone marrow cells. According to the clonogeneic colony-forming unit assay, we found approximately 20% of colonies showed successful insertion of *Il2rg* exon 2-8 cDNA in intron 1. Furthermore, we transplanted *ex vivo* genome-edited HSPCs to NOG mice (CD45.1) as a primary recipient in two groups (Cas9-RNP only treated; Cas9-RNP+insertion vector treated group). We followed each recipients after transplantation for 12 to 16 weeks. In primary recipients, CD3(+) (12.5%±2.1) and CD132+ (*Il2rg*) T cells detected at 12 to 16 weeks post-transplantation (n=3) in Cas9-RNP+insertion vector treated group which was significantly different from Cas9-RNP treated group ($p<0.01$). Notably, we observed the development of the thymus in primary recipients at 20 to 24 weeks post-transplantation, showing that transplantation of *ex vivo* genome-edited cells can phenotypically correct X-SCID. We then performed serial transplantation and found CD3(+) and CD132(+) cells in secondary recipients as well, which indicating a successful genome-editing of long-term repopulating *bona fide* HSCs. In summary, we demonstrated that the insertion of cDNA fragments to introns can restore gene functions in a natural manner regardless of mutations of the disease in HSPCs. In addition, our results show that the present method of non-viral genome-editing can cure X-SCID mice. It would be a promising therapy in X-SCID and other genetic hematopoietic disorders.

857. Manufacturing of Gene-Edited, Regulatory-Like, T Cells (edTreg) for Treatment of IPEX and Other Autoimmune Disorders

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IPEX (immunedysregulation, polyendocrinopathy, enteropathy, X-linked) is a severe congenital autoimmune disorder resulting from hemizygous inheritance in males of a mutant *FOXP3* allele. *FOXP3* encodes a transcription factor that governs the development, maintenance, and function of regulatory T cells (Treg). Tregs are a distinct T cell lineage that restrict autoimmune responses in healthy subjects by suppressing self-reactive effector T cell activation and proliferation. To date, more than 70 *FOXP3* mutations have been identified in IPEX patients. IPEX syndrome manifests as early and severe onset of multi-organ autoimmunity, including severe enteropathy, T1D, thyroiditis and dermatitis. Without stem cell transplant or aggressive immunosuppression, most of affected subjects will die within the first few years of life. Even if a stem cell transplant is available, achieving sufficient clinical stability to undergo the transplant procedure is challenging. Here, we aim to develop an autologous regulatory T cell therapy for IPEX and other severe autoimmune disorders using a modification of a previously reported gene-editing approach. In our previous proof-of-concept gene editing design we co-delivered TALEN nucleases and AAV6 donor template designed to introduce a constitutively active MND promoter into the *FOXP3* locus via homology-derived repair (HDR). This approach permitted us to bypass endogenous *FOXP3* promoter silencing and enforce FOXP3 expression in CD4 effector T cells (Teff). The resultant high level and stable expression of endogenous FOXP3 converted Teff to Treg-like cells with immunosuppressive activity. In the current study, to permit therapy for IPEX, we modified our gene editing of *FOXP3* to utilize co-delivery of CRISPR/Cas9 RNPs and an AAV6-delivered donor template designed to integrate an expression cassette containing the MND promoter driving expression of two coding sequences separated by a ribosome skip peptide: functional FOXP3 cDNA and a surface LNGFR tag. On-target donor template integration will provide both stable FOXP3 and selection marker expression for use in manufacturing and also simultaneously knock-down expression of the mutant *FOXP3* allele in IPEX. In parallel, we established a clinical cell manufacturing protocol enabling the transition to CMC process development. Using the current protocol, we obtained efficient HDR rates across multiple healthy donors. Edited cells were consistently enriched to >95% purity by a magnetic LNGFR antibody selection and expanded 50-fold in a week. Expression of FOXP3 cDNA in edited cells was sufficient to enforce Treg-like phenotypes including the up-regulation of Treg-associated markers (CD25, CTLA-4, and ICOS), and down-regulation of CD127 and inflammatory cytokines (IL2, IFNgamma, TNFalpha). Importantly, we demonstrate sustained *in vivo* suppressive activity of these edited Treg-like cells (edTreg) in a xeno-GvHD mouse model. edTreg (as well as expanded natural Treg) limited effector T cell expansion and tissue infiltration and significantly protected mice from xeno-GvHD induced by co-transferred autologous effector T cells. Along with preliminary data showing successful editing in CD4 T cells from IPEX patients, our data provide key pre-clinical proof-of-concept and safety data supporting use of edTreg in a clinical trial for IPEX and, potentially, for use other autoimmune diseases.

858. T Cell Populations in Dogs with Canine Leukocyte Adhesion Deficiency Treated by Gene Therapy Using a Foamy Viral Vector

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Dogs with canine leukocyte adhesion deficiency or CLAD, similar to children with LAD-1, suffer from recurrent bacterial infections and early death due to the absence of the CD18 integrin on leukocytes.

The longer life span of dogs allows longer follow-up than mice, and dogs have been used historically as a valuable model system for human transplantation studies. In older children and adults with LAD-1, revertant CD18+ T-cell populations often arise over time, due to the key role that CD18 plays as a co-receptor in T-cell activation. These CD18+ T-cells may assist in fighting bacterial infections prevalent in these LAD-1 patients, but may be sub-effective due to the lack of other complementary T-cell compartments. In CLAD dogs treated by gene therapy, peripheral blood analysis of T-cell subsets demonstrated an expansion of CD18+ T-cell populations over time. We present here an analysis of these expanded T-cell sub-populations to determine subset bias in long-term studies of treated CLAD dogs. All four dogs were treated as 6-8-week-old CLAD pups that received a reduced intensity conditioning regimen (e.g. IV Busulfex 8 mg/kg) prior to infusion of CD34+ cells transduced with a foamy viral vector DP-MSCV-cCD18 containing a canine CD18 cDNA. Peripheral blood obtained over time (to 8 years) was lysed of RBCs, and analyzed on a flow cytometer for the following leukocyte markers: neutrophils (CADO48A mAb); monocytes (CD14); B-cells (CD21); and T-cells (CD3). T-cell subsets were further analyzed using antibodies to: CD4, CD8, CD25, CD45RA, CD62L, and FoxP3. All leukocytes were assessed for gene correction by CD18. Overall CD18+ leukocyte levels in the peripheral blood ranged from 2% to 13% at 8 years. CD18+ T-cell levels in the peripheral blood were notable, with CD18+ T-cell levels at 14-19% at 1-year post infusion, expanding to 36-54% by 8 years. Effector memory (EM) T-cells (CD45RA- CD62L-), showed a population bias of CD18+ CD4+ cells (39% vs 17% CD18-). Central memory (CM) T-cells (CD45RA- CD62L+) also showed an increased bias in CD4+ CD18+ populations (14% vs 8%). Naïve cells (CD45RA+ CD62L+) were disproportionately represented in CD18- CD4+ subsets (68% vs 32% CD18+). T-regulatory cells (CD3+ CD4+ CD25+ FoxP3+) did not show significant differences in subpopulations between CD18+ and CD18- cells (40% to 47% CD18+). In conclusion, long-term follow-up of gene therapy treated CLAD dogs provide a unique model to assess the role of CD18 in long-term T-cell subset maintenance and interactions. The availability of anti-dog antibodies and cross-reactive anti-human antibodies allowed the definition of key T-cell subsets in treated dogs. Not surprisingly, memory T-cells in all cases were expanded in CD18+ T-cell compartments, whereas naïve T-cells were more highly prevalent in CD18- T-cell compartments. Although untreated CLAD dogs, like LAD-1 patients, have T-cells in the peripheral blood, antigen stimulation is impaired in CD18- T-cells. Although primarily a disease affecting the myeloid compartment, granulocytes in particular, LAD-1 does lead to dysfunction in the T-cell compartment, especially in rejection. With heightened gene recombination in T-cells, newly arising CD18+ T-cells receive a strong selective advantage in antigen stimulation, leading to the increased presence of CD18+ cells in T-cell memory compartments over time.

859. Immunomodulatory Capabilities of CDC-Evs In Vitro and Their Therapeutic Potential in a GVHD Mouse Model, Improving Survival and Clinical Score

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Background: Multiple studies have shown that cardiosphere-derived cells (CDCs) have immunomodulatory capabilities, limit fibrosis and promote tissue regeneration. Previously we have shown that extracellular vesicles produced by CDCs (EVs-CDCs) recapitulate most of the activities mediated by the cells, both *in vitro* and *in vivo*. It has also been reported that the immune system, mainly T cells and macrophages, play an important role in the healing and regeneration process in acute myocardial infarction models and other animal models. Our studies indicate that immunomodulation seems to be critical for the therapeutic activities elected by CDCs-EVs. **Aims:** To evaluate the ability of CDC-EVs to immunomodulate T-cells and macrophage activity *in vitro.*To study the therapeutic potential of CDC-EVs in a Graft Versus Host Disease (GVHD) mouse model, to compare CDC-EVs obtained with different isolation processes and to identify the minimal effective dose. **Methods:** EVs obtained from serum starved CDCs were isolated by ultrafiltration by centrifugation and added to human T lymphocytes activated by PHA. T-cells were labeled with CFSE and proliferation analyzed by flow cytometry. Mouse macrophages were isolated from peritoneal exudates from mice primed with 3% Brewer's Thioglycolate, treated with CDC-EVs or vehicle and phenotype tested by qPCR. GVHD was induced after delivery of allogeneic bone marrow cells and spleenocytes in a Balb/c mouse model. Once the disease was developed, mice were randomized and systemically injected with CDC-EVs or vehicle. Weight change, clinical score and animal survival were evaluated dayly or twice per week. Mice were sacrificed after 56 days of follow up. **Results:** CDC-EVs modulate in a dose dependent manner the immune response of *in vitro*activated human T cells. CDC-EVs were able to down regulate PHA-induced expression of CD69 in Tcells, resulting in a pronounced reduction of $CD4^+$ and $CD8^+$ T cell proliferation. We also observed that CDC-EVs upregulate the expression of anti-inflammatory genes in activated mouse macrophages. This immunomodulation was significantly higher than the one observed when EVs from MSCs were used. Repeated systemic dosing of CDCs-EV in a GVHD mouse model reverted the weight loss observed in vehicle treated mice, increased survival and reduced GVHD score. Moreover, this effect was observed with different isolation processes of CDCs-EVs. **Conclusions:** These data demonstrate that CDC-EVs have strong immunomodulatory capabilities on human activated T-lymphocytes and mouse macrophages. Repeat dosing of CDCs is effective increasing survival in a GVHD mouse model and reducing GVHD score. These data support the use of CDC-EVs for the treatment of inflammatory indications.

860. Engineering a WAS-Targeted megaTAL for High Efficiency Homology-Directed Repair (HDR)

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MegaTALs are monomeric hybrid nucleases comprised of a transcription activator-like effector (TALE) DNA binding domain linked to homing endonuclease (HE) cleavage domain. Their compact size coupled with high cleavage efficacy and specificity make megaTALs attractive reagents for clinical gene editing applications. Cleavage at the target site has three potential outcomes: (i) error-free repair via reannealing (ii) mutagenic NHEJ; or (iii) HDR using information contained within a suitably designed donor template (or sister chromatid). In previous work, we have shown that a substantial fraction of the megaTAL induced DSBs are resolved perfectly (resulting in no detectable genomic change) since co-expression of a 3' repair exonuclease (TREX2) results in a marked increase in the rates of gene disruption via NHEJ. Moreover, we have also shown that megaTAL-mediated DSBs are preferentially repaired by the HDR pathway in the presence of a DNA donor template suggesting that megaTAL editing might lead to reduced rates of undesired NHEJ events in the presence of a donor template. Here, we tested this concept using megaTALs designed to cut at a unique site in the human Wiskott-Aldrich syndrome (*WAS*) gene. The *WAS* gene is mutated in both Wiskott-Aldrich syndrome and X-linked thrombocytopenia and therefore represents an interesting target for HSC engineering. Using iterative yeast surface display (YSD), we engineered multiple novel HE variants that recognized and cleaved the intended target site *in vitro*. We used two selection approaches, either screening on the basis of HE cleavage alone or initial selection for high affinity HE variants followed by a cleavage screening step. Top performing WAS HE variants were converted to megaTAL format by combining with a TALE domain (containing a 12-repeat variable diresidue array designed to recognize a DNA target region upstream of the HE-target site). Cleavage activity of WAS megaTAL enzymes was initially screened in a HEK293T traffic light reporter (TLR) line, and the top candidates were subsequently evaluated in primary human $CD4^+$ T and $CD34^+$ cells using mRNA transfection. We show that megaTAL variants identified using the YSD cleavage screen were more active in the traffic TLR system compared to megaTAL variants identified through a combination affinity and cleavage YSD selection screen. In contrast, enzymes selected with the YSD cleavage screen were not optimal for megaTAL-driven HDR activity in primary $CD34^+$ cells. When CD34+ cells were transfected with WAS-specific megaTAL in the presence of an AAV6 donor template, the variant selected via combinatorial YSD affinity and cleavage screen exhibited the highest HDR rate compared to variants selected by cleavage screen alone. Strikingly, in the presence of an AAV donor, NHEJ rates in $CD34^+$ cells were reduced to background levels as assessed by TIDE/ICE sequencing. Taken together, these findings demonstrate that a combination cleavage and binding YSD screening approach selects for WAS-targeting HEs that drive efficient HDR in primary $CD34^+$ cells. These observations suggest that megaTALs engineered in this fashion may represent an optimal platform for transition to clinical genome editing applications in WAS and, potentially, other hematopoietic disorders.

861. Sustained Expression of C1 Esterase Inhibitor Using Recombinant AAV Gene Therapy for the Treatment of Hereditary Angioedema

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Hereditary angioedema (HAE) is an autosomal dominant disease characterized by unpredictable and excessively painful swelling attacks of the face, hands, feet, genitalia, or gastrointestinal tract, as well as possible life-threatening episodes affecting the throat and upper airway. HAE is caused by haplo-insufficiency at the SERPING1 locus which results in deficient activity of the SERPING1 protein product C1 esterase inhibitor (C1-INH). C1-INH is a serine protease inhibitor involved in the complement, contact, and fibrinolytic systems. Decreased C1-INH activity in HAE patients triggers swelling attacks via hyperactivation of proteases in the contact cascade, activation of B2 receptors on the endothelial cell surface, and resulting increases in vasodilation and vascular permeability in surrounding tissue. Additionally, it is speculated that hyperactivation of the complement cascade predisposes HAE patients to autoimmune disease. HAE is a promising candidate for AAV-mediated liver directed gene therapy since 1) C1-INH is a plasma protein synthesized in the liver, 2) protein replacement therapies are efficacious indicating that the effects of C1-INH are non-autonomous and also provides insight into efficacious target concentration, and 3) concerns regarding transgene immune response are mitigated as nearly all patients express wild-type C1-INH protein. To identify a rAAV-based gene therapeutic that can replace the defective allele's production of active C1-INH a cell based potency assay has been employed to screen through expression cassettes and optimized codon variants, with promising candidates then screened in wild-type mice. Antigen assays that specifically detect human C1-INH versus the murine ortholog demonstrate that human C1-INH can be expressed to a potentially therapeutic range even at low vector doses. Furthermore, no tolerability concerns from in life observations, gross pathology or cellular blood chemistry were observed in these animals or in animals expressing human C1-INH at levels substantially above a potential therapeutic level. Activity assays selective for the human C1-INH demonstrated that the majority of the human C1-INH synthesized in these animals is competent to interact with and inhibit target protease. Additional studies in mice to evaluate various transgene designs are ongoing. Assays to enable specific evaluation of human C1-INH levels in macaque are in development. These results illustrate the potential for an AAV-based gene therapy approach for the treatment of HAE.

862. Start Codon ELANE Mutations are Necessary and Sufficient to Induce the Promyelocyte Differentiation Block Observed in Severe Congenital Neutropenia

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Severe congenital neutropenia (SCN) is characterized by low neutrophil counts and differentiation arrest at the promyelocyte/myelocyte stage of granulopoeisis. Most SCN cases are associated with point mutations in a single allele of the neutrophil elastase (*ELANE*) gene. There is heterogeneity in phenotypes associated with ELANE mutations, even among those with the same mutations. The cellular pathophysiology of *ELANE* mutations is poorly understood due to a lack of animal genetic models that recapitulate the disease and compensatory innate immune events in patient bone marrow. We have reported that *ELANE* exon 3 mutations are necessary to induce a promyelocyte/myelocyte block due to mislocalization of the misfolded ELANE protein resulting in UPR/ER stress in promyelocytes derived from patient iPSC lines, correctable genetically and pharmacologically (Nayak et al, JCI 2015). It is unclear whether there are different mechanisms underlying the block in other classes of ELANE mutations. In particular, it is unclear how start codon mutations may result in SCN. We characterized start site mutations in the *ELANE* gene (-87 A>G) which result in the expression of alternatively translated peptides through alternative start codons and intragenic internal ribosomal entry sites (Tidwell et al 2014). We used two independent ATG mutant SCN patient derived iPSC (SCN110 and SCN54) and CRISPR/Cas9 gene editing to evaluate if ELANE mutations are both necessary and sufficient to cause neutropenia. First, we investigated the granulopoietic efficiency of ELANE ATG mutant iPSC. Both lines demonstrated, similar to the patients from whom they derive, a granulopoietic differentiation blockade at the myelocyte/metamyelocyte stage. CRISPR/Cas9 correction of the SCN110 iPSC (SCN110C) restored the normal differentiation and production of mature neutrophils similar to control iPSC demonstrating that the ELANE start site mutation is necessary to cause disease phenotype and demonstrates that ELANE mutation is sufficient to cause the differentiation defect seen in SCN. To investigate the necessity of *ELANE* mutations for disease phenotypes, we knocked in the start site GTG mutation into a normal donor iPSC line (ELANE GTG-KI). Granulopoetic differentiation of the ELANE GTG-KI phenocopied the differentiation arrest similar to SCN iPSC. Collectively, these data demonstrate that ELANE mutations are both necessary and sufficient to cause granulopoietic differentiation defects leading to neutropenia. ELANE ATG mutant promyelocytes have higher apoptosis (31% and 27% vs 11% and 22% respectively, in patient derived iPSC lines compared with their isogenic, corrected patient derived iPSC derived cultures 110C) or healthy donor control. There was also increased expression of proapoptotic proteins (Bim, Bak, Bax, and Bad) in ELANE mutant iPSC derived promyelocytes compared with their isogenic, corrected counterparts or controls. In summary, these studies have demonstrated that ELANE mutations are necessary and sufficient to cause granulopoietic differentiation defects in iPSC models of SCN. Ongoing studies are focused on identifying the cellular pathophysiology causing this increased apoptosis in promyelocytes with ELANE mutations.

863. Gene Therapy for Hemophagocytic Lymphohistiocytosis (HLH) Caused by Mutations in UNC13D

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Familial hemophagocytic lymphohistiocytosis (HLH) is a disease caused by mutations to genes involved in the lymphocytic killing of target cells by cytolytic vesicles that contain perforin and granzyme. The list of effected genes includes *PRF1, UNC13D*, *STX11*, *STXBP2*, and other genes which are involved in the intracellular trafficking of cytolytic vesicles and their exocytosis from the cytotoxic cell. Consequently, an otherwise innocuous immunological insult can result in an escalating inflammatory response, typified by fever, cytopenias, neurological symptoms, organ failure and death. Treatment of HLH focuses on reducing inflammatory responses with drugs such as etoposide and dexamethasone. Currently, the only cure for inherited HLH is an allogeneic bone marrow transplant to reconstitute the immune system with cells from a healthy transplant donor. In order to safely receive this transplant, a patient's inflammation must be adequately controlled, and an estimated 20-30% of HLH patients die before transplantation due to uncontrolled inflammation. Even of those that do receive a transplant, the 5-year survival rate is reported by the Histiocyte Society to be 54% +/- 6%. Post-transplant complications are common and include veno-occlusive disease, GVHD, infections, primary non-engraftment and mortality resulting from these conditions. Here we describe our work towards developing a lentiviral-based gene therapy for patients suffering from a lack of functional Munc13-4 protein (also known as "Familial HLH type III", or "FHL3" patients that have mutated copies of the *UNC13D* gene). To maximize Munc13-4 expression, we constructed several different lentiviral vector expression constructs which include either the canonical or a codon-optimized version of the *UNC13D* gene under the control of either the Ef1α or MND promoter, and some of which incorporated a Kozak sequence and/or GFP marker. We have validated that while these different constructs can all be manufactured with similar VSVG pseudotyped lentiviral vector titers, some generate higher levels of Munc13-4 expression in transduced cell lines. Western blot and PCR amplifications of the transgenes indicate that the gene is transferred without rearrangement and that the full length protein of 123 kDa is produced. Furthermore, within transduced cell lines the Munc13-4 protein expression levels surpass that of endogenously expressed Munc13-4 in PBMCs with a copy number of 0.2 or less. We next harvested Sca-1+ cells from Munc13-4 deficient mice (termed Jinx mice), and transduced these cells at an MOI of approximately 40. A methylcellulose-based assay showed there are no signs of HSC toxicity, as similar colony counts were observed for transduced and control/non-transduced cells. Genomic DNA isolated from the progenitor cells showed reasonable levels of gene transfer, with an efficiency of approximately 0.5 copies per cell. Transduced Sca-1+ cells were also transplanted into Jinx mice and transgene stability was validated in peripheral blood mononuclear cells genomic DNA for 12+ weeks

post-transplant. In addition, we have shown that mice with gene-corrected cells exhibit phenotypic correction in terms of the ability of their cytolytic cells to degranulate. We are currently analyzing the immune response of transplanted mice using LCMV-challenges. These data support the continued study of Munc13-4 gene therapy for type III familial HLH.

864. Intramuscular rAAV2/8 Mediated Expression of Etanercept in Mice with Collagen-Induced Arthritis

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Rheumatoid arthritis (RA) is one of the most prevalent chronic inflammatory diseases that affects up to 1% of the population worldwide. This disease involves primarily the joints and if inadequately treated, causes irreversible joint damage and disability. Development and use of disease-modifying anti-rheumatic biologics targeting tumour necrosis factor-α (TNF-α), which plays a pivotal role in RA pathogenesis, have significantly improved the clinical outcome for patients. However, the costly production, and repeated dosing regimen, required to achieve therapeutic effects in patients can be problematic. As an alternative, gene transfer for *in vivo* production of biologics, such as etanercept, which is a fusion of TNF-α receptor and IgG1 Fc, could help overcome these challenges. Here, we tested the hypothesis that gene transfer to the muscle, mediated by recombinant Adeno Associated Virus (rAAV), could result in robust expression of etanercept and improve disease outcome. Two rAAV2/8 genome configurations were designed to express etanercept under the control of the 'CASI' promoter (combination of CMV enhancer, ubiquitin enhancer and chicken β-actin promoter), one with the protein secretion signal sequence from TNF receptor 2 (TNFR2) and one from the human growth hormone (hGH). The vectors were delivered to BALB/c mice by single intramuscular (IM) injection (1E11 vector genome copies (GC) per animal; n=5/group). Robust levels of etanercept were detected in the serum with both vectors, irrespective of the protein secretion signal sequence, achieving approximately 5 μg/mL at the end of the study (day 56). These levels of etanercept exceed the serum trough level of 1.2 μg/mL associated with a marked clinical response in RA patients (Sanmarti *et al.* (2015), Annals Rheumatic Diseases, 74:8). To assess the effect of etanercept gene transfer on arthritis we used the collagen-induced arthritis (CIA) mouse model in DBA/1 mice, a commonly used murine model for RA. We administered rAAV2/8-CASI vectors expressing hGH-etanercept or an irrelevant transgene (IM: 1E11 GC per animal) to groups (n=7) of DBA/1 mice that had been immunized with bovine type II collagen in complete Freund's adjuvant 1 week previously. Robust levels of etanercept were observed in the serum (~2.2μg/mL, day 48 post-dose) whereas in control groups the level was below the limit of detection. In this pilot study, we also monitored the severity of arthritis in the limbs and observed a tendency for a reduced arthritis score in animals treated with the etanercept vector (24.0±4.9 area under curve of clinical score after onset of arthritis) compared with control animals (36.4±3.1). To build on these data, a repeat study to assess whether etanercept gene transfer can prevent disease onset is underway. Mice are dosed with rAAV2/8 hGH-etanercept vector and compared with groups dosed with vehicle, or a control rAAV2/8 vector expressing an irrelevant antibody (n=9 for all groups). The mice are monitored daily to measure the timing of disease onset and severity of the symptoms (currently at day 70 post-treatment). If rAAV2/8 is ineffective when administered in a prophylactic setting, future studies will focus on therapeutic administration of the vector.

865. Characterization of a Novel Rat Model of HIV Neuropathogenesis

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Over the past 30 years, the Human Immunodeficiency Virus (HIV) has been a disease that has been difficult to cure. Through the infection of immune cells, HIV weakens the host's immune system leaving the host vulnerable to secondary infections. If left untreated, HIV infection can make the progression towards acquired immune deficiency syndrome (AIDS), a disease that affects about 1.2 million people in the U.S. alone. HIV can also infect the resident immune cells in the central nervous system (CNS) such as astrocytes and microglia. Infection of these cells can lead to the development of HIV-associated neurocognitive disorder (HAND). While there are treatment options available for HIV such as highly active antiviral retrotherapy (HAART), they have not been successful in removing HIV in the CNS. To study HAND and similar diseases, we are developing a novel transgenic rat model of HIV infection, termed "iHIV". The iHIV transgenic rat contains a replication defective HIV provirus targeted to the Rosa26 locus. In this modified HIV provirus, the gag and pol genes have been replaced with a transcription termination signal flanked by loxP sites followed by a nanoluciferase reporter. The provirus contains the 5' and 3' LTRs along with HIV accessory proteins. This system contains a Cre-inducible provirus that expresses the nanoluciferase reporter and accessory proteins under control of the LTR promoter. Genotyping analysis confirms copy number and appropriate targeting of the iHIV construct. Injection with AAV-Cre Virus into iHIV animals shows an increase in detectable Nanoluciferase reporter activity. For cell specific expression of the iHIV provirus, a CX3CR1-driven CreERT2 transgenic rat expressing tamoxifen-inducible cre recombinase was developed to promote activation of the provirus in microglia, the primary host cell in the CNS. To evaluate the CX3CR1-CreERT2 rat, it was crossed with a rat containing a DIO-mCherry construct. Double-positive progeny were assessed for CreERT2 activity in microglia by looking for colocalization. In this transgenic rat line, intracranial injections of the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNFα) into iHIV positive animals elicits an increase in nanoluciferase activity. Further characterization of the rat as a model of HIV neuoropathogenesis is ongoing. This work was supported by the Intramural Research Program at NIDA/NIH.

866. A Situational Analysis of HIV and AIDS in Pakistan

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HIV (Human immunodeficiency virus) transmission has been reduced by protected sex and screening of blood products and other body fluids in the developed countries. It has been reported that Pakistan is at high risk of HIV/AIDS infection but presently the prevalence rate is considerably low. The number of reported cases of HIV/AIDS in Pakistan has been continuously increasing since 1987. By 2010 the total number of registered cases has reached to 6000 and this figure is on the rise with the passage of time. Some serious strategies must be implemented to control this deadly disease.

867. Finding the Minimum Vector Copies Per Cell Needed to Reach Phenotypic Correction in a Mouse Model of Erythrocyte Pyruvate Kinase Deficiency Using a Clinically Applicable Lentiviral Vector

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Pyruvate kinase deficiency (PKD) is a rare autosomal recessive disorder resulting from mutations in the *PKLR* gene. The disease is characterized by hemolytic non-spherocytic anemia of variable severity, which in some cases is fatal during early childhood. Additional complications secondary to hemolytic anemia include jaundice, cholelithiasis, splenomegaly, variable degrees of iron overload and reticulocytosis. Currently, splenectomy and periodic transfusions are the only palliative therapeutic options available. In selected and severe cases, allogeneic stem cell transplantation has been shown to correct the disorder; however this is associated with considerable toxicity and not considered a standard therapy in PKD. We have developed a gene therapy protocol based on the *ex vivo* correction of hematopoietic progenitors using a lentiviral vector (LV) which carries a codon-optimized version of the PKLR cDNA (coRPK) which may represent a potentially curative therapy for PKD. The vector has been successfully tested in a clinically relevant mouse model and has been designated as an Orphan Drug for the treatment of PKD by the EMA and FDA. However, the minimal ratio of corrected cells required to achieve a therapeutic effect needs to be finaly defined. With this purpose, in a first attempt, lethally irradiated PKD mice were transplanted with different ratios of wild-type and PKD bone marrow (BM) cells (ranging from 5 to 100%). 20-30% of donor wild-type healthy cells resulted in improvement in all erythroid parameters, with comprehensive reversal identified with 30-40% of non-deficient BM cells. In a second approach, mouse PKD hematopoietic progenitor cells were transduced with a GMP-like therapeutic lentiviral vector (LV) at different multiplicities of infection (MOI) that ranged from 0.3 to 50. Transduced cells were then transplanted into lethally-conditioned murine PKD recipients. Consistent with data obtained in the previous approach, the minimum vector copy number (VCN) required to correct the PKD phenotype was found to be 0.2-0.3, with full correction identified with VCNs at or above 0.5. These preclinical results offer new experimental evidence to help guide forthcoming clinical gene therapy programs for the treatment of severe PKD.

868. Targeting β-Globin Gene into α-Globin Locus in Human Hematopoietic Stem and Progenitor Cells

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β-thalassemia is one of the most common genetic diseases in the world, with a global annual incidence of 1 in 100,000. Disease pathology of β-thalassemia is believed to not only be due to loss of β-globin protein (*HBB* gene), reducing the ability of red blood cells to deliver oxygen, but is also caused by accumulation of the β-globin binding partner, α-globin (*HBA1/2* gene), leading to dramatic erythroid toxicity. Therefore, CRISPR/Cas9 was used to initiate a site-specific DNA double-strand break at the *HBA1* locus followed by delivery of a DNA repair template by AAV6 that the cell's DNA damage repair machinery can use to integrate a functional β-globin transgene in human hematopoietic stem and progenitor cells (HSPCs). We found that we were able to successfully replace the entire coding region of *HBA1* with our *HBB* transgene in HSPCs, which yielded functional hemoglobin tetramers when cells were differentiated into RBCs. Furthermore, edited cells were found to be capable of long-term engraftment and repopulation of the hematopoietic system in NSG mouse transplantation experiments. This work not only establishes a novel genome editing strategy for treatment of β-thalassemia, but also characterizes a RBC-specific safe harbor site that could be useful for treatment of a wide range of hematopoietic disorders.

Cell Therapies III

869. Targeted Disruption of HLA Genes by Genome Editing for Next GenerationTargeted Disruption of HLA Genes by Genome Editing for Next Generation Donor iPS Cells

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Induced pluripotent stem cells (iPSCs) have potential for regenerative medicine, however, immune rejection caused by HLA-mismatching is a concern. While B2M gene knockout and HLA-homozygous iPSC stocks are being investigated, the former approach may induce NK cell activity and fail to present antigens, the latter is challenging to

recruit rare donors. Here, we show two genome-editing strategies for next generation donor iPSCs. First, we generated HLA pseudo-homozygous iPSCs by editing one specific HLA haplotype of heterozygous iPSCs. Second, we generated HLA-C-retained iPSCs by disrupting both HLA-A and -B alleles and leaving at least one HLA-C allele to suppress the NK cell response and retain antigen presentation. Importantly, HLA-C-retained iPSCs could evade T cells and NK cells *in vitro*and *in vivo*. We estimated that 12 lines of HLA-C-retained iPSCs are immunologically compatible with over 90% of the world-wide population, greatly facilitating iPSC-based regenerative medicine applications.

870. Factors Affecting Src-Kinases May Reduce Efficacy of T Cell Based Therapeutic Products

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T cell activation (TCA) is required for T cell function. During *ex vivo* manufacturing of T cell based therapeutic products, various factors including T cell receptor (TCR) agonists, serum, different combinations of cytokines, and various inhibitors of cellular signaling pathways are used to improve cell expansion, prevent differentiation and enhance product potency. In T cells, Src-kinases (Lck, Fyn) are required for effector function, and factors that inadvertently affect Src-kinases may also affect efficacy of T cell based therapeutic products. Previously, we found that during *ex vivo* culture of human T cells, factors that inhibit Src-kinases inadvertently activate distal TCR signaling. Here, we characterized the mechanism for inadvertent activation of distal TCR signaling during inhibition of Src-kinases. We found that in resting human T cells, inhibition of Src-kinases increased calcium influx and activated the calcium-dependent transcription factor NFAT1 and NFAT1-dependent distal TCR signaling. Since ORAI1 is the major calcium channel in T cells, we assessed the role of ORAI1 in aberrant calcium influx induced by Src-kinase inhibitors. In resting human T cells, ORAI1 blocking antibodies rescued aberrant calcium influx induced by Src-kinase inhibitors. Bioinformatics studies identified a tyrosine residue at position 60 (Y60) in ORAI1 predicted to be phosphorylated by Src-kinases. Consistent with this, Src-kinase Lck phosphorylated ORAI1 at Y60. Together, these data suggest that phosphorylation of ORAI1 by Src-kinases may be critical in maintaining calcium homeostasis, and factors that inhibit Src-kinases during *ex vivo* manufacturing of T cells may activate calcium-dependent signaling pathways by interfering with ORAI1 phosphorylation and inducing aberrant calcium influx. Current studies are underway to assess the effect of inadvertent inhibition of Src-kinases during *ex vivo* manufacturing of chimeric antigen receptor (CAR) T cells on product stability, safety and efficacy. Since small molecule drug inhibitors often have off target effects, a greater understanding of their impact on TCR signaling pathways may help to eliminate use of undesired interfering factors and improve safety and efficacy of T cell based therapeutic products.

871. A Novel Allogeneic γδ T Cell Product Targeting CD20 for the Treatment of B Cell Malignancies

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Patient-derived autologous αβ T cells transduced with chimeric antigen receptors (CARs) targeting CD19, CD22, and BCMA have delivered impressive outcomes for the treatment of patients with various hematological malignancies. While potentially very effective, limitations of autologous cell therapies continue to pose challenges from various standpoints including manufacturability, availability, safety, consistency and cost of production. In order to overcome many of these challenges we are developing an alternative approach employing allogeneic gamma delta (γδ) T cells to create a universal T cell therapy for the treatment of cancer. γδ T cells represent a functional "bridge" between innate and adaptive immunity and are evolutionary highly conserved. γδ T cells are a minor lymphocyte population in the circulation but can be found in larger numbers in tissues such as skin and gut, where they exhibit potent immune monitoring, anti-viral, anti-tumor and anti-microbial functions. Importantly, γδ T cells recognize pathogen-stressed and transformed target cells in an HLA-independent fashion, thereby facilitating their use in an allogeneic setting. We have developed a robust manufacturing process for selective activation, expansion and engineering of various subsets of γδ T cells from tissues, peripheral or cord blood. Here we present a CAR-engineered γδ T cell product targeting CD20 antigen for the treatment of B cell malignancies. A Vδ1 subset of cells was selectively expanded from peripheral blood of normal healthy donors and engineered with 2nd generation CAR construct featuring fully human scFv targeting domain to create an allogeneic CAR T cell product. The level of expansion, purity and anti-tumor potency following cryopreservation were tested and support creation of a substantial number of doses (e.g. >100) of well characterized uniform product that are available immediately in an "off-the-shelf" manner. CAR transduction, accomplished using γ-retrovirus-based gene transfer, substantially enhanced the innate anti-lymphoma activity of these cells. We have demonstrated that in response to target cells CD20 CAR γδ T cells secrete effector cytokines, induce apoptosis and clear lymphoma cells in vitro and that these functions can be further potentiated by providing exogenous cytokines to support proliferation. When tested in vivo, CD20 CAR γδ T cells demonstrated potent antitumor activity in both disseminated (Raji) and subcutaneous (Raji and Mino) models of B cell lymphoma without evidence of graft versus host disease. CD20 CAR γδ T cells rapidly localized to subcutaneously implanted tumors and showed robust activation and proliferation profiles within days of infusion. This activation and proliferation were dependent on the presence of CD20 target as no such activation occurred outside the tumor microenvironment, in contrast to CD20 CAR αβ T cells. Methods to improve the performance and persistence of γδ T cell products will be discussed. Overall, these data show selectively

expanded Vδ1 T cells represent a unique, safe and effective platform for therapeutic intervention in various cancers and support further clinical investigation of a CD20-targeted CAR γδ T cell product drug candidate.

872. Natural Resin Using BMP2 Induces Osteogenic Differentiation of Dental Follicle Stem Cells

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Background: Bone regeneration through tissue engineering one of the main focuses of attention of experts in a wide range of sectors, including maxillofacial surgery. Natural resin that is highly sticky and has high physical strength and thermal resistance to use as a scaffold in bone. **Methods and Materials:** Mesenchymal stem cell derived from Human third molar is a powerful cell source for stem cell therapy and bone tissue engineering. Therefore, it is essential to optimizing the ability of these cells osteogenic differentiation on the new scaffold named nano natural resin for bone tissue engineering in vitro. In this experimental study, the biological behavior and osteogenic capacity of the third molar tooth derived MSC were compared on 2D and 3D cell culture on nano resin scaffold in present of BMP2 as an inducer of MSC differentiation into osteoblast. **Results***:*There was a significant difference between the rate of differentiation markers in 2D and 3D cultures of MSc. During osteogenic differentiation, ALP activity was demonstrated to be significantly higher in 3D. The authors concluded that 3D culture on nano resin showed osteogenic differentiation. It is the first time that we introduced natural chewing resin scaffold for osteogenic differentiation and bone tissue engineering. **Conclusion:** Dental follicle of impacted third molar containing stem cells with high potential for differentiation, which is available source of stem cells to be used in cellular treatment and tissue engineering specially in repair of bone damages. **Keywords:** nano resin, Tissue Engineering, Mesenchymal Stem Cells

873. Closed and Automated Cell Processing Using Rotea™ to Advance Cell Therapy

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The manufacturing cost and complexity has been one of the biggest obstacles for delivering cell therapy products. Here we present Rotea™, a compact closed automation system with counterflow centrifuge technology, which enables gentle cell separation, wash, and concentration with controllable input and output volumes. The 10ml chamber on the single use kit can sustain billions of cells as a fluidized bed and can be discharged multiple times if needed. The kit can be easily connected to other closed systems for upstream and downstream applications, for example Finesse bioreactor system, making Rotea a flexible modular instrument to fit in different workflows. Using human primary T cells as a model, we have demonstrated that across different input and output settings, Rotea contributes superior processing time, recovery, and viability for cell wash, concentration, and buffer exchange as compared to manual centrifugation. More importantly, Rotea is capable of facilitating lymphocyte separation from leukopheresis products, as well as bead removal after expansion, and therefore could shorten manufacturing time of cell therapy products.

874. A Straightforward and Efficient Strategy to Generate Universal CAR T-Cells

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Allogeneic CAR-T therapy, which uses donor-derived T cells instead of patients' own T cells, aims at being a more convenient, accessible approach compared to the conventional autologous CAR-T therapy. However, the allogeneic nature of this "off-the-shelf" therapy poses the risk of GvHD (Graft versus Host Disease), where the donor cells can potentially view the host as foreign and react against host tissues. This effect is largely mediated through the activation of TCRs (T cell receptors) on the donor cells. Fortunately, the TCRs can be genetically inactivated from donor cells via gene editing approaches, such as TALEN(r) or CRISPR/Cas9 system. Nevertheless, such intervention requires an additional purification step to remove residual TCR+ cells, which could hamper productivity and CAR-T cell fitness. Here, we developed a straight forward method to improve this process. With transient expression of an anti-CD3 CAR in addition to the stably expressed "therapeutic CAR" in the donor T cells, we programed the cells to self-eliminate the TCR+ cells population and obtained ultrapure TCR - population (99%-99.9%) at the end of CAR-T production. We demonstrated that the fitness of the produced cells was not affected by the transient expression of the anti-CD3 CAR. Anti-CD3 CAR transient expression did not have significant impact in the CAR-T cell growth rate, T cell differentiation or exhaustion level as compared to the non-CD3 CAR counterpart. Both *in vitro* and *in vivo* T cell killing assay results suggest that the CD3-CAR treatment did not affect the CAR-T cell killing function. This novel and easy-to-implement procedure removed a tedious purification step in the TCR- CAR-T cell manufacturing. Furthermore, it can potentially minimize or prevent the risk of GvHD at the same time preserve production yield and cell fitness.

875. Highly Purified Human Extracellular Vesicles Produced by Stem Cells Alleviate Aging Cellular Phenotypes Through Peroxiredoxins Delivery

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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. 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 16-fold more EVs than MSCs. Furthermore, we investigated the activities of iPSC-EVs and found that EVs from both stem cell types alleviate aging phenotypes of senescent MSCs that were induced by three different methods, including a progerin-induced premature aging model. When highly purified iPSC-EVs were applied in culture to senescent MSCs that have an elevated level of reactive oxygen species (ROS), they reduced cellular ROS levels and alleviated aging phenotypes of senescent MSCs. The human iPSC-EVs are more potent in anti-senescence than MSC-EVs when equal numbers of EVs were applied. Our discovery reveals that EVs from human stem cells can alleviate cellular aging in culture, at least in part by delivering intracellular peroxiredoxin antioxidant enzymes. Overall, the delivery of human iPSC-EVs attenuated cell aging and promoted cell proliferation, suggesting that purified EVs from human iPSCs may represent a cell-free approach for alleviation or reversal of aging.

876. Probing the Effects of Interpatient Variability on Autologous Engineered Neural Stem Cell Therapy for Glioblastoma

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Neural stem cells (NSC) engineered to deliver drugs are a promising new approach to treating glioblastoma (GBM). In the clinic, the ideal NSC therapeutic should be easy to isolate and autologous to avoid immune rejection. Induced neural stem cells (iNSCs) can be transdifferentiated from fibroblasts using a rapid, single-factor transduction strategy and engineered to express fluorescent reporters and the cytotoxic protein TRAIL. However, interpatient variability in transduction efficiency, conversion efficiency, migratory capacity, and therapeutic efficacy of these cells for GBM treatment remains unexplored. The goal of this ongoing study is to generate and characterize GBM patient cells to optimize our rapid transdifferentiation protocol and dosing regimen in a patient-specific manner. The isolation process began by collecting small skin punch biopsies from the surgical border of human GBM patients undergoing tumor debulking at UNC Hospitals. We then processed the tissue to isolate and bank fibroblast lines from 6 different patients. We first discovered using cell viability assays that the different lines grew at different rates, with the slowest increasing only 1.27-fold and the fastest 2.98-fold over 6 days. Viral transduction is a central component of iNSC generation and using the same viral titer of a fluorescent reporter on each patient line resulted in marked differences in the percentage of positive cells, ranging from only 22.45% positive to 87.22% positive depending on the patient sample. ELISA revealed even larger differences in terms of TRAIL output; patients with the highest transduction efficiency secreted up to 100-fold the amount of TRAIL per cell as patients with the lowest. This was validated by whole transcriptome RNA sequencing as well as therapeutic efficacy studies *in vitro*. Using a novel organotypic brain slice assay, 4 different patient-derived iNSC-TRAIL were co-cultured at differing ratios with primary GBM cells in a physiologically relevant 3D model, and EC50 was determined for each patient after 24 hours. In the most efficacious case, tumor viability was reduced by half when the homogenous spheroid included 15% iNSC-TRAIL, while the same effect could only be achieved when 75% of the spheroid was composed of iNSC-TRAIL in the weakest case. 2 patients were selected for further study to represent a fast-growing, high TRAIL-secreting population and a slow-growing, low TRAIL-secreting population, respectively. Then, human GBM cells were implanted in nude mice surgically resected 3 days after establishment, immediately prior to implantation of GBM-patient iNSC on scaffolds into the surgical cavity. Dosing was determined based on results from the TRAIL ELISA and tumor recurrence was measured by BLI, with significant attenuation of tumor volume depending on patient and dose as demonstrated in Table 1. Together, these results provide the first evidence that iNSCs can be generated from cancer patient tissue, reveal differences between patient lines, and demonstrate the efficacy of this approach in mouse models of surgical resection in mice. This suggests that generation of therapeutic iNSCs from patient-derived tissue has the potential to be an efficacious approach to treating cancer.

Table 1. Attenuation of GBM Recurrence through Treatment with iNSC-TRAIL

Experimental Group	Patient	Patient Cell Characteristics	Cell Number Dosed	Median Survival	Number of Days in Remission Post-Resection
1	Sham	--	--	20	11
2	GP6	slow-growing, low-secreting	400K	25	12
3	GP6		1M	24	19
4	GP2	fast-growing, high-secreting	400K	34	29
5	GP2		1M	39	42

877. A Versatile Platform for Generation of Single-Cell Derived, Genetically Engineered, Fully Characterized Pluripotent Master Cell Lines as a Renewable Source for Off-the-Shelf Cell Therapy

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Adoptive cell transfer-based therapy has become the most promising approach against cancer with the success of T cells engineered with chimeric antigen receptor (CAR). Unfortunately several concerns are inherent when developing such a product including off-target editing, random integration of the transgene, and chromosomal aberrations. We previously demonstrated that induced pluripotent stem cells (iPSC) can be used to create single-cell derived clonal lines genetically engineered with desired modalities. Through a series of proof-of-concept experiments, we show that our platform is capable of reprogramming and engineering universal donor lines to produce single-cell derived homogeneous master iPSC lines suitable for mass production of an off-the-shelf cellular therapeutic. FT819 is the first-of-kind off-the-shelf human iPSC-derived CAR T-cell product candidate. Here we engineer reprogrammed human peripheral blood-derived T-cells with a biallelic integration of a novel, modified CD19 CAR into the T-cell receptor α constant locus. This approach will simultaneously confer CD19 antigen specificity and preclude graft versus host disease. Molecular characterization reveals a biallelic targeting efficiency of between 10-15% with about 50% of those possessing random integration of the targeting construct. Clones were also screened for stability of pluripotency and clearance of reprogramming vectors. Cytogenetics revealed a majority of karyotype-normal clones with occasional trisomy 12 and copy number variation aberrations. Potential off-target editing sites were determined by SITE-Seq but no off-target mutations were detected in selected clones. Despite the low efficiencies and abnormalities associated with multi-pronged genetic engineering, we successfully produced many biallelic-targeted, vector-free, and cytogenetically normal engineered IPSC master cell lines. FT538 is a human iPSC-derived NK cell product candidate developed for use in combination with an anti-CD38 monoclonal antibody for treatment of myeloma. It is designed to have a targeted integration of two transgenes with a knockout of CD38. These modifications were made in a single engineering event into an already fully characterized single-cell-derived iPSC clone to precisely integrate a constitutive promoter-driven bicistronic cassette into the CD38 locus. Such a large cassette impaired targeting efficiency to less than 5%. But constitutive expression of the transgenes allowed for enrichment of targeted cells during single-cell sorting. This yielded about 40% of isolated clones with targeted insertion of the transgenes, half of which showed evidence of correct genome targeting without random insertions. Our approach shows it is possible to target a large expression cassette into a locus that is otherwise silent in iPSC. Furthermore, this editing of an established iPSC clone suggests our platform is capable of supporting multi-round genetic engineering. By strictly monitoring the cellular integrity of single-cell derived edited clones, it is now possible to exclude genomic abberations and variability that are commonly associated with engineering and subculturing at the population pool level. Collectively, we demonstrate for the first time that a single cell engineering platform can be utilized to develop high-quality precision-engineered master cell lines for the mass production of off-the-shelf cellular therapeutics.

878. Therapeutic Induced Neural Stem Cells as a Treatment for Brain Metastases

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Introduction: Non-small cell lung cancer (NSCLC) and breast cancer are the most common primary cancers to spread to the brain. The median survival for patients with these metastases is less than 9 months. A new therapeutic agent is desperately needed in order to seek out and eradicate these metastases. To address this need, we propose using neural stem cells (NSCs) as a targeted drug delivery system to scavenge for metastases in the brain. NSCs have demonstrated a remarkable, innate ability to selectively migrate to tumors. When engineered to produce cytotoxic proteins such as TRAIL, NSCs have been proven to migrate to and kill glioblastoma tumors. We have previously developed a method of rapidly transdifferentiating human fibroblasts into induced neural stem cells (hiNSCs) using a lentivirus containing the gene to induce expression of the multipotency transcription factor, Sox2. The hiNSCs are a personalized, tumor-homing therapeutic cell line. Here, we describe the first efforts to use hiNSCs to treat cancer that has metastasized to the brain. We utilize intracerebroventricular (ICV) injections to deliver hiNSCs to brain tumors. ICV devices that allow for long-term, repeated dosing are well-established in the clinic. Additionally, ICV injections distribute cells throughout the brain, reducing the distance the migration distance to the tumor and allowing for the possibility of treating multiple tumor foci. **Methods:** In order to assess the potential of therapeutic hiNSCs as therapy for cancer that has metastasized to the brain, we performed an *in vitro* efficacy co-culture assay, used *in vivo* studies to determine the migration, persistence, and efficacy of therapeutic hiNSCs against H460 NSCLC and triple-negative breast cancer MB231-Br tumors in the brain. Following implantation of tumors into the brains of nude mice, hiNSCs were injected into the ventricle contralateral to the site of tumor implantation. The migration and persistence of hiNSCs was investigated by following the bioluminescent signal of the hiNSCs as well as by sectioning following sacrifice. The therapeutic efficacy of the hiNSCs was determined by following the bioluminescent signal. **Results/Conclusion:** Co-culture results demonstrated that hiNSC therapy reduced the viability H460 and MB231-Br 95% and 82% respectively compared to non-treated controls compared to non-treated controls. To explore the persistence of ICV-administered hiNSC serial imaging show a maximum level of cells 4 days after injection that persisted for more than one week in both models, Investigating migration, fluorescent analysis of post-mortem whole-brain tissue sections showed that hiNSCs migrated from ventricles and co-localized with contralateral tumor within 7 days. Using both H460 and MB231-Br models, kinetic tracking of

intracranial tumor volumes showed therapeutic hiNSCs reduced the growth rate of brain tumors by 31-fold and 3-fold, respectively. This work demonstrates for the first time that we can safely and effectively deliver personalized tumor-homing cells through the ventricles to target brain metastases. This will be important foundational work in the development of a new, much-needed therapeutic agent to scavenge for metastases in the brain, thus reducing the risk of tumor recurrence and decreasing the mortality of NSCLC and breast cancer.

879. Regulation of Differentiation of Neural Stem Cells from Human Mesenchymal Stem Cells by MicroRNA

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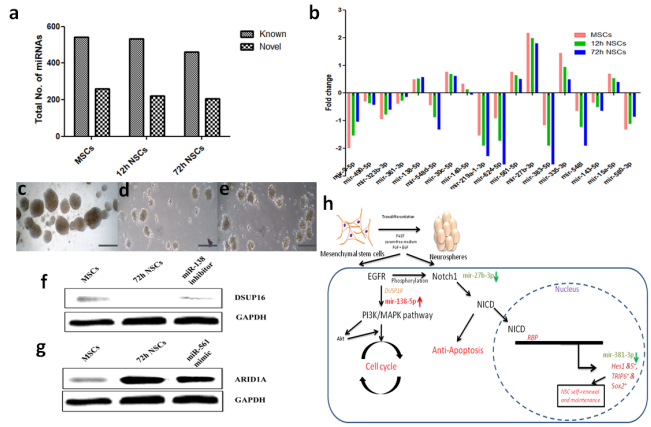
MicroRNAs (miRNAs) are non-coding, small RNA molecules that play decisive roles in cell proliferation/differentiation by targeted inhibition of mature mRNAs. In this study, complete profiling and identification of the significant miRNAs was done which have been implicated in NSC differentiation from human mesenchymal stem cells (hMSCs) induced by EGF and FGF under serum-free conditions. The profiling of miRNAs was done using Next-Generation sequencing technology. Both known and novel miRNAs were identified in the 12h NSC (early) and 72h NSC (late) samples when compared with the hMSCs (control) as retrieved from the miRBase tool (**Fig. 1a**). To understand the regulatory action of individual miRNAs on NSCs differentiation, time-series analysis was also performed using miRNA-TA tool. Only 18 (4%) miRNAs out of 431 conserved miRNAs were found to be differentially regulated with respect to time (**Fig. 1b**). Among the 18 miRNAs, miR-561-5p and miR-138-5p were observed as important miRNAs based on their significant ($p<0.05$) expression and cellular function. The expression levels of their target genes: ARID1A (for miR-561-5p) and DUSP16 (for miR-138-5p) were determined using western blot & quantitative PCR. In order to validate the predicted functions of the 2 miRNAs, respective mimics and inhibitors were used during NSC differentiation. On treating the culture with miR-138-5p inhibitor, the morphological sizes of neurospheres were found to be significantly reduced in the cultures. Expression of the target gene DSUP16 was also observed to be upregulated when compared with the control (without inhibitor). Upon overexpression of miR-561-5p, the neurosphere size, numbers and its target gene (ARID1A) expression were significantly reduced and/or downregulated in the differentiated culture when compared with the control (**Fig. 1c-g**). These results suggest the synergistic opposing action of miR-138-5p and miR-561-5p which could help in enhanced differentiation of NSCs from hMSCs (**Fig. 1h**). These results may possibly help not only in developing new strategies for rapid and efficient generation of hNSCs but also potential use in the treatment of various neurodegenerative diseases in near future.**Figure 1: MicroRNA signature changes during NSCs differentiation. a.** Total number of known and novel miRNAs identified in 12h, 72h NSCs and hMSCs (control). **b.** Differentially regulated significant ($p<0.05$) miRNAs using retrieved using the mirnaTA tool. **c.** hNSCs (no treatment) **d.** transfected with miR-138-5p inhibitor. **e.** overexpressed with miR-561-5p mimic (magnification: 10x; Scale bar: 4.18mm). **f & g.** Western blot analysis of target genes DSUP16 & ARID1A of miR-138-5p and miR-561-5p respectively. **h**. Proposed schematic representation on regulation of miR-138-5p and miR-561-5p via Notch signaling pathway. When hMSCs were treated with EGF + FGF, the level of miR-138-5p was found to be significantly upregulated which inhibited its target gene DUSP16. Inhibition of DUSP16 can potentially inactivate dephoshorylation of EGFR in the cytoplasm. The activated EGF and Notch receptor can in turn activate the downstream targets of Notch signaling which is known to play a major role in NSC differentiation and self renewal. The downregulated levels of miR-561-5p promote increased expression of the target gene ARID1A which aids in proliferation of neural stem cells into neurospheres by potentially activating notch-target genes like Hes1&5, Sox2, TRIP6 etc).



880. Optimizing Gamma Delta T Cells Expansion for Cell-Based Immunotherapies

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Gamma delta (γδ) T cells have the ability to kill tumor cells through multiple cytotoxic mechanisms, making them attractive candidates for cancer immunotherapy. γδ T cells have been investigated as an immunotherapy in multiple clinical trials, which attempted to stimulate the expansion of γδ T cells using either *in vivo* or *ex vivo* methods. *In vivo* stimulations have successfully increased the number of circulating γδ T cells. As a consequence of this in vivo expansion, CD4+ regulatory T cells also increased, indicating administration of an *ex vivo* expanded product could be a more effective therapy. *Ex vivo* expansions of γδ T cells are currently being tested using peripheral blood or apheresis products as the starting cellular sources. This method is patient specific and relies on how well a patient's cells expand. We have shown that there is variation in γδ T cell expansion from different donors. While some donors achieve final γδ T cell percentages of 90%, others only achieve 40%, suggesting some donors have characteristics that make them better expanders. The development of an allogeneic product, where a third party donor could donate cells to be expanded ex vivo and stored for future treatments, would reduce the time from patient diagnosis to treatment by eliminating the possibility of a poor expansion. We have previously shown that γδ T cells can be expanded from peripheral blood mononuclear cells (PBMCs) using zoledronate

and IL-2 in serum-free conditions. Using this method, we aimed to determine if there was a population of donors optimal for the expansion of γδ T cells and to develop an allogeneic donor product. After 14 days of expansion, we found that donors who exercised 3-6 times per week had a 5-fold increase in the number of γδ T cells ($p< 0.05$) and a 2-fold increase in the percentage of γδ T cells ($p< 0.005$) compared to sedentary donors who exercised 0-1 time per week. Additionally, γδ T cells from exercise donors had increased cytotoxicity against K562 cells ($p< 0.005$) in comparison to sedentary donors. To develop a relevant third party product and reduce the possibility of graft versus host disease, it is necessary to remove αβ T cells from the expanded γδ T cell culture. αβ T cells were depleted from the cultures on days 0, 3, 6 or 9 of expansion and compared for overall cell growth and cytotoxicity. γδ T cell cultures depleted of αβ T cells on day 6 had a 3.3-fold expansion from day 6 to day 12, while cultures depleted of αβ T cells on day 9 had a 1.1-fold expansion from day 9 to day 12 ($p <0.05$). On day 12, there was no difference in cytotoxicity between day 6 or day 9 αβ-depleted cultures. Although there was no difference in the total number of γδ T cells on day 12 for day 6 or day 9 depleted cultures, cell growth for 4 of 6 donors depleted on day 9 decreased by day 12, suggesting it is optimal to deplete αβ T cells on day 6 of expansion. To determine the effects of freezing and storing the expanded cells, γδ T cells were αβ-depleted on day 6, expanded in culture and frozen on day 12. The post-thaw viability averaged 90±4.8% directly after thawing, decreased to 73.3± 8% on post-thaw day 1, and increased to 79.1±8% on post-thaw day 2. Cytotoxicity of frozen γδ T cells against K562 cells decreased by 30-50% from pre freezing values directly after thawing, but recovered to their pre freezing values by post-thaw day 2. In addition, to test the potential to scale up a third party product, αβ depleted γδ T cells from three different donors were mixed on day 6 at ratios of 1:1:1 and 1:2:3 and grown through day 12. Total cell growth and γδ T cell growth increased similar to non-mixed cells, indicating it is possible to drastically increase cell numbers by combining cells from different donors. Overall, these data suggest there are characteristics that make some donors more optimal for the expansion of γδ T cells and that an allogeneic donor approach is feasible.

881. Regulatory T Cell Differentiation via Engagement of the PD1/ PDL1 Co-Inhibitory Pathway

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According to epidemiological data, the incidence of autoimmune diseases includingType 1 diabetes (T1D) has steadily increased in the US and throughout the world in the last few decades. Development of an autoimmune disease is characterized by the breakdown in immune tolerance mechanisms leading to an imbalance between autoreactive CD4+ effector T cells and Foxp3+ regulatory T cells (Treg). In T1D, this impairment leads to the T cell-mediated destruction of pancreatic β cells, the progressive decline in insulin secretion and ultimately an inability to regulate blood glucose homeostasis. Though T1D can be managed by regularly monitoring blood glucose levels and administering exogenous insulin, patients are still susceptible to complications from fluctuations in blood glucose as insulin replacement therapy is not as efficient for blood glucose control as natural secretion. The best way to maintain natural insulin secretion is to reduce or stop the destruction of the pancreatic β cells and thus slow or halt the progression to T1D. One approach to restore immune tolerance and preserve β cell mass is to augment the number and/or function of regulatory T cells. Previously, we showed that conventional T cells overexpressing PDL1 via a lentiviral vector were able to convert T helper type 1 (Th1) cells into Treg through the induction of the PD1/ PDL1 co-inhibitory pathway. Here, we evaluated whether a truncated form of PDL1 affixed to beads could also alter the Th1 cell phenotype. We have designed a construct which contains the truncated extracellular domain of PDL1 as well as polyhistidine and biotin tags. The recombinant PDL1 protein was produced in a mammalian expression system, purified via nickel affinity chromatography and then biotinylated in an enzymatic reaction. The biotinylated PDL1 protein was immobilized onto streptavidin-coated beads and incubated with primary human Th1 cells. Two days later the cells were analyzed by western blot and flow cytometry for expression of the Treg- and Th1-specific transcription factors, Foxp3 and Tbet respectively. The expression of Foxp3 was significantly increased in cells incubated with PDL1-coated beads while that of Tbet was significantly reduced. This data suggests that the truncated PDL1 construct is able to direct Th1 cells to differentiate to Treg. We are currently investigating the phenotype, function and stability of these differentiated cells by examining the expression of extracellular markers as well as the intracellular signaling elicited by the PDL1-coated beads. The cells generated using the PDL1-coated beads will subsequently be evaluated in vivo utilizing a mouse model of T1D to determine the efficacy in ameliorating the pathogenesis of the disease.

882. A Novel Platform Combining Chemical and CRISPRa Approaches for the Generation of Human iPSCs

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Cellular reprogramming of somatic cells into induced pluripotent stem cells (iPSC) has traditionally relied on the ectopic expression of specific transcription factors. Although proven effective, the process is often inefficient requiring unique quantities and combinations of transgenes necessary to faithfully activate the endogenous pluripotency circuitry. To circumvent this indirect method and directly activate endogenous master pluripotency genes, a CRISPR-based gene activation system (CRISPRa) incorporating an inactive Cas9 enzyme (dCas9) and SunTag-recruited transcriptional activators (VP64) to reactivate the endogenous expression of several "reprogramming factors" was recently developed to generate iPSCs from mouse fibroblasts. Here we describe the generation of iPSCs from human neonatal foreskin fibroblasts using this dCas9-SunTag-VP64 system, targeting specific

loci in the promoter regions of endogenous *NANOG*, *OCT4*, *SOX2*, *KLF4*, *MYC*, and *LIN28*. To enable cellular reprogramming, we combined the CRISPRa system with our previously reported stage-specific reprogramming media containing small molecule inhibitors targeting signaling pathways regulating stem cell pluripotency. Using this combined approach, iPSC colonies were detected as early as 7 days post-induction. Accordingly, a steady increase in expression for pluripotency genes *NANOG*, *OCT4*, *SOX2*, *LIN28* as well as *REX1* (which was not targeted for CRISPR activation) was observed. iPSCs generated through this approach also expressed alkaline phosphatase and pluripotency-associated surface markers SSEA-4 and TRA-1-81. The reprogramming efficiency was improved (reaching 5.0% of the total cell population) by targeting an Alu-rich motif over-represented in promoter regions of genes involved in early embryonic genome activation. The derived iPSCs are being further characterized for their pluripotent state and lineage propensity. Collectively, these data support a novel combined chemical and CRISPRa platform for the generation of human iPSCs by directly activating the endogenous pluripotency circuitry without ectopic expression of reprogramming factors. This work highlights the future potential of our combined chemical and epigenetic approach in a wide range of applications to modulate cell identity toward cell types of therapeutic interest.

883. Enhanced Neovascularization Effects of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells Encapsulated in Catechol-Modified Hyaluronic Acid Hydrogel on Ischemic Tissues

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Background: Neovascularization is an essential process for improving microcirculation in the ischemic tissues, and human induced pluripotent stem cell-derived endothelial cell (hiPSC-EC) has emerged as a therapeutic source cell for neovascularization. However, poor survival and low retention rate of engrafted hiPSC-EC have been major huddles on its application for clinical reality. We aimed to enhance cell survival, vessel formation, and therapeutic potential by encapsulating hiPSC-EC with mussel-inspired catechol-modified hyaluronic acid (HA-CA) hydrogel. **Methods**: We induced endothelial-lineage differentiation from hiPSCs, followed by sorting for CDH5 (VE-Cadherin) as described in our previous report. We characterized hiPSC-CDH5+ cells 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 tissue, hiPSC-EC was encapsulated in HA-CA hydrogel. The cytoprotective effect of HA-CA hydrogel on hiPSC-EC was examined by LIVE/DEAD assay under oxidative stress in vitro. We then evaluated whether the encapsulation of hiPSC-CDH5+ cells in HA-CA hydrogel (hiPSC-EC/HA-CA) could enhance long-term cell survival and vascular regenerative effects in a hindlimb ischemia model of nude mouse by imaging and histological analysis. **Results:** The highly enriched hiPSC-EC was generated, having genuine EC characteristics and proangiogenic activities. Biocompatible HA-CA hydrogel showed cytoprotective effect for hiPSC-EC against H_2O_2-induced oxidative stress in vitro, and also enhanced engrafted cell survival and retention in vivo. When we injected hiPSC-EC/HA-CA into adjacent area of hindlimb ischemia, the recovery of functional microcirculation was improved, resulting better prevention from limb loss after 8 weeks of hindlimb ischemia compared to PBS- or HA-CA-control group. hiPSC-EC/HA-CA showed more robust and longer cell survival in ischemic tissue, with contribution to dynamic neovascularization process; the engrafted hiPSC-EC/HA-CA was 1) perivascularly localized with linearly aligned parallel to host vessels, 2) having a putative guiding role for new vessel formation, and 3) progressively incorporated into host vessel over 8 weeks after hindlimb ischemia. **Conclusions**: This study demonstrated that a biocompatible HA-CA hydrogel substantially improved long-term survival of hiPSC-ECs in an ischemic environment, and enhanced therapeutic effect via neovascularization, suggesting a clinical potential of hiPSC-EC/HA-CA. Furthermore, this novel platform of cell-based therapy combined with bioengineering technique can also help investigate the underlying mechanism of neovascularization in ischemic tissues.

884. Large Scale Manufacturing Process Development for CAR-37 T Cell Therapy using CliniMACS Prodigy System

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CAR T cell therapy is a promising therapeutic modality for treatment of hematologic malignancies. A novel autologous CAR T cell product (CAR-37) targeting CD37 antigen was recently developed by our group (Scarfo et al., 2018). A controlled, robust, and reproducible manufacturing platform is essential for the successful clinical applications of T cell therapy. We developed a large-scale manufacturing process at the Dana-Farber/Harvard Cancer Center's Connell & O'Reilly Families Cell Manipulation Core Facility using the CliniMACS Prodigy (Miltenyi Biotec) under GMP conditions. We selected this system based on the benefits of using a single device with a closed system for GMP manufacturing. As part of the CAR-37 process development, four training runs were conducted using the same process and equipment intended for GMP manufacturing, but using cells obtained from healthy donors. For all runs, PBMCs were collected via leukapheresis; T cells were purified by CD4/CD8 isolation and activated with TransAct CD3/CD28 beads (Miltenyi Biotec). The phenotype analysis of healthy donors PBMCs from these training runs pre- and post- CD4/CD8 selection was consistent across all manufacturing runs. Post-selection median percentages were 93.2% CD3, 58.0% CD4, and 33.9% CD8, with a median CD4/8 ratio of 1.7. Development/research-grade lots of anti-CD37 CAR lentiviral

vector were used to transduce the cells for all training runs. T cells were expanded in the CliniMACS Prodigy® TS 520, under stabilized culture conditions with automated feeding and media exchange. The process was continuously monitored to determine kinetics of T cell expansion, transduction efficiency and the phenotype of CAR T cells in comparison to prior small-scale manufacturing. The T cell phenotype and transduction efficiencies were comparable to small-scale manufacturing, consistent throughout manufacturing runs, and overall T-cell yields were sufficient for anticipated clinical dosing. Median values for final product CD3, CD4 and CD8 composition were 97.7%, 55,4%, and 40.0%, respectively with a median CD4/8 ratio of 1.5. Efficiency of transduction of CD3 cells was 95.1% by EGFR staining and median VCN was 2.68. The final CAR T cell product was tested *in vitro* and *in vivo* in comparison to small-scale manufacturing. To define the functionality of CAR-37 T cells, luminescence-based cytotoxicity assays and Luminex cytokine release assays were performed against a panel of lymphoma cell lines. CAR-37 T cells demonstrated antigen-specific cytotoxicity against CD37 expressing tumor cells after *in vitro* stimulation. Consistent with the cytotoxicity assays, CAR-37 T cells also demonstrated antigen-specific Th1-type cytokine production *in vitro* in response to different target-expressing tumor cell lines. Furthermore, antitumor efficacy of CAR-37 T cells was tested *in vivo* using NSG mouse model. The results demonstrated that T cells engineered with CAR targeting CD37 antigen as manufactured in the Prodigy are potent therapeutically and can eradicate CD37-expressing tumors *in vivo*. These data, in combination with the ongoing experiments, indicate the successful implementation of the CAR-37 manufacturing process using CliniMACS Prodigy® system. The successful completion of the four large-scale training runs demonstrate reproducibility of the CAR-37 manufacturing process and the applicability to production for clinical trials. Additional validation runs are planned to support upcoming IND filling.

885. Evaluation of the Safety and Efficacy of piggyBac Transposon-Mediated Chimeric Antigen Receptor T Cells Using a Non-Human Primate Model

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Introduction: Chimeric antigen receptor (CAR) T-cell therapies are in the spotlight for treatment of relapsed/refractory tumors. Numerous CAR T cells have been developed for hematological and solid tumors with promise of clinical efficacy. However, immune-related serious adverse events such as cytokine release syndrome (CRS) and neurotoxicity remain a concern due in part to on-target/off-tumor or off-target toxicities, via engagement of the target antigen on normal tissues. Although humanized mouse models play important roles in preclinical trials of CAR T-cell therapies, addressing their on-target/off-tumor toxicities remains a challenge due to significant divergences in terms of immune systems and antigenic homology between mice and humans. Non-human primate models provide a more informative platform for on-target/off-tumor toxicities as their immune system and antigenic homology are similar to those of humans. Preclinical studies on CAR T cells have been increasingly conducted in non-human primates, such as cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*M. mulatta*); however, no reports to date investigated non-virally engineered CAR T cells in non-human primates. We have engineered several CAR T-cell therapies using non-viral *piggyBac* transposon system by electroporation, some of which are being prepared for first-in-human trials, including GM-CSF receptor (GMR) CAR T cells for myeloid neoplasms and EPHB4 CAR T cells for sarcomas. With the support of the Japan Agency for Medical Research and Development, we have developed an infrastructure for preclinical safety studies to evaluate non-virally engineered CAR T cells in non-human primate models. **Methods:** $17 \pm 3 \times 10^6$ mononuclear cells were collected from 11 ± 6 ml blood samples of cynomolgus macaques, and the mononuclear cells were electroporated with 7.5 μg of a transposon plasmid encoding GMR CAR and 7.5 μg of a *piggyBac* transposase plasmid using the NEPA21 Super Electroporator. The electroporated T cells were cultured in a defined T-cell medium supplemented with recombinant human interleukin (IL)-7, human IL-15. The cells were collected on day 14 after culture initiation. The final simian GMR CAR T cells were counted, and CAR expression and immunophenotype were determined. Additionally, simian T cells without genetic modification were cultured as control. To test the efficacy of CAR-T cells, CAR-T or control T cells were cultured with the human acute myeloid leukemia cell line MV4-11 at an E:T ratio of 1:1. Next, autologous CAR-T cells were administered to three cynomolgus macaques, followed by a close monitoring of the clinical manifestation of each recipient for specific symptoms of CRS, and a sequential sampling of blood for complete blood count, blood chemistry, coagulation tests, and cytokine profiles. **Results:** We obtained simian GMR CAR-T cells with 11 ± 3% of CAR positivity and modest cell expansion ($8.7 \pm 3.5 \times 10^6$ CAR-T cells at day 14). Nevertheless, CAR-T cells almost completely killed MV4-11 cells, whereas control T cells had a minimal effect. Additional analyses were ongoing at the time of the abstract submission, with the collected data and analyses to be presented at the annual meeting. **Conclusions:** We successfully generated non-virally modified CAR T cells from cynomolgus macaques using the *piggyBac* transposon system with electroporation. The results of the safety study along with the data derived from the administration of GMR CAR T cells in non-human primates will be available shortly.

886. Generation of Isogenic Human iPSCs Utilising Autonomously Replicating Episomal S/MAR DNA Nanovectors

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A significant advantage of **induced pluripotent stem cells (iPSCs)** is that they can be derived from somatic cells, which can be isolated using non-invasive techniques. This eliminates not only ethical concerns associated with embryonic stem cells but also the risk of immune rejection. Therefore, iPSCs are an attractive tool for personalised medicine, drug screening and the generation of disease

models. Typically, the modification of pluripotent cells is done by using **integrating viral vectors**, which are the most effective gene delivery systems in use today. However, their efficacy of gene transfer is tempered by their potential integrative genotoxicity. Alternatively, **non-viral DNA vectors** are attractive alternatives to viral gene delivery systems because of their low toxicity, relatively easy production and great versatility. However, their efficiency is still regarded as below the requirements for realistic *in vivo* gene therapy due to deficient delivery exacerbated by the merely transient gene expression of plasmid DNA *in vivo.* Thus, the development of safer, more efficient, economical and easily prepared persistently expressing genetic vectors remains one of the main strategic tasks of gene therapy research and is the crucial prerequisite for its successful clinical application.An ideal vector for the genetic modification of cells should deliver sustainable therapeutic levels of gene expression without compromising the viability of the host in any way. Permanently maintained, episomal and autonomously replicating DNA vectors, which comprise mainly human elements, might provide the most suitable method for achieving these goals. We developed a non-viral, non-integrating and autonomously replicating DNA vector system based on the use of a Scaffold Matrix Associated Region (S/MAR), for the persistent **genetic modification and reprogramming** of human induced pluripotent stem cells (hiPSCs). Although this DNA Vector is among the best of its class, one of its limitations is that as it is produced in bacteria, it comprises a large proportion of bacterial sequences which are unnecessary and undesirable for clinical application. Accordingly, we developed minimally sized-antibiotic free Nanoplasmids™ as a novel technology to persistently genetically modify human stem cells without causing any molecular or genetic damage. We showed that both S/MAR vectors and Nanoplasmids are capable of simultaneously reprograming and genetically modifying human fibroblasts. Molecular and genetic analysis of S/MAR-labelled cells revealed that the vectors are kept at low copy numbers, are present in their episomal forms and do not modify or genetically damage the cells or their progeny, as the cells fully retain their pluripotent capabilities and can differentiate into representatives of the three germ layers while sustaining high levels of transgene expression. Additionally, we investigated the effect of S/MAR vectors on the cell's genome and compared it to the widely used episomal reprogramming system based on EBNA/OriP vectors. We found that EBNA alone is responsible for the significant dysregulation of 10 genes, including the oncogene *Fos*, compared to only four genes deregulated due to S/MAR vectors. Additionally, by removing bacterial sequences, we found that the genetic perturbation is reduced to a single gene being upregulated when Nanoplasmids are used. For the first time, this work shows that human cells can be simultaneously modified and reprogrammed using S/MAR vectors. This vector system provides robust transgene expression which is sustained through the reprogramming and differentiation process and results in the generation of isogenic hiPSC.

887. Transplantation of Human Myoblasts in Non-Human Primates as a Model for Studies of Cell Therapy in Skeletal Muscle

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Background: Different cells isolated from human tissues were proposed for cell therapy of muscle diseases, essentially to restore dystrophin expression in Duchenne muscular dystrophy, but their myogenic properties were reported only after xenotransplantation in immunodeficient mice. Given that cell therapy protocols developed in non-human primates (NHPs) were better translated to Duchenne muscular dystrophy patients than those that were not verified in this model, restoring dystrophin in up to 26%, 27.5% and 34.5% of the muscle fibers in myoblast grafted regions, and considering that NHPs are crucial in preclinical transplantation research, we wanted to test the feasibility of xenotransplant human myogenic cells in NHP muscles. **Material and methods**: Human myoblasts (CD56+ cloned human cells derived from muscle samples obtained post mortem in a normal 13-month-old baby) were transduced with the LacZ gene using a replication defective retroviral vector. They were injected into muscle regions of 1 cm^3 (around 25 x 10^6 viable cells per site) in four cynomolgus macaques immunosuppressed with oral tacrolimus and dexamethasone. Allogeneic LacZ-labeled myoblasts were transplanted similarly in other muscles as a positive control for engraftment. Cell-grafted regions were sampled 1 month later and analyzed by histology in cryostat serial sections using ß-galactosidase histochemical detection, hematoxylin and eosin stain, and CD8 and CD4 immunodetection. **Results**: Abundant ß-galactosidase-positive muscle fibers were found in all regions grafted with human myoblasts (an average of around 25/mm^2). Histological analyzes showed absence of specific cellular immune responses in three monkeys and minimal focal lymphocytic infiltrates in the monkey that had the lowest tacrolimus blood levels. Similar patterns of ß-galactosidase-positive muscle fibers were observed in all regions grafted with cynomolgus myoblasts, in the absence of specific immune responses. **Conclusions**: We demonstrated that human myoblasts can form hybrid muscle fibers in NHP muscles and that a conventional tacrolimus-based immunosuppression is sufficient to control rejection in this case (which seems to be similar to allogeneic rejection). This opens the door to NHP studies with other human cells in which myogenic properties were found by xenotransplantation in immunodeficient mice, validating the myogenicity of these cells in a more appropriate model than mice for clinical translation and investigating the administration parameters necessary in humans.

Vector and Cell Engineering, Production or Manufacturing II

888. The Ambr 15 System as a Screening Tool for Lentiviral Vector Process Development

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bluebird bio is a clinical stage gene therapy company focusing on severe genetic diseases and T-cell based immunotherapies. Some of our therapies use a lentiviral vector (LVV) system for *ex vivo* primary cell transduction. Adherent cell culture-based LVV production processes can robustly produce supply for current programs but may be limited in their ability to scale. Thus, volumetrically-scalable suspension cell culture-based processes are needed to meet future demands for indications with large patient populations. Traditional stirred tank bioreactor (STR) development is performed at the bench-scale (2-5 L) for eventual scale-up. This quickly becomes cost-prohibitive when executing design of experiment (DoE) or screening studies with numerous replicates given the high cost of reagents and consumables in transient transfection-based processes. The work is also operationally intensive and requires substantial lab infrastructure to run multiple systems. With the goal of lowering cost while increasing throughput during development, a mini/micro scale bioreactor system was sought which could reproducibly perform the LVV production process to screen for changes that significantly affect productivity and consistency. Examples of this type of screening are studies focusing on operational process variations/perturbations, additives, or reagent/consumable alternatives. The ambr® 15 (advanced microbioreactor) instrument mimics the characteristics of classical STRs at the miniature scale (10-15 mL). It uses cost-effective, disposable microbioreactors that are controlled by an automated workstation. Twenty-four vessels can be operated simultaneously with the benefit of independent gassing for DO/pH control and built-in liquid handling. Given its potential utility for screening, we developed and refined an ambr 15 LVV production process until it achieved culture characteristics and productivities comparable to bench-scale. Specifically, we sought to achieve similar cell growth rates, culture viability, transfection efficiencies and infectious titer compared to the bench-scale. Herein we present an overview of how we established, implemented and optimized an ambr 15 LVV production process for screening. We demonstrate that the process cannot be linearly or methodically scaled-down from a bench-scale STR due to system differences, which include vessel geometry, the lack of analogous mini/micro scale medium exchange technologies, liquid handling mechanisms, and constraints in system component availability. Instead, we adjusted to system differences by developing novel process steps and techniques. The implementation of these techniques produced results which closely aligned with the bench-scale process in culture characteristics, transfection efficiency and productivity. They also lowered the variability between vessels in the workstation. Altogether, this work demonstrates the power of the ambr 15 system and its suitability as a process screening tool for scalable LVV production.

889. Development of a Suspension-Based Scalable Platform for Lentiviral Vectors

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Lentivirus is one of the most powerful vectors used in cell based therapies so far. It allows permanent therapeutic effects as the delivered gene can be maintained for the life time of patients. While Lentiviral vectors can be directly used for in- in vivo gene therapy, with the recent commercial approval and stunning successes of various CAR-T therapies in many clinical trials, the demand for lentiviral vectors is higher than ever. However, the low productivity and limited scalability of lentiviral manufacturing platform have been a major bottleneck in the field of gene and cell therapy. The limited scalability of lentivirus production is often associated with adherent nature of production cell, contributing to high cost-of-goods. To overcome this, as a first step, we are establishing the suspension based lentivirus production process. To that end, we have obtained access to adherent HEK293T cells and adapted them to suspension culture. Single cell clones (SCC) were isolated from this parental, suspension-adapted HEK293T cell by a limiting dilution followed by a subsequent expansion and banking of isolated clones. The isolated clones were tested for lentiviral vector production and some of the single cell clones surpassed the parental cell productivity resulting in higher titer of lentivirus compared the parental cells. To further improve lentiviral vector production, substantial effort was made to optimize the process parameters to increase productivity. Some of parameters tested include newly designed plasmids, use of cell culture supplements and boosts, cell density at the time of transfection, plasmid DNA amount and ratio in transfection, and type of production media, etc. The data from these experiments, including the results from the scale up process will be presented. In addition, we also developed and optimized the analytical methods to reliably measure the lentiviral titers and the potency of the vectors in primary cells. This process will offers a scalable lentiviral vector production platform with a high productivity which employs a suspension-adapted, clonal HEK293T cell line with a GMP compliant manufacturing process.

890. High Titer Lentivirus Production in Suspension Cell Culture

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A major bottleneck in recombinant viral vector manufacturing is the labor-intensive, expensive process currently required to meet growing clinical demand. Most production processes make use of adherent cultures amenable only to scale out (increase in identical units) for manufacturing rather than scale up (increase in vessel size). To meet anticipated viral vector demand and reduce costs, a scale up, suspension process that yields high titer virus is required. Here, we describe the development of a suspension-based transient lentivirus production process at an industry-relevant scale. We screened HEK293T clonal derivatives for improved growth and production characteristics to create a cell line specifically optimized for lentiviral production. This cell line was suspension adapted and shown to exhibit high cell density and viability in suspension formats including single-use bioreactors. In addition, we developed an animal origin-free, chemically defined media that is compatible with PEI-based transfection in suspension

platforms. Finally, optimal process parameters were defined for growth and lentivirus production phases, and lentiviral production in single use bioreactors has been shown to yield similar functional titers to the traditional adherent, serum-containing lentivirus process.

891. Advances in Large Scale Cell Manufacturing: Smart Perfusion and Quick Dissolving Microcarriers

Yoni Levinson
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Scalable bioreactor platforms for large scale expansion of cell therapy products, including Mesenchymal Stromal Cells (MSCs), Pluripotent Stem Cells (PSCs) and primary cells, are becoming widely adopted in industry. Bioreactors offer critical advantages to manufacturing: they can produce commercially relevant quantities of cell product in a manageable footprint, they are closed systems that lend themselves to streamlined unit operations, and by tightly control cellular environments they can produce robust, repeatable results. Previously, we have presented a bioreactor platform which featured two novel capabilities: continuous media exchange through perfusion, and streamlined cell harvest through dissolvable microcarriers. Here we present advances in both of these areas. The first is a Raman spectroscopy probe whose data can be modeled to provide inline, real time measurements of biomolecules such as glucose, lactate and ammonia. We have successfully integrated this Raman probe into our bioreactor system and modeled the aforementioned molecules for an MSC, microcarrier based expansion process. Furthermore, we have demonstrated the ability to use the real time Raman measurements to control perfusion; with our Smart Perfusion system, media and nutrient solutions can be fed only as needed in order to maintain set points. We have demonstrated two examples of Smart Perfusion: one in which glucose is fed into the bioreactor to automatically maintain a given set point in response to cellular consumption, and a second in which fresh media is perfused through the bioreactor to limit the buildup of waste products such as lactic acid and ammonia. The second is a novel dissolvable microcarrier based off of the Cultispher technology. While traditional Cultispher is dissolved enzymatically, the enzymatic activity can be a rate limiting step in manufacturing. Our novel carrier has been designed to dissolve in response to a chemical, rather than enzymatic stimulus, the kinetics of which are significantly faster than Cultispher. We have demonstrated that, using this quick dissolving microcarrier, MSCs can be harvested from a bioreactor in less than half the time.

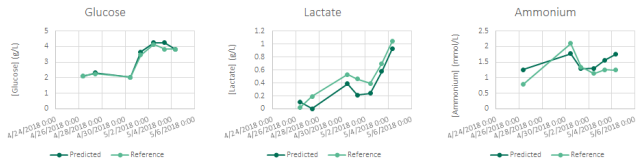

Figure 1: Raman-predicted versus reference values for glucose, lactate and ammonium in a bioreactor based MSC expansion process. RMSEp values for glucose, lactate and ammonium are 0.18 g/L, 0.16 g/L and 0.36 mmol/L, respectively.

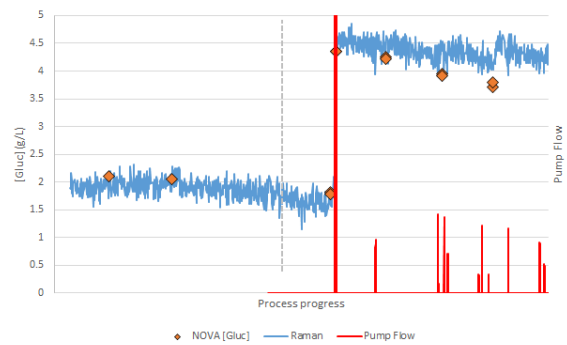

Figure 2: Partway through a bioreactor MSC expansion process, the glucose is adjusted from 2g/L to 4g/L, which is automatically performed via Smart Perfusion. As the cells consume glucose, a pump automatically feeds in fresh glucose to maintain the set point (red peaks).

892. Large-Scale Production, Purification, and Concentration of Therapeutic 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 is 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 methodology to scale-up production of high-titer LV using HYPERStack technology, a vessel consisting of many layers of gas-permeable tissue culture-treated growth surface in a fully closed, disposable system. Following LV packaging and harvest, we describe a method for LV purification by way of Mustang Q anion-exchange membrane chromatography, and a method for LV diafiltration and concentration using tangential flow filtration (TFF). Research labs typically generate LV vector by way of a plate-based culture strategy. We routinely generate LV stocks of over 10^8 transduction units (TU) per ml using such methods. Utilizing therapeutically relevant LV vectors we compared laboratory plate-based to large-scale HYPER technology-based LV production. HYPERStack LV culture resulted in similar LV yield per surface-area while requiring significantly reduced overall incubator space and hours of labor. We also report an increase in LV production when collection steps are completed in serum-free media (10-20% increase in LV production). Mustang Q anion-exchange membrane chromatography was used to purify and concentrate the LV resulting in a 1000-fold volume reduction with excellent viral recovery (60-70% of starting LV). Tangential flow filtration was used to further concentrate the LV down to 15-20 mL and to perform a PBS diafiltration. The HYPERStack LV packaging strategy, Mustang Q chromatography, and TFF purification regiment described here is a

practical method for LV scale-up to early-stage clinical trial volumes using affordable disposable laboratory supplies. We will adapt these approaches in our Facility at the Medical College of Wisconsin as we generate GMP-grade vector for clinical trials.

893. GMP Manufacturing of Lentiviral Vectors: Scale-Up Considerations

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The number of highprofile products based on cell and gene therapies has increased dramatically over the past few years. Consequently, advanced therapeutics now attract significant interest from the wider Biotech/Pharma and investment communities. A number of gene and cell therapy products have recently been approved, illustrating their potential. For the past 20 years, Oxford BioMedica (OXB) has been a pioneer in the development of products based on lentiviral vectors. OXB is using this broad CMC, clinical and regulatory experience and know-how to facilitate development of lentiviral vectors for OXB pipeline products and for those of our strategic partners. To support this, OXB has developed a suspension cell culture manufacturing process capable of generating lentiviral products with suitable quality attributes and at the capacity and cost of goods required to provide security of GMP supply. Initial process development in shake flasks was translated to 5 L laboratory scale bioreactors in order to understand parameters that were important for scale-up. Modelling methodologies were utilised at 500 mL and 5 L bioreactor scales to determine the limitations of the scaleup. Following this, scale-up was first verified at 50 L pilot scale and then at 200 L manufacturing scale using processes exclusively based on single-use technologies. The suspension platform process has now been demonstrated successfully at 200 L scale under GMP conditions for a variety of lentiviral vector based products. Recent innovations in process and scale up will be discussed.

894. Chromatography Purification Methods for Rhabdoviral Vectors

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The field of genetic vaccines and oncolytic viruses has matured at a stage whereby further progress is hindered by the establishment of effective manufacturing processes that can deliver high yields of purified, high quality viral vectors with efficient removal from the final product of host-cell debris, proteins, nucleic acids, as well as inactive virus particles and virus aggregates. The most commonly used purification strategies for recombinant proteins such as chromatography face numerous obstacles due to the large size of virus particles. We are currently investigating two chromatography purification methods that utilize a different chromatography matrix and flow configuration to identify the optimal procedures for purification of Rhabdoviral vectors. The first method is through the use of a polymethacrylate CIMmultus monolithic column functionalized with hydroxy (OH) groups (BIA Separations). Monolithic columns possess a uniform, continuous network of large-sized pores and channels that are suited for the purification of large biomolecules such as virus particles. Compared to conventional packed-bed resins, monolithic columns allow convective mass transfer which leads to reduced virus processing time. We have recently established a step and linear gradient elution method with a 1 mL hydrophobic CIMmultus OH column which resulted in >60% virus recovery and removal of >70% impurities. In contrast, purification using a CIMmultus monolithic column functionalized with quaternary ammonium (QA) groups resulted in <20% virus recovery. The second method is via membrane chromatography which, similar to monolith chromatography, is comprised of a matrix with a network of large interconnected pores that is suitable for the passage and purification of virus particles. Most commercial membrane chromatography devices as well as the monolith columns operate under a radial flow configuration, which results in non-uniform flow distribution along the matrix and ultimately poor usage of binding sites and the dilution of samples. We have developed a process using a laterally-fed membrane chromatography (LFMC) device that drives a homogeneous flow distribution along the device. This flow configuration has been shown to result in better resolution and lower product dilution during protein purification. We have recently evaluated the LFMC device with a Sartobind Q membrane for Adenovirus purification in comparison to the radial-flow Sartobind Nano capsule that contains the same 1 mL amount of the Sartobind Q membrane functionalized with QA groups. We confirmed the advantage of the LFMC device in terms of virus recovery, better resolution of virus-containing peaks, lower sample dilution and shorter run times. We have now extended this study to investigate the purification of Rhabdoviral vectors with the Sartobind Q membrane either housed in a commercial capsule (Sartobind Nano) or in the LFMC device. Preliminary results show <10% virus recovery with the Sartobind Q membrane. We therefore plan to test a hydrophobic membrane to assess if it will outperform the Sartobind anion exchange membrane similar to what was observed with the monolithic columns. Future work will involve scaling up both methods utilizing the 8 mL monolithic CIM OH column and the 10 mL LFMC device along with the 7 mL commercial membrane chromatography capsules.

895. Latest Advancements in Process Intensification for Viral Vector Manufacturing

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The hopes are high for gene therapy to improve the quality of life across the globe. Biotech companies have therapies in their pipelines that may be transformative, possibly curing genetic diseases with just one or several treatments. However, the current manufacturing costs and availability of the viral vectors needed for these therapies turn out to be a large obstacle. Viral vector manufacturing typically requires large and expensive manufacturing facilities that result in high Cost of Goods. One of the strategies to address this issue, is to intensify the viral vector production processes. This presentation will give an overview of the latest process intensification technologies to dramatically increase process efficiency, decrease production scale

and the CoGs. Combined, these new technologies like, cell lines optimized for virus propagation, media capable of supporting high cell density cell growth, high cell density single-use bioreactors and high efficiency and single step purification technologies provide a novel viral vector manufacturing platform. The platform enables viral vector yields to be significantly increased, which in turn allows commercial manufacturing in a small-footprint, low cost micro-facility capable of delivering at low operational costs. The platform is currently being established by using Sabin IPV as a case study. Current status is that the Vero cell line has been selected and successfully cultured in high cell density, single-use bioreactors up to 40 million cells per mL. Following infection with Sabin poliovirus vaccine strains, a single chromatographic step using a novel membrane has resulted in high recoveries and the required purity for IPV vaccines. For IPV, these yield intensifications mean that the entire commercial scale process can be operated in isolators in a footprint of around 30 m2. Combination of four of these manufacturing units would be capable of delivering more than 40 million doses of trivalent sIPV per year. Performance of the manufacturing process in isolators also allows the manufacture of viral vectors that currently require high biological safety containment in an inexpensive facility design. This presentation will discuss the technologies used in manufacturing platform and data obtained to date on Sabin IPV in more detail.

896. Scale up of Iodixanol Density Gradient Continuous Flow Ultracentrifugation Used for AAV Purification

Sandra Merino
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Adeno-associated virus (AAV) is increasingly used in Gene Therapies and the scale up of production processes is an essential step in the transition from small scale laboratory procedures to a large scale process. There is a need to generate large quantities of purified vector in sufficient amounts for clinical trial and manufacturing of final therapies. Transition to the manufacturing process requires a process that is rapidly and easily scalable and preferably can be used for a number of gene therapy presentations without the need for substantial redesign. Laboratory processes are readily effected using ultracentrifuges and column chromatography methods. Both methods will prepare AAV effectively at small scale and both can be transitioned to the larger manufacturing scale. To scale up laboratory Ultracentrifugation methods we tested the Alfa Wassermann Promatix 1000™ Ultracentrifuge using the continuous flow mode for its capability to purify AAV vectors. The resulting purifications using Iodixanol density gradient centrifugation show a high recovery rate with a single step purification with a yield exceeding 50%. This Iodixanol density gradient method has been tested further using the Alfa Wassermann PKII and KII ultracentrifuges which are used in cGMP manufacture of gene therapies and vaccines. Compared to the laboratory systems the cGMP ultracentrifuges have a scale up range of 3x laboratory scale up to 27x laboratory scale. Analysis of ultracentrifuge separations was completed using Iodixanol gradients. Results showed equivalent gradient shapes in the KII Large-scale, PKII Pilot-scale and the AW Promatix 1000™ scale ultracentrifuge systems. The presented figures describe equivalent Iodixanol gradient shapes across the Alfa Wassermann range of ultracentrifuges. Both scalability and linearity are achieved. Scalability is demonstrated because the run parameters remained the same, even though rotor assembly volume varied. Linearity was confirmed; comparable gradient formations are achieved in the KII Large-scale, PKII Pilot-scale and the AW Promatix 1000™ scale. The Iodixanol gradient remains identical throughout the volumetric differences between each separation. The Alfa Wassermann ultracentrifuges retain the same separation parameters in a process in which the volume of the product sample centrifuged is to be scaled up or down.

897. Mitigating the Risks of Adventitious Agents in Serum: Elimination or Viral Inactivation

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Human AB serum (hABs) is used in culture medium for some cell therapies, such as chimeric antigen receptor T cell (CAR T) or engineered T cell receptor therapies, to provide the necessary growth factors and nutrients for cell proliferation. While hABs has been demonstrated to effectively sustain primary cell cultures, hABs has several drawbacks including lot-to-lot variability, supply constraints, and, above all, the risk associated with adventitious agents. hABs is derived from either whole blood or plasma donations that are subject to stringent sourcing controls such as viral screening on individual donations. The addition of a viral clearance step can further mitigate the risk associated with potential adventitious agents. Celgene is exploring two strategies to mitigate the risk associated with adventitious agents: γ-irradiation of hABs to inactivate viruses and serum-free medium (SFM) formulations to eliminate the use of complex animal-derived materials. γ-irradiation has been proven to be an effective viral reduction method in animal sera, but its application to human serum is currently limited. γ-irradiation is known to impact serum composition, thus risking potential changes to process performance and critical product quality attributes. Additionally, there is a limited number of hABs vendors with γ-irradiation experience, ultimately requiring close collaboration between company and vendor to establish a robust supply chain. Adoption of a SFM formulation can eliminate some of the challenges associated with serum. SFM formulations are commercially available, well defined, and can serve to replace hABs entirely. Implementation of a new SFM formulation in an existing process risks changes to critical quality attributes of the cell product and may require significant development efforts to achieve comparability. In addition, many commercially available SFM formulations still contain human- or animal-derived materials, such as human serum albumin or transferrin, which also can present viral disease transmission risk and must undergo appropriate viral reduction treatments. Celgene's strategy is to evaluate both γ-irradiated hABs and SFM for cell therapy applications. Both media containing γ-irradiated hABs and SFM support T cell expansion, though differences have been observed when compared with typical hABs containing media. Moreover, both γ-irradiated hABs and serum-free alternatives may result in differences in CAR T cell quality attributes, such as phenotype and activity. Investment in development and comparability is critical when planning to incorporate γ-irradiated hABs or SFM in cell therapies in

order to reduce the risk associated with adventitious agents. Careful selection of media formulation for future programs should also be a consideration given the challenges of changing media formulations for an existing product.

898. High Titer Recombinant Lentivirus and Adeno-Associated Virus Production for Therapeutic Applications

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Mirus Bio LLC, Madison, WI

Transient transfection is a robust and reliable tool for the manufacture of recombinant lentivirus and adeno-associated virus (AAV). The therapeutic promise of these technologies through the modification of relevant immune cells or overexpression of a target gene drives the demand for enhanced virus production methodologies. Recognizing this need, Mirus Bio has developed a novel transfection formulation, *Trans*IT-VirusGEN®, specifically for high titer manufacture of recombinant lentivirus and AAV in 293-derived cell types. In addition, movement of recombinant virus production into the gene and cell therapy manufacturing arena increases the focus on quality parameters such as reproducibility and raw material testing. To address some of these criteria, lot-to-lot consistency, identity and safety testing of the transfection formulation were assessed. To further support high yield recombinant lentivirus and AAV production, we identified novel enhancers and a complex formation solution that increase functional virus titers 2-3 fold over previously optimized high titer conditions. These parameters were tested with multiple serum-free media formulations and examined using different plasmid DNA concentrations to better understand the compatibility within a workflow. In addition, head-to-head comparisons of this novel system were performed with commonly used transfection technologies to accurately assess the recombinant virus production capabilities. Our data demonstrate that we can push the limits of high titer recombinant lentivirus and AAV production to increase manufacturing capabilities for gene and cell therapy applications.

899. Viral Clearance for rAAV Products in a Sf9/Baculovirus Manufacturing Process

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Voyager Therapeutics, Cambridge, MA

Although the risk of infection with viruses pathogenic for humans is lower for insect cell lines than mammalian cell lines, the risk of contamination from adventitious and endogenous viruses is still present when producing recombinant adeno-associated virus (rAAV) vectors with a Sf9/baculovirus system. For this reason, gene therapy manufacturers must minimize the risk of viral contamination to ensure the safe and continuous supply of clinical and commercial products. We have implemented a comprehensive viral risk mitigation strategy that includes prevention of adventitious viruses entering the upstream process, detection of contaminants in raw materials and process intermediates, and removal of viruses by downstream purification processes. This work focuses on the latter and will outline a stage-appropriate viral clearance strategy for rAAV vectors derived from a Sf9/baculovirus production system. In this work, we show the significant reduction of multiple model viruses for four orthogonal purification steps, which demonstrates the capacity of Voyager's downstream platform process to effectively remove a relevant panel of viral contaminants.

900. Development of a Formulation to Improve Stability of Herpes Simplex Virus Type 1

Jeong Oh[1], Woojin Shin[2], Byeong Chang[1], Jae Jung[2], Ki Jeong Lee[1]
[1]Vaccine Stabilization Institute, Camarillo, CA,[2]University of Southern California, Los Angeles, CA

There are increasing demands of viruses and viral vectors due to recent successes in cell and gene therapies. Manufacturing and preserving intact biological activities of viruses and viral vectors, however, have been challenging because they have high-ordered complex structures and are labile to routine environment for pharmaceutical applications, i.e. manufacturing, storage, and administration. One approach to preserve the stability of virus and viral vector based products is lyophilization. Here we report developments of a stable formulation of a herpes simplex virus (HSV). HSV encoding a green fluorescence protein (GFP) was used for optimizations of formulation and expression of GFP was monitored as a measure of biological activities of the virus. Addition of mixture of amino acids in formulation improved stability of HSV during lyophilization. The stability of the virus is further improved by addition of sugar, a salt, and a protein in the presence of the amino acids. With the formulation, close to full recovery of virus was achieved after lyophilization and reconstitution. In addition, the biological activity of the virus before lyophilization also appeared to be greater than that of other commercial formulations. These results suggest that the combination of amino acids, protein, sugar, and salt not only stabilizes the virus during lyophilization but also stabilizes during analytical procedures in liquid state. The formulation technology and a screening process established at Vaccine Stabilization Institute can be further applied for other viruses, viral vectors, and vaccines to enhance the productivity of related therapeutic products.

901. Capability Creation Through Collaborative Innovation. Development of Production Understanding to Facilitate the Rapid Development and Industrial Manufacture of AAV Based Viral Vectors

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Cobra Biologics, Keele, United Kingdom

Production of AAV vectors for gene therapy applications presents many scientific and technical challenges for rapid process development and large scale GMP compliant manufacture. To address these challenges we have built a portfolio of collaborative innovation projects, which build and enhance knowledge of the underpinning science of AAV production, coupled with process and technology understanding that when combined facilitate industrial AAV vector manufacture. The presentation/poster will highlight the innovation collaborations, and

provide an insight into the complexity and challenges of transient AAV production. Specifically it will cover some of the key challenges for AAV production in anchorage-dependent, tethered and suspension cell systems, focusing on improving the AAV genomic particle yield by developing a media that maintains cell health during the process. Also covered will be the essential requirement for accurate and robust process and product characterisation to support rapid process development and manufacture, with a key focus on understanding transgene packaging efficiency and the ability of AAV to package off-target sequences. Finally, recent progress on the understanding of innovative purification approaches that employ new technologies for chromatography-based systems, and early stage innovative novel separation approaches utilising structure-function relationships of the different AAV serotypes. We have tried to remain agnostic to the type of AAV vector produced, therefore a diverse range of AAV serotypes are used as model vectors within these projects, ensuring a broad serotype understanding and serotype-specific process challenges.

902. Scalable Single-Use Technology for Viral Vectors Production

Alex Chatel, Jean-Christophe Drugmand, Yael Dohogne, Florence Collignon, José Castillo
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Adherent cells for the production of viral vectors are widely used in the development and commercialization of gene therapies and will become even more so in the future. Traditional processes for adherent cell culture use static methods (e.g. multi-tray plastic ware) for process development and industrial production, but these suffer from a number of limitations. Static methods lack precise environmental control (pH, Dissolved Oxygen (DO), media composition), are heavily dependent on manual operations and can only be scaled-out as opposed to scaled-up. To address these challenges, Univercells has developed a portfolio of single-use, fixed-bed bioreactors - the scale-X™ systems - accommodating viral production from process development, pilot scale, to medium and large scale industrial production. The scale-X bioreactor systems are operated in fed-batch or perfusion modes, with in-line clarification and capture processes operated in simulated continuous & automated mode reducing therefore the number and complexity of operations compared with traditional equipment. Direct linear scalability is ensured by applying concepts similar to those found in the scale-up of chromatography columns, whereby the height of the reactor is kept the same with its diameter increased as a function of the scale. In addition, the physical and chemical conditions are kept the same across scales, ensuring a smooth and risk-free scale-up. Costs of manufacturing of viral gene therapy and cell-based vaccines are significantly reduced and local production at affordable costs is possible in emerging countries thanks to this innovative solution. Based on case studies using VERO and HEK293 cells for sIPV and adenovirus production, this presentation will demonstrate how scale-X system can improve reproducibility, reduce both capital and operating costs and remove the scalability bottleneck between clinical trials and full commercial production. Results such as cell culture dynamics, productivity, and key process quality indicators will be demonstrated as well.

903. Closing the Fill/Finish Step for Reduced Risks to cGMP Virus or Cell Production

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Fill/finish is an especially risky step of the production of any biological therapeutic. Absolute sterility has to be accomplished without compromising live product quality. Here we report media fill tests for this final step in the aseptic Xvivo GMP System to test the sterility of conditions. This system provides a continuously HEPA filtered ISO 5/ Class A processing space without using any room air or external HVAC systems. It can be charged with dry Nitrogen for an inert atmosphere or maintain a constant 37 C/ 5% CO2/ physiologic O2 to avoid suboptimal transients for a cellular product. Personnel, and the bioburden associated with them, are separated from the aseptic space by a soft, flexible glovefront. We used a highly permissive color-changing microbial broth to simulate a typical vial fill process of three batches of 100 vials each. We used an air sampler to draw processing chamber air across a contact plate during filling. Before and after each batch, we used contact plates to assess microbial contamination of probable risk surfaces like gloves, sleeves, floor, and doorknobs. We incubated all positive controls, test vials and plates for up to 14 days, evaluating them at 1, 5, 7, and 14 days. Positive control plates and vials all showed ample evidence of contamination within 5 days while none of the test samples showed signs of contamination. Environmental monitoring plates for air and probable risk surfaces also showed no signs of contamination (less than 1 CFU). We conclude that the Xvivo GMP System is an aseptic environment that can reduce risks for the critical fill/finish step of any cGMP viral or cellular production process.

904. Optimization, Validation, and Implementation of Retroviral Retentive Filters for Gene Therapy Vectors

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The most recent guidelines established by the FDA (July 2018) for Human Gene Therapy Investigational New Drug Applications (INDs), recommend that information be provided on viral safety studies for viral adventitious agents. Viral retentive filtration is a well-established practice in traditional biologics manufacturing, where it is necessary to identify and mitigate adventitious and endogenous viral agent risk. A similar approach can be used for Adeno-associated Virus (AAV)-based gene therapy, using retentive retroviral filters to mitigate viral contamination risk. This approach takes advantage of the small diameter of AAV (20 - 25 nm) to pass through a retentive retroviral filter, while retaining larger adventitious and endogenous viral agents. The work to be presented focuses on the initial development process of the retroviral retentive filter Viresolve® NFR for an AAV gene therapy feed. Results suggests a retroviral retentive step that provides high capacity and high AAV yield. Also, we will discuss the selection of an IND enabling viral clearance panel that best represents the potential

adventitious and endogenous viral agents found in a AAV based gene therapy process. Finally, the downstream impact of implementation of Viresolve® NFR in a GMP setting will be considered.

Next Generation RNA Virus Vector Technologies

905. The Impact of Vector Integration on Chromatin Architecture

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Self-Inactivating (SIN) Lentiviral Vectors (LVs) have demonstrated great efficacy and safety in preclinical models and clinical trials. Still, SIN LV integrations are not entirely neutral to the cell genome and can interfere with physiological gene expression by various genotoxicity mechanisms. In this work, we study the impact on chromatin architecture of SIN LV integrations carrying chromatin insulators (CI) and/or strong enhancers, features able to interact with distant genomic loci and whose effects on the 3D conformation of the genome are so far unexplored. To this aim, we devised a LV-specific Circular Chromosome Conformation Capture (LV4C) method, able to retrieve the LV integration site (IS) linked by proximity ligation to the corresponding host-genomic long-range interacting site. As internal control, we captured chromatin interactions of the MYC-locus that we compare with published interactions for that locus (Correlation with HiC datasets R2 = 0.9). We applied LV4C on K562 cell clones marked by multiple (30-60) LV IS of either a SIN LV with a strong enhancer or of the same construct armed with an *in vivo* characterized CI bound by the CCCTC-binding-factor (CTCF). In an *in vivo* genotoxicity study based on tumor prone mice (n=178 mice) in which we studied five different insulator-harboring SIN LVs, we show this CI to improve the mouse survival by reducing vector-mediated oncogene activation. By LV4C, both LVs display long-range interactions with gene-dense regions, spanning mostly 100-500Kb from the IS. For 50% of the LV IS we observe the LV to interact with multiple (2 to 5) genomic targets. Moreover the LV without CI interacts throughout many different Topologically Associated Domains (TADs) at distances greater than one Mb. Differently, the interactions engaged with the CI-harboring LV confine within the one Mb-scale and occur inside the same TAD targeted by the vector integration site. Interestingly, while the LV without CI interacts with genomic loci upstream and downstream the IS, inducing gene deregulation at such sites, the vector harboring the CI fully redirects long-range interactions towards genomic loci upstream the LV IS that are enriched for CTCF-motives in convergent orientation to the ones present in the LV ($p<0.01$). At these interaction-sites, the genes upstream the IS are not deregulated, while for genes downstream the IS, where no chromatin-loops are formed, we observe vector-mediated aberrant transcription of host genes.Ongoing HiC and ChIP experiments on CTCF binding and different histone marks, together with our gene-expression and interaction-data, will allow studying in a concerted way the effects of LV integrations globally on the cellular genome. Taken together our results show how regulatory features within LVs can affect host chromatin architecture, lastly impacting gene expression. We also highlight the mechanism underlying insulators' function in LVs, promoting their use to improve the safety of gene therapy applications. Finally, our approach can serve to study any regulatory element, carried by LVs, on the host chromatin conformation.

906. Identifying Novel Erythroid Specific Enhancers to Optimize Transgene Expression in Beta Globin Gene Therapy

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Hematopoietic stem cell gene therapy using lentiviral vectors is a promising avenue for treating beta globin disorders. Currently all lentiviral vectors for the gene therapy of hemoglobinopathies utilize core elements of the beta globin locus control region (LCR) to enhance human beta-globin transgene expression. While these lentiviral vectors have been deployed successfully in the clinic, several challenges remain regarding their efficacy in severe β^0/β^0 thalassemia. The current solution to these challenges have involved removing vector safety features or further truncating the large LCR enhancer to achieve better transduction rates. These strategies are problematic because the removal of insulator elements in combination with the high vector copy numbers seen lately in the clinical products have raised potential safety concerns and the sizes of these truncated LCR enhancers span in average 3000bp, a length that still adversely affects the titers of the globin vectors. Furthermore, there is evidence that the canonical and truncated LCRs are not erythroid specific but rather active in multipotent hematopoietic progenitors. In the current study we sought to discover novel, strong and small sized enhancers for high and erythroid specific transgene expression. Towards these ends, we assayed the chromatin accessibility using DNase I along the *ex vivo* erythroid differentiation from adult human CD34+ cells to identify putative erythroid-specific cis-regulatory elements. We then selected 5703 distinct elements and dissected them into overlapping tiles of <200bp length to generate a screening library of 15000 total sequences. The library was synthesized, cloned into a GFP reporter lentiviral vector and subsequently tested in a human erythroid cell line. Enrichment analysis in the high GFP expressing population revealed 682 elements with enhancer activity, corresponding to 570 DHSs several of which were located in close proximity to erythroid specific genes, such as GYPC, GATA1 and CDH1. We observed that enhancing activity of a DHS is located within certain tiles which are found enriched for erythroid transcription factor binding sites, such as (GATA1, TAL1,

KLF1). The compendium of 570 enhancing elements served as a basis for identification and design of truncated concise erythroid-specific enhancers that are currently being evaluated in CD34+ derived erythoid cells. Collectively, our data not only provide further insight into enhancer biology but can be used for constructing more efficient vectors for the gene therapy of hemoglobinopathies.

907. High-Resolution Functional Dissection of Enhancers within the B-Globin Locus Control Region using a Lentiviral Vector-Based Massively Parallel Reporter Assay

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Objective: Autologous hematopoietic stem cell transplantation combined with ex-vivo gene therapy is a promising approach in treating disorders of the hematopoietic system. Identifying combinations of strong lineage-specific control elements that do not impede packaging or transduction efficiency when included in lentiviral vectors has proven challenging. Candidate enhancers or promoters must be tested against a litany of performance criteria until high-performing combinations can be found. A current limitation in designing enhancers of minimal-length is a lack of knowledge regarding the exact boundaries of "sequence intrinsic" enhancers (the actual DNA sequence that provides enhancer function) for a given cell type. Current technologies such as ChIP-Seq and its variants provide vague boundaries of enhancer location when specific combinations of protein binding can be observed within a region. Furthermore, technologies based on protein binding may fail to assist in identification of enhancer regions when proteins transiently bind, modify local chromatin structure, and dissociate before they can be fixed in place by DNA-protein crosslinking. We have developed a method termed LV-MPRA (Lentiviral Vector-based, Massively Parallel Reporter Assay), to generate targeted enhancer maps spanning large DNA regions that provide boundaries of "sequence intrinsic" enhancers at near base-pair resolution. **Methods:** Microarray generated DNAs spanning a targeted region (~16kb) are cloned into a lentiviral vector resulting in placement of, 1). A "Query" sequence upstream of a promoter and, 2). A barcode in both the 3'UTR of a reporter gene and upstream of a polyadenylation signal. The library is then transferred to a cell-line and the strength of a given "Query" sequence can be ascertained by quantifying the abundance of mRNA barcodes by next-generation sequencing (NGS). **Results:** We employ LV-MPRA to elucidate the boundaries of the previously unknown "intrinsic enhancer" sequences of the β-globin Locus Control Region (LCR). We observe that enhancer activity peaks fall well within "classical" enhancer boundaries as defined by the literature. **Conclusion:** We have harnessed the power of massively parallel automated DNA synthesis and NGS to simultaneously analyze thousands of synthetic DNA fragments in parallel to identify "sequence intrinsic" enhancers of the LCR at near base pair resolution. Our approach can be applied to map any genomic locus and facilitates the rapid identification of enhancers for therapeutic vector design.

908. Developing Strategies to Improve the Titers and Gene Transfer of Complex Lentiviral Vectors

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Introduction: Lentiviral vectors (LV) are used to deliver genetic materials to achieve long-term stable expression in target cells to treat many monogenic blood cell diseases. However, some complex LV, like β-globin vectors, have low titers and defective gene transfer, which restrict the clinical translation and commercialization for using these LVs in ex vivo gene therapy. Our overall objectives are to elucidate the mechanism(s) of the titer and gene transfer reduction in complex vectors that compromise lentiviral activities at each step of the viral lifecycle, and to develop strategies to improve the vector design and production to create effective LVs for gene therapy. **Method:** We characterized the efficiencies of viral RNA (vRNA) transcription in packaging cells and viral particle formation during the production of LVs of a "well-behaved" vector, EFS-ADA (4 kb), and a poorly-performing vector, CCL-βAS3 (8.7 kb). 293T cells and unconcentrated viral supernatant were harvested three days after transfection. RNA was extracted, reverse transcribed by random primers, and quantified by droplet digital PCR (ddPCR). Because RNA is transcribed from 5' to 3', we used primers and probes spanning the 5' LTR (R/U5), the PBS (Psi), and the 3' LTR (U3/R) to quantify early, intermediate, and fully transcribed vRNA. The efficiency of viral particle formation was determined by P24 ELISA in viral supernatant. The gene transfer efficiency was measured by transducing CD34+ cells with the vectors at various MOI and determining VCN by ddPCR after 2 weeks in vitro myeloid differentiation culture. **Results:** We observed that vRNAs of the complex vector, CCL- βAS3, with a low titer and defective gene transfer were significantly more truncated in both packaging cells and LVs than vRNAs of the simple vector, EFS-ADA. Only 9.3% vRNA of the complex vector were complete vRNA that can be fully reverse transcribed and integrated into the host cell genomes compared to 75% for EFS-ADA. We further showed that we can improve titers by increasing the level of complete vRNA when packaging LVs with Tat, an HIV accessory protein that enhances the processivity of RNA Pol II. Furthermore, we observed that the complex vector with a reverse-orientation β-globin gene cassette had defective viral particle formation with reduced P24 production. We showed that knocking out protein kinase R (PKR), which is known to inhibit protein translation, in the 293T cells used for vector packaging rescued the translation of viral proteins and improved the titers of vectors with reverse-orientation gene cassettes by 5-7-fold. **Conclusions:** Our findings uncover an important role of complete vRNAs as substrates for each step of lentiviral lifecycle, and reveal that knocking out PKR abolishes the antiviral response against vectors in reverse orientation. Current studies are underway to increase the production of complete vRNA by deleting the problematic sequences or manipulating cellular factors.

909. Use of Syncytins for the Transduction of Murine and Human B Cells

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Syncytins are fusogenic placental glycoproteins derived from endogenous retroviruses. Syncytins mediate several fusogenic biological processes such as the development of syncytiotrophoblast and the generation of skeletal myofibers. This fusogenic activity prompted us to explore the use of syncytins for pseudotyping recombinant HIV-1-derived lentiviral vectors (LV). Previous attempts by others had failed to obtain infectious particles. We decided to test these new LV in the presence of vectofusin-1, a transduction adjuvant for several other retroviral envelopes. For the first time, LV pseudotyped with murine syncytin-A or -B became infectious when vectofusin-1 was added during transduction. While these LVs poorly transduced most cell types *in vitro,* they were highly effective in murine or human CD19+ B lymphocytes. High levels of transduction were achieved *in vitro* in the A20 murine B lymphoma cell line (88±10% transgene+ cells). Transductions of both human (64 ±5%) and murine (89 ±4%) primary CD19+ B cells were also obtained without prior cellular activation. *In vitro* transduction of murine bone marrow cells showed transgene expression in B cell precursors including pro-B, pre-B, immature B cells with increasing intensity throughout their differentiation towards mature B cells. Evaluations were conducted *in vivo* in mice following intravenous administration of syncytin-A Luc2 LV without vectofusin-1. We observed the transduction of spleen cells and the provirus was detected in CD19+ spleen B cells three weeks after LV infusion. This is the first report that syncytins can efficiently pseudotype LV and interact with B cells. Ongoing studies explore receptors and entry mechanisms as well as possible therapeutic applications of these pseudotypes in B cell-mediated immunotherapy or vaccines.

910. Analysis of Human iPSCs Generated by a Non-Integrating Measles Virus Vector

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By the ectopic expression of reprogramming genes OCT, KLF4, SOX2 and MYC (OKSM), somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs). Human iPSCs are considered to be a promising cell source to provide an invaluable resource for the basic investigation and the advanced medicine including gene therapy and regenerative medicine. To minimize risk of insertional mutagenesis in iPSC generation, the use of non-integrating vectors including Sendai virus (SV) is very useful. However, efficient transduction and reprogramming for unstimulated T cells remains one of the challenges of SV vectors. We, therefore, developed a new non-integrating measles virus (MV) vector to improve efficiency of T-cell engineering. In our MV vectors, F gene was deleted to eliminate cell membrane fusion-associated cytotoxicity. MV vectors transduced genes widely through MV receptors including CD46 and signaling lymphocyte activation molecule (CD150/SLAM) and nectin-4. In this study, we examined transduction efficiencies of MV vectors in hematopoietic cells by using GFP expression vector (MV-G). MV-G allowed highly efficient gene transfer into most hematopoietic cell type including T cells (>90%), B cells (>90%), monocytes (>90%), hematopoietic progenitor cells (HPCs, >90%). At the same multiplicity of infection (MOI) of viral transduction, MV-G induced less apoptosis in T cells compared to SV vectors due to slower amplification of viral RNA in the transduced cells after transduction. Next, we developed MV vectors harboring four reprogramming genes (MV-OKSM) and compared with SV vectors harboring the same genes (SV-OKSM). By using the MV-OKSM, we could generate high-quality iPSCs with the similar morphology, pluripotency markers, karyotype and differentiation capacity as human embryonic stem cells. Upon the less cytotoxicity, iPSC generation efficiency of MV-OKSM was much higher than that of SV-OKSM in unstimulated T cells (0.47 ± 0.25% vs 0.008 ± 0.009%). In addition, unlike iPSCs generated by conventional reprogramming vectors, MV-induced iPSCs maintained high OKSM genes expression and showed naive-like morphology when cord blood $CD34^+$ cells were reprogrammed. The whole-genome sequencing also revealed less CpG methylation compared to primed MV-iPSC. We are now studying how the MV-induced iPSCs are sharing naive and primed iPSC properties based on gene expression profiling. Considering the safe history of MV vaccine, carrying capabilities of multiple genes, and higher transduction efficiency for resting T cells, our exclusive MV vector would be a potential gene transfer system for reprogramming and T-cell immunotherapies such as CAR-T.

911. Assessing Functionality and Potential of the Next Generation BET-Independent Integrase-CBX MLV Vector for Safer Gene Therapy

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Background: Integrating retroviral vectors have proven to be a powerful tool for long-term correction of genetic defects in a variety of severe blood and immune disorders. However, in a small subset of patients therapeutic benefits were compromised by vector-induced dysregulation of cellular genes and leukemia development. To develop safer MLV-based vectors we uncoupled the interaction between the MLV integrase and its cellular tethering factors, BET proteins, and fused the chromodomain of the CBX1 protein to the uncoupled integrase[1,2]. The resulting BET-independent IN-CBX MLV ($BinMLV^{CBX}$) vector showed efficient and redistributed integration, away from typical gamma-retroviral markers and towards heterochromatin regions. In addition, $BinMLV^{CBX}$ showed a reduced transformational potential compared to wild-type MLV (MLV^{WT}) in a serial colony-forming assay, suggesting an improved safety profile. **Experimental approach**: In this study we assessed the functionality and the safety of the redistributed

BinMLV^{CBX} vector compared to MLV^{WT} using a clinically more relevant vector design with a self-inactivated (SIN) LTR and a weak internal Elongation Factor 1 alpha short (EFS) promoter driving GFP reporter gene expression (EFS.GFP). As a reference, we used the same vectors carrying the potent but genotoxic spleen focus forming virus (SFFV) promoter (SFFV.GFP). Transgene expression and stability over time were compared following transduction in hematopoietic (HSC) and mesenchymal stem cells (MSC), together with the potential of transduced MSC cells to differentiate in smooth or skeletal muscle cells (differentiation index). **Results:** The BinMLV^{CBX}-EFS.GFP vector produces at high titers and efficiently transduces CD34^{+} hematopoietic stem cells in line with MLV^{WT}, without any apparent transgene silencing over time. Although transduction efficiency and stability of expression were comparable, the expression level (GFP fluorescence) was lower for BinMLV^{CBX}-EFS.GFP compared to BinMLV^{CBX}-SFFV.GFP, corroborating the more subtle modulation of transgene expression when using the EFS promoter. In addition, human MSCs were transduced and differentiated in smooth or skeletal muscle cells. All MLV preps transduced MSCs to comparable levels, with EFS-containing vectors resulting in lower expression levels compared to vectors using SFFV as internal promoter. However, following differentiation in smooth or skeletal muscle cells, BinMLV^{CBX} transduced MSCs showed a differentiation index more in line with untransduced cells, that was two-fold better than for cells transduced with MLV^{WT} vector, both with the EFS and the SFFV promoter. The fact that MLV^{WT} transduction affects the MSC differentiation potential, while the redistributed BinMLV^{CBX} vector does not, suggests that BinMLV^{CBX} is less genotoxic than MLV^{WT} in the mesenchymal stem cell model. **Conclusion:** We demonstrate high transgene expression that is stable over time and maintains after differentiation, without affecting the differentiation potential of MSCs for BinMLV^{CBX}-EFS.GFP, suggesting a reduced genotoxicity compared to MLV^{WT}. 1. El Ashkar S, De Rijck J, Demeulemeester J, et al. BET-independent MLV-based Vectors Target Away From Promoters and Regulatory Elements. *Mol Ther Nucleic Acids*. 20142. El Ashkar S, Van Looveren D, Schenk F, et al. Engineering Next-Generation BET-Independent MLV Vectors for Safer Gene Therapy. *Mol Ther Nucleic Acids*. 2017

912. Towards Optimising Lentiviral Vectors Through Structure Informed Genome Modification

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Lentiviral vectors are being successfully used as therapeutic agents in a series of clinical applications including gene therapy, genome editing and cancer immunotherapy. Current HIV-1 derived lentiviral vectors are encoded on 4 independent plasmids. Here, we focus on the transfer vector that contains the therapeutic gene and the *cis*-acting elements that drive its expression including the packaging signal (*psi*). Lentiviral vector particles carry two copies of transfer vector RNA linked non-covalently as a dimer and important for efficient encapsidation. We are focussing on improving the infectivity of vectors by targeting their dimerisation and packaging properties based on the hypothesis that WT HIV-1 regulates genome encapsidation tightly by recognising dimeric RNA. The gRNA is known to act as a riboswitch between the monomeric conformation optimised for translation and the dimeric conformation linked with packaging. We created mutants in the 5'LTR targeting regions that play important roles in the process of dimerisation including the Dimerisation Stem Loop (DSL), the U5-AUG duplex formed by sequences located at the beginning of the U5 region and nucleotides surrounding the start of the *gag* gene, and the polyA stem loop, critical for regulation of polyadenylation. The mutations aim to create vectors whose RNA is more likely to adopt the dimeric conformation and therefore will enhance their packaging. To evaluate this we developed a novel cotransfection based competitive RT-qPCR assay to measure their relative packaging efficiencies. Biochemical characterisation showed that transduction and packaging efficiencies were highly influenced by the region where the mutations were introduced. Northern blots confirmed that the propensity of mutated vector RNA to dimerise was increased in the mutants as we hypothesised. Here, we report the effect of the dimerisation-stabilising mutations on infectious and physical titres of lentiviral vectors, as well as on their packaging efficiency measured with our novel competitive qPCR assay. Our data suggest that there is a fine balance between RNA stability and its propensity to dimerise, and that enhancing dimerisation does not automatically lead to better packaging of vector RNA. Finally, we explored by SHAPE (Selective 2′-Hydroxyl Acylation Analyzed by Primer Extension) the structure of *psi* in our vector RNAs, in particular studying the influence of regions adjacent to *psi* on dimerisation and packaging. SHAPE identifies the RNA backbone flexibility, which is an indication of whether nucleotides are base-paired or not. Our single nucleotide level structural analysis revealed that the presence of *gag* sequences stabilise the *psi* element, suggesting their role in supporting a stable RNA conformation that can be packaged and offering a potential explanation for their requirement in the transfer vector plasmid for maintenance of infectious titres. These findings will give us better insights into the biology of lentiviral vectors and enable us to design more efficient vectors for clinical applications.

Neurological Diseases

913. Normalization of Fmr1RNA and Protein Expression in the Brains of Mice that Model Fragile X Associated Tremor/Ataxia Syndrome (FXTAS) Using CRISPR-Cas9

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The Fragile X Mental Retardation 1 (*FMR1*) gene encodes the ubiquitously expressed translational repressor protein FMRP. Within the 5' untranslated region of *FMR1* is a CGG trinucleotide repeat that, when expanded from normal repeat length, is the predominant cause of Fragile X Syndrome (FXS), and the only

known cause of Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) or Fragile X-associated Primary Ovarian Insufficiency (FXPOI). Both FXTAS and FXPOI are incompletely penetrant disorders due to trinucleotide repeats within the premutation range (55-200 CGG repeats), while FXS occurs with expansions surpassing 200 CGGs, which causes epigenetic gene silencing. Here, we evaluated Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas9 for its potential to correct expanded *FMR1* trinucleotide repeats in human cells and a mouse model harboring a premutation knock-in allele of CGG repeats. We tested multiple guideRNA sequences for cutting efficiency with SpCas9 in HEK 293 cells and patient derived fibroblasts. Effective editing resulted in complete or partial deletion of the CGG repeats as well as nucleotides proximal to the repeats. Two guideRNAs were further evaluated *in vivo* in CGG knock-in mice FXTAS model. A dual-AAV1 vector strategy was used, with AAV-guideRNAs and AAV-SpCas9 injected into mice striatum. Three weeks later, tissues were harvested and isolated DNA was subjected to PCR amplification and Sanger sequencing to map the CRISPR-induced deletions. We found complete or partial deletion of the CGG repeats and deletion of 3-48 nucleotides upstream and downstream of the target site. In all cases the transcriptional start site and the start codon were intact, elements required for functional gene expression. In RNA isolates assessed for *Fmr1* mRNA levels by qRT-PCR, we found that transcripts were normalized from 3-fold elevation in control treated animals to indistinguishable from normal after gene editing. Additionally, the protein encoded by *Fmr1*, FMRP, which typically has lower translational efficiency in *Fmr1* CGG knock-in mice, showed expression recovery to levels similar to those seen in WT mice. These results are the first *in vivo* report of editing the *Fmr1* trinucleotide repeat with CRISPR. Importantly, our data demonstrate that CRISPR mediated deletion of the CGG repeats in *Fmr1* can rescue both transcript upregulation and FMRP down regulation, and provides an important path forward for FXTAS treatment.

914. CRISPR/Cas9 Specific Editing of the Mutant HTT Allele

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Huntington disease (HD) is a fatal dominantly inherited neurodegenerative disorder caused by CAG repeat expansion within the first exon of the huntingtin gene. Although mutant huntingtin (mHTT) is ubiquitously expressed, the brain shows robust and early degeneration. Current gene silencing approaches for lowering mHTT expression, including RNA interference or antisense oligonucleotides, have been efficacious in mouse models, but basal mutant protein levels are still detected. To fully mitigate expression from the mutant allele, we hypothesize that allele specific genome editing can occur via prevalent promoter-resident single nucleotide polymorphisms (SNPs) in heterozygosity with the mutant allele. This approach would also avoid reducing normal HTT protein levels. Here, we identified SNPs in *HTT* that either cause or destroy protospacer associate motifs critical for CRISPR targeting, using either fully active Cas9 or a nuclease dead Cas9 to induce epigenetic silencing. Importantly, in both cells from HD patients and a transgenic HD model harboring the human allele, we show selective targeting of the mutant allele, resulting in mHTT lowering while avoiding silencing of normal HTT.

915. A GABA-Selective AAV Vector-Based Approach to Up-Regulate Endogenous Scn1a Expression Reverses Key Phenotypes in a Mouse Model of Dravet Syndrome

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Dravet syndrome (DS) is a severe epileptic encephalopathy that begins in infancy and is characterized by prolonged, intractable seizures, developmental delay, and an increased risk of sudden unexpected death in epilepsy (SUDEP). DS affects approximately 1:16,000 individuals worldwide, with over 80% of cases attributed to loss-of function mutations in the Scn1a gene. Scn1a encodes for the sodium channel subunit Nav 1.1, which is expressed in a cell-type selective manner within inhibitory GABAergic interneurons. Fundamental to the pathophysiology of DS, Scn1a haploinsufficiency leads to impaired GABAergic transmission, runaway excitation, and intractable seizures. Currently, there are no disease-modifying treatments for DS due to the requirement for cellular selectivity, as well as the large size of the Scn1a transgene (6kB). To address these limitations, we designed an AAV vector incorporating highly conserved, human regulatory sequences to constrain vector expression to GABAergic inhibitory interneurons. Furthermore, we designed an engineered transcription factor to potently and specifically up-regulate Scn1a within the GABAergic population. AAV-ETX exhibited cell type-selective up-regulation of SCN1A in human iPSC-derived GABAergic neurons, mouse CNS, and non-human primate CNS. A single intracerebroventricular (ICV) infusion of AAV-ETX into P1 Scn1a +/- mice resulted in widespread expression within hippocampal and cortical regions, with a corresponding increase in SCN1A mRNA and Nav1.1 expression levels. Furthermore, Scn1a +/- mice treated with AAV-ETX exhibited a significant improvement in sensitivity to hyperthermic seizures, as well as a reduction in the frequency and severity of spontaneous seizure events measured via v-EEG. Importantly, AAV-ETX treated Scn1a +/- mice were indistinguishable from wildtype (WT) littermates when observed for SUDEP for up to 5 months. By comparison, Scn1a +/- control treated mice experience ~50% mortality in that time. Our findings support the development of a one-time, disease-modifying therapeutic, capable of restoring Nav 1.1 channel homeostasis within the affected cell type, correcting for GABAergic circuit dysfunction, and reversing multiple hallmark symptoms of DS.

916. Reversal of Chronic Neuropathic Pain by Functional Neurotransmitter Rewiring of Spinal Nociceptive Neurons after Spinal Segment-Targeted Subpial Dual Gene (GAD65 and VGAT) Delivery

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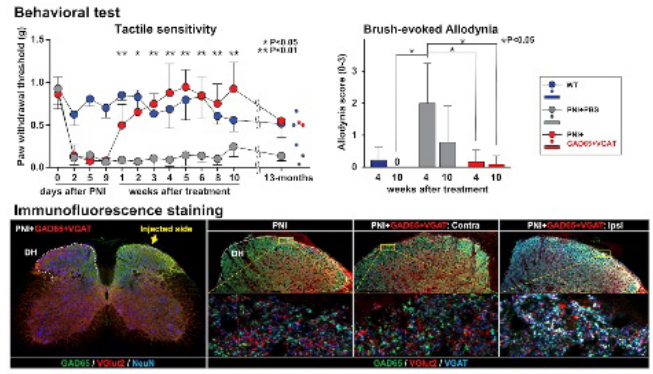
Purpose: Persisting somato-topically defined neuropathic pain resulting from spinal cord or peripheral nerve injury represents a major clinical problem. At present no clinically-effective anti-nociceptive therapy which would target selected spinal segments and be without systemic side effect is available. Decrease in segmental GABAergic tone associated with peripheral or spinal injury has been postulated to play a role in the evolution and maintenance of neuropathic pain states. By using a novel spinal subpial gene delivery technique, we studied the anti-nociceptive potency of unilateral, and segment(s)-targeted dual gene(s), GAD65 and VGAT, delivery into dorsal horn nociceptive neurons in mouse neuropathic pain model. In addition, a dosing study to define a targeted viral dose to be used in perspective human clinical trial was tested in adult pig. **Method:** i) Wilde-type mice (C57BL/6, 3-months old) with established neuropathic pain induced by partial sciatic nerve ligation (PNI) received unilateral lumbar (L2-L4) subpial injection of 0.5 µl of a mixed AAV9-UBI-GAD65/VGAT vectors or vehicle. Before and after treatment, animals were tested for tactile hypersensitivity, brush-evoked nociception, open-field motor performance and paw placement pattern. After 12-weeks or 13-months survival, immunofluorescence staining (IF), immunofluorescence in situ hybridization (FISH) and electron microscopy (EM) were performed. ii) In a separate experiment, patch clamp recording (PC) from acutely-prepared spinal cord slices were performed. iii) vgat-IRES-Cre and vglut2-IRES-Cre mice with PNI received subpial injections of AAV9-Lox-PLox2272-GAD65/VGAT and were tested for tactile hypersensitivity and brush-evoked nociception for 10 weeks. iv) Eight adult pigs received progressively increased volumes of AAV-anc80-Rpl22-3xHA injected into subpial space at L4-L6 segments. After 48-h survival, the distribution of HA expression was analyzed using IF. **Result:** i) Treated mice showed a progressive and complete reversal of nociceptive responses 1-2 weeks after treatment. This treatment effect continued for the duration of study (10 weeks) and was still present at 13 months post-treatment. In contrast, no change in vehicle-injected animals was seen. IF, FISH and EM showed a potent upregulation of GAD65 and VGAT expression in inhibitory interneurons and appearance of mixed excitatory-inhibitory phenotype in endogenous excitatory interneurons and DRG neurons.



ii) PC showed significant increase in postsynaptic inhibitory activity in dorsal horn neurons. iii) Subpial injections of scAAV9-Lox-PLox2272-GAD65/VGAT in vgat-IRES-Cre or vglut2-IRES-Cre mice with neuropathic pain showed a potent reversal of pain behavior in vglut2-IRES-Cre mice but not in vgat-IRES-Cre mice. iv) IF showed a dose-depended spread of HA expression in adult pig. **Conclusion:** The present study shows that unilateral subpial delivery of GAD65 and VGAT genes has a potent and long-lasting anti-nociceptive effect and that the primary mechanism associated with the anti-nociceptive activity is the induction of inhibitory phenotype in endogenous excitatory neurons. Favorable safety profile seen in large animal models show that this treatment approach can readily be implemented in human patients with chronic neuropathic pain.

917. Intrathecal scAAV9/SUMF1 Gene Therapy for Multiple Sulfatase Deficiency

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Multiple Sulfatase Deficiency (MSD; OMIM #272200) is a rare, autosomal recessive pediatric-onset neurodegenerative lysosomal storage disease, characterized by seizures, developmental delay, ichthyosis, scoliosis, and hepatosplenomegaly, and death within a few years of symptom onset. Currently no specific treatments exist for this disorder. The disease pathology is due to homozygous loss-of-function mutations in the *SUMF1* gene that encodes for the formylglycine-generating enzyme, which is required for post-translational modification and activation of all known sulfatase enzymes. As such, pathogenic mutations in the *SUMF1* gene impact the function of all 17 human sulfatase enzymes, leading to a broad phenotypic spectrum that overlaps with known inherited sulfatase disorders, such as MLD, MPS subtypes, and others. We have developed a novel self-complementary vector encoding a codon-optimized human SUMF1 gene (scAAV9/SUMF1), the unaltered design of which could be appropriate for human use. Pre-clinical studies were conducted in a severe Sumf1 knock-out (KO) mouse model where ~35% of KO pups die between 7 and 11 days of age and the median age of survival is ~20 days. Intracerebroventricular (ICV) delivery of scAAV9/SUMF1 in neonates resulted in 75% of treated Sumf1 KO mice surviving past 175 days. Neonate-treated Sumf1 KO mice were functionally indistinguishable from age-matched wildtype controls

as assessed by rotarod, open field and spontaneous alternation tests. To test the boundaries and limitations of a gene therapy for MSD, we intrathecally-injected escalating doses of scAAV9/SUMF1 in 7 day-old Sumf1 KO mice. Encouragingly, even when treating Sumf1 mice at an age when they begin to die as part of the disease course, we found that intrathecal-delivery of scAAV9/SUMF1 significantly improved survival, with the highest dose resulting in 60% of Sumf1 KO mice living > 100 days of age (follow up ongoing). Functional, biochemical and histological studies are ongoing to assess the therapeutic benefit. Additionally, the safety profile of the MSD vector has been excellent with no adverse effects noted even in treated normal mice. Overall, our preclinical results predict that intrathecally-delivered scAAV9/SUMF1 could provide a meaningful benefit to MSD patients.

918. Respiratory Directed Gene Therapy to Silence SOD1 Prolongs Survival in the SOD1 ALS Mouse

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease with no cure and limited therapies. Patients with ALS develop progressive loss of limb function, an inability to speak, swallow and breathe, and they ultimately succumb to respiratory failure. Gain of function mutations in the SOD1 gene accounts for approximately 2% of ALS patients, and a well characterized mouse model SOD1^{G93A} has been created which recapitulates many aspects of ALS in humans including respiratory phenotypes. We hypothesized that reducing expression of mutant SOD1 with respiratory targeted gene therapy will improve respiratory function and enhance survival. Therefore, we injected adult SOD1^{G93A} mice with rAAVrh10 expressing a microRNA against mutant SOD1 (AAV-miR) through intralingual and intrathoracic injections. Animals were longitudinally assessed post injection until end stage disease. Respiratory function was measured using whole body plethysmography and respiratory mechanics were measured using forced oscillometry (Flexivent, Scireq). Behavior, strength, weight and survival were also assessed. Post mortem studies included vector genome assessment and knock-down of SOD1 RNA, as well as histological examination of motor neurons, nerves and neuromuscular junction. At study end point, we observed systemic viral distribution, with a majority of vectors within the liver, but a considerable number of vectors were detected within the respiratory muscles of the intercostals, tongue and diaphragm. Vector genomes were correlated with knock-down of RNA of SOD1 with 64% reduction in the tongue and 80% reduction in the diaphragm. Systemic reduction was also observed within the liver, heart and hindlimb with 86%, 87% and 89% respectively. Interestingly, a significant enhancement in survival, approximately 50 days, was observed in the treated group with significant improvement in weight maintenance over time. Decline in motor strength was improved as determined by inverted screen and 4-limb grip strength, and neurological screening revealed significant improvement in function in treated compared to untreated animals. Spontaneous breathing was measured longitudinally in awake animals. Treated animals responded more similarly to wildtype animals than untreated animals with significant differences from untreated animals in frequency and minute ventilation at days 150-169. However, by humane endpoint, pulmonary mechanics were not different between untreated and treated animals. Histological assessment revealed that the axonal integrity of the hypoglossal and phrenic nerves was better maintained in treated animals than untreated at end-stage. The neuromuscular junctions within in the tongue and diaphragm were also histologically evaluated and improvement was noted in innervation and organization of diaphragm intermuscular junction of treated animals when compared to untreated. Our data suggests that reduced expression of SOD1 in the tongue may increase muscle strength allowing for weight maintenance which aided enhanced survival, emphasizing importance of nutritional maintenance. Moreover, AAV-miR gene therapy maintained axonal survival and morphology of the neuromuscular junction, improved overall breathing but ultimately restrictive lung disease was still evident at end-stage. Overall, this study demonstrates the importance of targeting the respiratory system for the treatment of neurodegenerative and neuromuscular diseases.

919. A NeuroD1-Based Gene Therapy for Brain Repair

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There is a huge unmet medical need to treat severe neurological disorders such as stroke and Alzheimer's disease. Human brain has largely lost the neuroregeneration capability in the adult stage. We have recently developed an innovative *in vivo* cell conversion technology to directly convert reactive glial cells into functional neurons inside adult mouse brains through expressing a single neural transcription factor NeuroD1 (Guo et al., Cell Stem Cell, BEST of 2014 article). In an ischemic stroke model, we demonstrate that our NeuroD1 AAV-based gene therapy can regenerate ~40% of lost neurons plus protect ~40% of injured neurons, leading to the reconstruction of a damaged motor cortex with layered structures. The NeuroD1-converted neurons are fully functional, showing repetitive action potentials and robust synaptic activities, and sending out long-range axonal projections to global brain regions. Interestingly, after high efficiency astrocyte-to-neuron conversion (90% conversion rate), the remaining astrocytes can regenerate themselves in parallel with the regeneration of new neurons, accompanied by a significant reduction of microglia and neuroinflammation. Most importantly, our NeuroD1-mediated *in vivo* astrocyte-to-neuron conversion technology can successfully rescue both motor and cognitive functional deficits caused by ischemic injury. Furthermore, we also converted striatal astrocytes into GABAergic neurons in a Huntington's disease mouse model and significantly improved motor functions and extended their life span. We are currently completing a series of preclinical studies in mice, rats, and monkeys in order to translate our highly efficient *in vivo* astrocyte-to-neuron conversion technology into successful clinical therapies. This project was supported by grants from NIH, Alzheimer's Association, and Charles H. Skip Smith Endowment Fund. G.C. is Verne M. Willaman Chair in Life Sciences at Penn State University.

920. AAV Gene Transfer Halts Disease Progression in Sheep with CLN5 Batten Disease after Pre- Or Post-Symptomatic Administration

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Neuronal ceroid lipofuscinoses (NCLs; Batten disease) are the most common childhood degenerative brain diseases and 13 different causal genes have been implicated (*CLN1-8*, *10-14*). Despite this genetic diversity, similar clinicopathological features define the NCLs; the near-ubiquitous accumulation of lysosome-derived storage bodies, progressive neuronal loss, retinal degeneration, seizures and psychomotor decline culminating in premature death. Currently there are few effective treatments. Sheep with naturally occurring CLN5 disease (*CLN5*$^{-/-}$) are ideal animals in which to test gene therapies. Their gyrencephalic brain is anatomically similar to the human brain and of similar size to a non-human primate, thus they provide a good approximation for dose requirements and vector distribution. The disease course and neuropathological features in these sheep closely mimic that seen in affected patients and a set of *in vivo* assessments have been developed for longitudinal non-invasive monitoring of disease progression. Previously we showed that the single intracranial administration of either adeno-associated viral (AAV9) or lentiviral vectors encoding ovine *CLN5* into 3-month-old pre-symptomatic *CLN5*$^{-/-}$ sheep provided long-term protection against stereotypical disease. These studies were extended by testing intracerebroventricular delivery of different doses at different ages. No difference was seen when pre-symptomatic *CLN5-/-* sheep received either 2 x 10^{12} or 5 x 10^{12} viral genomes of scAAV9.*CLN5*. Sheep in both cohorts remain clinically normal at 29 months, the only disease sign being delayed-onset visual loss. Longitudinal computed tomography (CT) scans show stabilisation of brain volumes. Therapeutic intervention at the earliest possible time is desirable, however the diagnoses of human NCLs is often prolonged. Hence, in a more clinically relevant setting, seven- and nine-month old *CLN5*$^{-/-}$ sheep with established disease symptoms and neurodegenerative changes also received gene therapy. Treatment at seven months halted any further decline in motor, neurological or behavioural capability, there has been little post-injection brain atrophy and the treated sheep remain healthy in the field at 40 months of age. Clinical disease progression and brain volume was stabilised in only one of the animals treated at 9 months. In contrast, untreated *CLN5*$^{-/-}$ sheep developed advanced disease symptoms, with manifest seizure activity, and do not survive beyond 21 months. Monitoring of these sheep continues, to determine any late-onset disease and to see if another dose of gene therapy may be warranted, and ocular gene therapy trials are underway. To date, the pre-symptomatic delivery of 4 x 10^{11} viral genomes of scAAV9.*CLN5* to the vitreous humour of the affected sheep eye has prevented retinal dysfunction to 15 months. Together, these data provide a strong rationale for clinical translation to CLN5 affected humans. *This project is funded by Neurogene Inc, the Batten Disease Support & Research Association, the Canterbury Medical Research Foundation, the New Zealand Neurological Foundation and CureKids New Zealand.*

AAV Vector Biology I

921. Identification of VP-Interacting Cellular Proteins in AAV Capsid Assembly by Proximity Proteomics

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AAV assembly activating protein (AAP) plays a key role in the capsid assembly process. Despite the increasing knowledge about the roles of AAP in capsid assembly, what and how cellular proteins play roles in this process still remains elusive. To better understand the mechanisms of AAV capsid assembly, we have recently started seeking to identify cellular proteins that play roles in assembly using BioID, a proximity-based proteomics approach. To begin to identify AAP-interacting cellular proteins important for capsid assembly, we have previously established an approach where AAP2 tagged with BioID biotin ligase (BirA*) is expressed in cells as a bait and successfully identified a nuclear deubiquitinating enzyme as a key player in effective AAV2 capsid assembly. With our success in the BioID-AAP approach, we have started exploring a reciprocal BioID approach where we perform the proximity-based proteomics study in a reversed direction, i.e., BioID-VP, to make our approach complementary and more robust. In this BioID-VP approach, any proteins residing in close proximity to the capsid VP protein in cells are expected to receive biotin tags and therefore can be effectively pulled down by streptavidin beads under stringent conditions for the downstream mass spectrometric (MS) analyses. Here, we demonstrate for the first time the proof-of-concept (POC) of the BioID-VP approach and show our preliminary proteomics data on AAV2 VP3-inetracting cellular proteins that potentially play roles in AAV2 capsid assembly. To this end, we utilized the BioID2 biotin ligase (233 aa) as opposed to the traditional BirA* (321 aa) enzyme and constructed a BioID2-VP3 bait protein by fusing a Flag-tagged BioID2 with the N-terminus of the AAV2 VP3 protein. We assumed that BioID2's smaller size is important for the VP-BioID approach because an addition of a bulky protein to the N-terminus of the VP3 protein may not be tolerated. As expected, it was not possible to produce BioID2-VP3 only capsids. However, when BioID2-VP3 and VP3 were expressed at a 1:9 ratio, AAV2 capsids could be produced at a level comparable to that of VP3 only capsids. To assess if the capsids produced together with BioID2-VP3 indeed has the BioID2-VP3 incorporated into the capsids, we performed immunoblotting of 2xCsCl ultracentrifugation-purified capsids made of either VP3 alone or a mixture of BioID2-VP3 and VP3 at a 1:9 ratio, confirming the incorporation of BioID2-VP3 in the assembled capsids. We also confirmed the BioID2 biotin ligase activity by immunoblotting. With

these reagents in hands, we performed an BioID2-MS experiment in which we expressed in HEK293 cells by transient plasmid transfection (1) BioID2-VP3 [10] + AAP2 [10], which cannot form capsid and (2) BioID2-AAV2VP3 [1] + AAV2VP3 [9] + AAP2 [10], which can effectively assemble into capsids (note: the numbers in brackets represent relative ratios). A preliminary LC-MS/MS analysis of BioID2-labeled proteins revealed that the proximity proteomes are significantly different between these two samples in that unassembled VP3 proteins interact with many host cellular proteins including those involved in the p53 pathway while such an interaction is much less pronounced when VP3 proteins assemble into capsids. This indicates that the assembly process moves VP proteins into a cellular microenvironment where interactions between viral capsid proteins and cellular proteins are limited. In summary, our study establishes POC of the VP-AAP reciprocal and complementary BioID approaches to study the cellular interactome in AAV capsid assembly.

922. A Single Amino Acid Residue in the VP1 Unique Region Plays an Essential Role in AAV Capsid Formation

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Our knowledge of how AAV VP protein subunits assemble into capsids has made a remarkable progress with the discovery of AAP that plays a key role in this process. The AAV capsid consists of the following three VP proteins, VP1, VP2, and VP3, at an approximately 1:1:10 ratio. The VP1 protein, the minor constituent of the AAV capsid, harbors the VP1 unique region (VP1u) of approximately 200 amino acids at its N-terminus that is not present in the VP3 protein. The VP1u region carries a catalytic phospholipase A2 (PLA2) domain and several clusters of basic amino acids that are essential for virus infectivity. VP3 only capsids devoid of VP1u can be effectively produced when the AAP protein is supplied in trans, demonstrating that VP1u is dispensable for AAV capsid assembly, and therefore it has been believed that VP1u has no role in promoting virion formation. Here we show provocative evidence that a single amino acid residing in the middle of VP1u is essential for virion formation. In our ongoing AAV Barcode-Seq projects, we have created a set of AAV8 and AAV9 capsid double alanine (AA) mutants that can cover the N-terminal half of VP1 including the entire VP1u region of each serotype (120 AAV8 AA mutants and 150 AAV9 AA mutants). To better understand how AAV capsids assemble, we here produced these mutant and the wild-type capsid vectors individually and on the same scale in HEK293 cells. An equal fraction of the produced vector particles was pooled into a single tube from which vector genome DNA was extracted. Each vector contained a double-stranded (ds) AAV genome tagged with a pair of 12-nucleotide-long unique DNA barcodes. In this experimental scheme, a fitness landscape of the tolerance of alanine mutations in genome-containing AAV vector production can be drawn by our AAV Barcode-Seq technology. As a result, we identified four AA positions in the VP1u region that exhibit significant impairment of the production of genome-containing viral particles and are shared with both AAV8 and AAV9 capsid proteins. Intolerance of three out of the four AA mutations could be expected because two are consecutive mutations near the N-terminus that destroy the splice acceptor (SA) site consensus motif, presumably resulting in dysregulation of the p40 promoter-driven transcription encoding VP2 and VP3 proteins, and because one mutation destroys the VP3 translation start codon. Interestingly, one of the four intolerant AA mutations shared with both AAV8 and AAV9 resides in the middle of VP1u between the PLA2 motif and the basic amino acid-rich region that does not appear to disturb either mRNA processing or protein translation. The two amino acids mutated to AA in the AAV8 and AAV9 mutants showing the unexpected intolerance are non-charged and highly conserved across different serotypes. To determine which or both of the two amino acids are functionally important, we created AAV9 single alanine mutants, and quantified the total amount of the assembled capsids and only genome-containing capsids by an AAV9 capsid ELISA and a quantitative dot blot assay, respectively. These assays revealed that impaired formation of genome-containing AAV particles is attributed to impaired capsid formation and not due to defective genome packaging and that only one amino acid out of the two plays this functional role. We are currently investigating the mechanism of how a single amino acid residue in the middle of the VP1u region impacts on AAV capsid formation, including the possibility that the impairment of capsid formation could be mediated by a qualitative and/or quantitative change in viral gene transcripts. In summary, our unexpected observation described here highlights the importance of studies investigating potential new roles of VP1u in capsid formation that have not previously been appreciated.

923. A CRISPR Screen Identifies Apical Polarity Determinant Crumbs 3 as an AAV Host Restriction Factor

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Adeno-associated viruses (AAVs) utilize a variety of attachment factors to bind the cell surface and enter the host cell. It is well known that cellular polarity can serve as a barrier for AAV entry due to localization of attachment or entry factors; however, the molecular factors that dictate such restricted entry mechanisms are not well understood. Here, using a genome wide CRISPR/Cas9 screen, we identify several host factors that restrict AAV transduction in human hepatocyte cultures *in vitro.* In particular Crumbs 3 (Crb3), a tight junction component implicated in apical polarity determination, was identified as a significant hit. CRISPR knockout (KO) and overexpression (OVX) lines were derived for Crb3. While Crb3 KO lines demonstrated increased transduction with several AAV serotypes, Crb3 OVX reduced AAV transduction, consistent with the putative role of Crb3 as a host barrier. Importantly, we observed a 2-log fold increase in AAV9 capsid binding to the surface of Crb3 KO cells. To investigate this further, we interrogated the effects of Crb3 KO on the expression of the AAV9 glycan receptor, galactose, as well as the universal AAV receptor, AAVR through a battery of assays. Lectin staining demonstrated increased galactose expression on the cell surface. In contrast, AAVR expression was unaltered as evidenced by western blot and confocal microscopy. Further immunofluorescence characterization of Crb3 KO cells demonstrated disruption of tight junctions, corroborated by

immunostaining of occludin and ZO-1, as well as disruption of cell polarity, as observed by mislocalization of e-cadherin. Our observations shed light into the molecular underpinnings of AAV cellular entry in polarized cells and host factors that are associated therein.

924. Comparative Analysis of the Capsid Structures of AAVrh.10 and AAVrh.39 and Their Antigenic Interactions and AAVrh.39 and Their Antigenic Interactions

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The use of vectors based on Adeno-associated virus rhesus isolate 10 (AAVrh.10) for gene therapy applications has grown rapidly over the past few years. The advantages of AAVrh.10 include its high transduction efficiency in the CNS in vivo and the low percentage of pre-existing neutralizing antibodies in the human population. Similar to AAVrh.10, AAVrh.39 is also capable of crossing the blood-brain-barrier and efficiently transduces neuronal cells and keratinocytes. However, AAVrh.39 is described to possess an alternative tissue tropism to different neuronal cell types compared to AAVrh.10. Here we present the AAVrh.10 and AAVrh.39 capsid structures determined by cryo-electron microscopy (cryo-EM) and three-dimensional image reconstruction to 2.75 and 3.27 Å resolution, respectively. In parallel, we determined the capsid structure of AAV8, the closest serotype, by cryo-EM to 3.10 Å resolution to enable comparison. For all three density maps the side chain densities for most amino acids are well ordered allowing unbiased modeling of their VP3 and subsequent structural comparison. AAVrh.10 and AAVrh.39 are 98% identical in amino acid sequence, with Cα coordinates differing by a root mean squared deviation (RMSD) of 0.3 Å when superposed. The capsid structures of AAVrh.10 and AAVrh.39 are similar to that of AAV8, with which they share an amino acid sequence identity of 94%. The overall RMSD is 0.5 and 0.6 Å, respectively, from AAV8. The structures conserve the VP topology described for all structures of AAV known to date. Surprisingly, only minor differences are observed among the three viruses at the previously described AAV capsid surface variable regions VR-I to VR-IX. Due to the structural homology between AAVrh.10 and AAV8 anti-AAV8 capsid antibodies were observed to cross-react with AAVrh.10. The AAVrh.10 capsid surface was then rationally engineered to create vectors that escape these antibodies while maintaining infectivity. In addition, a test of individual human serum samples produced two groups: those that did not bind and those that recognized AAVrh.10 capsids but did not prevent cell transduction. Further analysis using transduction assays with purified IgG from the binding sera showed significant enhancement of transduction whereas the Fab portion was unable to enhance transduction. These observations indicate a possible antibody-dependent enhancement of infection mechanism via Fc receptor interaction. Similar studies for AAVrh.39 are underway. Our structural comparisons will be presented along with implications of the conserved structures despite phenotypic differences and the information of the antigenic structure of these promising vectors.

925. Mass Spectrometric Identification of Host Factors that Restrict AAV Vector Genome Transcription

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Transcriptional silencing of AAV vector genomes in different tissues is a potential challenge facing human gene therapy clinical trials. Such molecular events could result in sub-therapeutic levels of transgene product resulting in decreased efficacy and possibly warrant repeat dosing. Recent studies involving AAV capsid mutants showing transcriptional defects have corroborated the notion that the AAV capsid itself plays an integral role in vector genome transcription in *cis,* although the mechanisms underlying such remain unclear. Further, the interplay between host restriction factors and the AAV capsid in regulating vector genome transcription remains poorly understood. Previously, we characterized RNF121, a host factor essential for both recombinant AAV and wild-type AAV transcription. Here, we present affinity-purification mass spectrometric data of the AAV capsid interactome in the presence and absence of RNF121. In RNF121 KO cells, we discovered an abundance of host factors relative to wild-type cells that include multiple components of the U2 snRNP pathway as well as other transcriptional modulators. RNF121 KO capsid binding partners were subject to Ingenuity Pathway Analysis and highlighted involvement of RNA processes, translation, viral infection, and DNA damage. Amongst these, RNA processing and splicing elements were particularly enriched with p-scores of 3.12e-65 and 1.11e-44, respectively. The impact of several individual targets on AAV transduction and vector genome transcription was established through siRNA inhibition and a battery of biochemical assays. Characterization of representative examples from the U2snRNP complex, helicases, and other factors with links to innate transcriptional repression will be presented. Our observations are a step towards better understanding AAV genome transcription and provide the foundation for development of strategies to address transcriptional silencing of AAV vector genomes.

926. AAVR Independent AAV Serotypes Require Neuraminidase 1 and Cathepsin A for Cellular Entry

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Adeno-associated virus has broad utility as a gene therapy vector, yet cellular factors that determine targeting and tropism are poorly understood. We recently published that a subset of divergent AAVs, comprised of AAVrh32.33 and AAV4, do not require and cannot bind to the canonical AAV entry receptor, AAVR. Here we employed a genome-wide CRISPR/Cas9 screen in Huh7 AAVR KO cells to determine cellular factors required for entry of these divergent serotypes. We identified two proteins that exist in complex together,

NEU1 and CTSA, which are specifically required for AAV4 and rh32.33 entry. NEU1 or CTSA knockout (KO) in MEF cells demonstrate a 10 to 100-fold decrease in AAV4 or rh32.33 transduction, respectively. Chemical inhibitors demonstrate a requirement of NEU1 enzymatic activity for entry of AAVR independent capsids, but show no effect on AAVR dependent capsids, including those who use sialic acid as an attachment factor. Cellular binding assays demonstrate no difference in cellular attachment of AAV4 or rh32.33 in NEU1 KO or CTSA KO cells compared to WT cells, suggesting a role in a bona-fide receptor complex. By understanding the cellular determinants of AAV entry for different capsid variants we aim to further improve rational design and targeting strategies of AAV vectors.

927. The Role of miRNA 17-92 Cluster in AAV2 Vector-Mediated Transgene Expression

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We have previously reported that infection of human cells by AAV2 vectors leads to activation of the glucocorticoid receptor (GR) signaling pathway, and leads to binding of GR to the D-sequence within the inverted terminal repeat of the AAV2 genome, which shares partial homology to the consensus half-site of the glucocorticoid receptor response element (GRE) (*Mol. Ther*., 18: S276, 2010). We have also reported that substitution of the D-sequence with the authentic GRE further increases AAV2 vector-mediated transgene expression (*Mol. Ther*., 1: S6, 2016). Thus, it is clear that GR activation plays a role in AAV2 vector-mediated transgene expression. More recently, it was reported that micro RNA-17-92 (miR17-92) mediates activation of the GR pathway (*Cell Rep*., 16: 1653-1663, 2016). In the present studies, we systematically evaluated the role miR17-92 in AAV2 vector-mediated transgene expression. In the first set of experiments, we performed transduction of HeLa cells with AAV2 vectors (500 vgs/cell) expressing the mCherry reporter gene, in the absence or the presence of increasing amounts (0, 0.1, 0.3, 0.5 μg) of a plasmid containing the miR17-92 cluster. A clear dose-dependent increase in mCherry expression was observed with increasing amounts of the miR17-92 plasmid, suggesting that miR17-92-mediated GR activation led to the observed increase in transgene expression. In the second set of experiments, both ssAAV2 and scAAV2 vectors expressing miR17-92 were generated. HeLa cells were transduced with either ssAAV2 or scAAV2 vectors (500 vgs/cell each) expressing the EGFP reporter gene, either alone or co-transduced with either ssAAV2-miR17-92 or scAAV2-miR17-92 vectors (5,000 vgs/cell each). In both cases, the extent of EGFP expression increased by ~7-fold in the presence of miR17-92 vectors. In the third set of experiments, we wished to determine whether expression of miR17-92 in the same vector genome could also lead to increased transgene expression in cis. To this end, the transduction efficiencies of ssAAV2-EGFP vectors, with or without miR17-92 cluster, were analyzed following transduction of HeLa cells at various vgs/cell (500-2,000 vgs/cell). In every instance, the presence of the miR17-92 cluster led to ~2-fold increase in EGFP expression. Taken together, these data suggest that insertion of the miR17-92 cluster in ssAAV genomes is an attractive strategy to achieve improved transgene expression, such as with ssAAV3-hF.VIII vectors, which has implications in the potential use of these vectors in gene therapy of hemophilia A in humans.

928. Assembly Activating Protein Affects the Association between Ubiquitin and Adeno-Associated Virus Viral Proteins

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Adeno-associated virus (AAV) vectors are one of the leading in vivo gene therapy delivery technologies. Improving vector design and production are primary challenges in the field as we strive to improve therapy safety and efficiency and reduce costs for patients. Pivotal to these challenges is gaining a deeper understanding of AAV's assembly mechanisms. The Assembly-Activating Protein (AAP), a non-structural protein expressed by AAV, allows viral protein (VP) levels to remain sufficiently high for vector assembly by blocking VP degradation in host cells. To study these interactions in depth, we have examined the ubiquitin-dependence of VP degradation, and AAP's ability to block it, by co-immunoprecipitation (co-IP) and western blot. Our results show that VP monomers co-immunoprecipitate with HA-tagged ubiquitin in the absence of AAP, an effect which is enhanced by the addition of the proteasomal inhibitor Bortezomib. In contrast, the addition of AAP in trans blocks the co-IP of VPs. These results suggest that VPs may be ubiquitinated prior to degradation during capsid assembly and that AAP's role in preventing VP degradation may involve directly or indirectly blocking VP ubiquitination. We also test the effects of AAV8 lysine to arginine mutants on protein levels and ubiquitin co-immunoprecipitation to determine key ubiquitin conjugation sites on AAV8 VPs.

Neurosensory Diseases

929. Therapeutic Efficacy of ARCUS Meganuclease Gene Editing for 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), a disease that progresses from night blindness to tunnel vision and, eventually, complete blindness in patients, often starting around 30 years of age. This autosomal dominant genetic disease is not amenable to conventional gene replacement strategies due to the need to eliminate a toxic gain-of-function mutation. Here we report the preclinical development of an allele-specific genome editing approach for P23H adRP that offers the potential for selective elimination of the causative mutant allele while leaving the wild-type allele intact. This gene editing approach is designed for sustained reduction of mutant RHO following a single administration, avoiding the need for regular subretinal injections. Selective targeting the P23H RHO point mutation was accomplished using an engineered I-CreI homing endonuclease (ARCUS). Specificity of ARCUS for the mutant hRHO allele was demonstrated in vitro and in vivo using murine models that express wild-type and/or mutant P23H hRHO. In cultured cells and in transduced human retina explants, the nuclease efficiently edits the P23H hRHO allele and exhibits little to no activity toward the wild-type allele. We further demonstrate that allele-specific knock out of the P23H hRHO rescues retinal morphology in a transgenic mouse model of P23H adRP. Subretinal injection of scAAV5-ARCUS in a P23H hRHO transgenic mouse model shows reduced markers of retinal stress (GFAP) and enhanced survival of both rod and cone photoreceptors. In a well characterized transgenic swine model of P23H adRP, functional rescue of physiological rod and cone function was evaluated. This model harbors 6 copies of transgenic P23H hRHO that results in catastrophic loss of rod photoreceptor function as early as P3. In ongoing experiments, we are screening a range of scAAV5-ARCUS titers by subretinal injection and evaluating retinal function by full field ERG (ffERG) at several postsurgical intervals through at least 19 weeks post injection (wpi). Notably, eyes treated with scAAV5-ARCUS (n=10) show a significant increase in rod-specific function (scotopic ERG flash = 0.001 cd/m2) at 6wpi (p=0.0016) and at 9wpi (p<0.0001) relative to untreated littermates (n=18). 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 P23H adRP. These results suggest that this scAAV ARCUS-based gene editing of P23H RHO may be applicable to treat adRP patients with the same P23H mutation.

930. Early Evidence of Safety and Efficacy of AAV8-RPGR Gene Therapy for X-Linked Retinitis Pigmentosa

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Purpose: An ongoing 2-part, Phase 1/2/3 interventional study investigates the safety and efficacy of an adeno-associated viral vector (AAV8) encoding retinitis pigmentosa GTPase regulator (RPGR) for treatment of X-linked retinitis pigmentosa (XLRP). **Methods:** This study (Clinical trials.gov ID NCT03116113) is being conducted in 2 parts: an open-label dose escalation (N=18) and a dose-expansion (N=45) of a high (2.5 x 10^{11}genome particles [gp]) and low (5 x 10^{10} gp) dose, with addition of an untreated control group randomized in a double-masked fashion 1:1:1. Subjects with genetically confirmed RPGR-associated XLRP are administered a sub-retinal dose of AAV8-RPGR. The vector genome is comprised of a rhodopsin kinase promoter to drive expression of a codon-optimized sequence that has been shown in nonclinical studied to generate the correct full-length RPGR protein. A 3 + 3 escalation scheme was used in Part I, with 3 subjects/dose (5 x 10^{9}, 1 x 10^{10}, 5 x 10^{10}, 1x10^{11}, 2.5 x 10^{11}, and 5 x 10^{11} gp). Part I endpoints are primarily safety (adverse events (AEs), dose-limiting toxicities (DLTs), ophthalmic assessments, viral shedding and immunogenicity). Part I subjects are followed for up to 24 months; Part II for 12 months. The Part II dose expansion includes the improvement in retinal sensitivity assessed with MAIA (Macular Integrity Assessment, Centervue, Padua Italy) microperimetry (MP) at 3 months as the primary efficacy endpoint, with the durability of response assessed at 12 months. Secondary efficacy and safety assessments are both anatomical (autofluorescence, spectral-domain optical coherence tomography [OCT], adaptive optics OCT [AO-OCT]) and functional (microperimetry, best-corrected visual acuity, multi-luminance mobility test). **Results:** 18 male subjects (mean age 32.4 + / - 9.37 years) have been enrolled and treated in Part I. AAV8 RPGR has been shown to be well tolerated, with no occurrence of serious AEs or DLTs. In this ongoing study, a total of 45 treatment-emergent events (TEAEs) occurred in 15 of 18 subjects. No subjects have been withdrawn due to a TEAE. Early indications of improved retinal sensitivity have been observed with microperimetry in all but the lowest 2 dose cohorts. As early as 1-month post-treatment, 3/3 (100%) subjects treated with the cohort-3 dose achieved the threshold of at least 7 dB improvement in retinal sensitivity in at least 5 of the central 16 loci. **Conclusion:** With a single dose of AAV8-RPGR, improvements in retinal sensitivity have been observed in XLRP patients as early as 1 month. These positive signals may be an early precursor to improvements in other efficacy outcomes. Part I subjects continue to be followed and treatment of subjects in the Part II dose expansion is ongoing.

931. Ocular Safety of Long-Term Suppression of VEGF by Intravitreally-Administered Gene Therapy, ADVM-022, in Non-Human Primates

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Repeated intravitreal (IVT) administration of anti-VEGF medications (e.g. aflibercept) is the mainstay of therapy for wet age-related macular degeneration (wAMD), maintaining and improving vision for many patients. Although direct causation between long-term VEGF suppression and adverse effects on the retina have not been established, pre-clinical animal work and limited clinical observations suggest that anti-VEGF medications may result in progression of geographic atrophy. With the continued development of long-lasting anti-VEGF treatment modalities, including gene therapy approaches, long term studies are needed to address potential long-term safety of continuous suppression of VEGF activity. Adverum Biotechnologies has developed ADVM-022, an adeno-associated virus (AAV) gene therapy vector encoding aflibercept, a current anti-VEGF standard of care for the treatment of wAMD. ADVM-022 contains the 7m8 capsid, and has been optimized for intravitreal delivery and robust protein expression.

We recently demonstrated that a single IVT administration of ADVM-022 resulted in durable aflibercept expression in the retina and provided long-term protection against laser-induced CNV in the non-human primate model of wAMD. ADVM-022 is currently in a phase I clinical trial in patients with wAMD under active anti-VEGF treatment. In the current study, we investigated the safety of ADVM-022 on the retina tissue following long term expression (2+ years) of aflibercept following intravitreal injection of ADVM-022 (2×10^{12} vg/eye). Retinal structural integrity and function were assessed by optical coherence tomography (OCT) and electroretinography (ERG), respectively, in African green monkeys (*Chlorocebus sabaeus*). The results indicated long term durable aflibercept expression in eyes treated with a single ADVM-022 IVT injection. Aflibercept expression in the vitreous (1.2 and 2.3 μg/mL) and retina (3.7 and 10 μg/g) from 2 eyes harvested 21 months post-dose and from one eye (vitreous: 5.6 μg/mL; retina: 14 μg/g) harvested 30 months post-dose was in the range previously demonstrated to suppress CNV in the animal model of wet AMD. Longitudinal analysis of retinal morphology by OCT in the long-term ADVM-022-treated animals found no changes in retina thickness. Specifically, RPE atrophy was not observed, and retinal thickness in the fovea, pericentral, and peripheral regions showed no changes from the baseline. Functionally, full field scotopic ERG conducted 19 months post-dose showed no statistically significant changes in a- and b-wave values between vehicle and ADVM-022 treated groups. In addition, assessment of the retinal activity in different retinal sectors assessed by multifocal ERG demonstrated that all responses were within the limits of the responses recorded in the treatment-naïve animals. In conclusion, ADVM-022 (2×10^{12} vg/eye) provides durable expression of aflibercept in the non-human primate retina, with no deleterious effects on retina structure and function observed out to 30 months post-dose, supporting a single IVT administration of ADVM-022 as a safe and effective long-term treatment option for patients with wAMD.

932. Restoration of Hearing in Tmc1-Deficient Mice Depends on Early and Well-Regulated Gene Delivery

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Tmc1 is believed to be the primary ion channel responsible for auditory hair cell depolarization in response to mechanical stimulation. Functional Tmc1 is necessary for hearing, and both humans and mice without Tmc1 fail to develop hearing. Previous work has shown that restoration of functional Tmc1 to Tmc1-deficient mice can partially rescue hearing when delivered neonatally via AAV containing a ubiquitous promoter. We explore here the effect of promoter specificity and mouse age at delivery on hearing rescue and on preservation of auditory hair cells. We delivered Anc80-CMV-Tmc1 at P2-P3 to TMC1 knock out (KO) mice. Consistent with previous reports, we observed partial rescue of auditory brainstem response (ABR) thresholds (~70 dB SPL) in TMC1 KO mice that were treated with AAV-based gene therapy. By contrast, no ABR response could be measured at equipment limits in untreated controls. We also observed preservation of cochlear hair cells in treated TMC1 KO mice as compared to untreated animals. In contrast, when we delivered the same gene therapy vector to TMC1 KO mice at P17, we observed minimal benefit, with ABR thresholds between 80 and 105 dB SPL. Interestingly, our observed onset of outer hair cell (OHC) loss in Tmc1-deficient animals is around P17, suggesting that most hair cells are present at the time of delivery, but cannot be rescued with the Anc80-CMV-TMC1 vector. We then assessed outer hair cell function using DPOAE in the TMC1 KO mice rescued at P2-P3. By this measure, we observed almost complete rescue of response for F2 frequencies at and below 16 kHz, suggesting better recovery of outer hair cell function than implied by the ABR threshold responses alone. We hypothesized that non-optimal levels of transgene expression, either in inner hair cells or in another cell type, may have limited ABR threshold recovery. To test this hypothesis, we delivered an Anc80-Tmc1 vector driven by a hair cell-specific promoter to P2-P3 TMC1 KO mice. When ABRs were assessed in these animals, we observed near normalization of thresholds at 16 kHz and below. We conclude that both age of delivery and specificity of expression are both tightly linked to functional rescue in Tmc1-deficient mice.

933. An AAV-CRISPR/Cas9 Gene Editing Approach for GUCY2D-Associated Cone Rod Dystrophy (CORD6)

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Purpose: Autosomal dominant mutations in *GUCY2D,* the gene encoding retinal guanylate cyclase-1 (retGC1), are the leading cause of cone-rod dystrophy (CORD6). Given our proven ability to deliver therapeutic *GUCY2D* to photoreceptors (PR) via AAV to treat recessive disease, an attractive treatment approach for CORD6 is to combine AAV-CRISPR/Cas9-based knockout of *GUCY2D*with complementation of a 'hardened' wt*GUCY2D in trans*. We recently showed selective and efficient somatic knock out of *GUCY2D* and *Gucy2e*, in macaque and mouse PRs, respectively, with AAV-CRISPR/Cas9 results in a subsequent loss of retinal structure/function resulting from reduced retGC1 expression. The purpose of this study is to establish 1) whether AAV-CRISPR/Cas9- based editing of *GUCY2D* is therapeutic in the R838S transgenic mouse model of CORD6, and 2) whether editing of the target locus and successful complementation with hardened wt*GUCY2D* can prevent loss of retinal structure/function. **Methods:** *S. aureus* Cas9 gRNAs were designed to target exon 4 of *GUCY2D* and exon 2 of *Gucy2e*. Relevant constructs were packaged in AAV capsids/variants with high PR transduction efficiency. Transgenic mice containing human *GUCY2D,* with the CORD6-causing R838S mutation, under a PR-specific promoter exhibit loss of retinal structure/ function similar to CORD6 patients. CORD6-Tg mice were subretinally injected (SR) with AAV-hGRK1-Cas9 and AAV-U6-*GUCY2D*-gRNA-hGRK1-GFP (right eye) or AAV-U6-*GUCY2D*-gRNA-hGRK1-GFP alone (left eye). Fundoscopy, electroretinogram (ERG), and optical coherence tomography (OCT) analyses were performed at 4, 10, and 20 weeks post-injection (p.i). $GC1^{+/-}$:$GC2^{-/-}$ mice have a single wt*Gucy2e* allele and no *Gucy2f* (the gene that encodes retGC2). Thus, ablation of *Gucy2e* via CRISPR/Cas9 should lead to a reduction in rod/cone function and PR degeneration over time, as is observed in GC1/2 double knock-out mice. $GC1^{+/-}$:$GC2^{-/-}$ mice were SR in both eyes with AAV-hGRK1-Cas9 and AAV-*Gucy2e*-gRNA-hGRK1-GFP, with right eyes being additionally injected with AAV8-hGRK1-'hardened'GC1. Fundoscopy, ERG, and OCT analyses were performed at 4, 10, and 20 weeks p.i, with immunohistochemistry and RT-qPCR analyses performed at 20 weeks p.i. A separate cohort of $GC1^{+/-}$:$GC2^{-/-}$ mice were SR with AAV-hGRK1-Cas9 and either AAV-U6-*Gucy2e*-gRNA-hGRK-GFP (right eye) or AAV-U6-*Gucy2e*-gRNA-hGRK1-'hardened'GC1 (left eye). Fundoscopy, ERG, and OCT analyses were performed at 4, 8, 12, and 20 weeks p.i. **Results:** In CORD6-Tg mice, PR structure/function were preserved through targeted editing/disruption of human mutant *GUCY2D*(R838S). This effect persisted for at least 20 weeks p.i. In $GC1^{+/-}$:$GC2^{-/-}$ mice, the 'knock-out + complementation *in trans*' approach using either a two- or three-vector system significantly preserved PR structure/function relative to eyes that received editing (knock out) reagents alone. This was observed as early as 4 weeks p.i. ERG responses and retinal structure in 'knock-out + complementation *in trans*' eyes were comparable to those of control eyes injected with vehicle alone. Preservation of structure/function persisted for at least 20 weeks p.i. in mice treated with the three-vector approach. Analyses of mice injected with the two-vector approach is still underway. **Conclusions**: Preservation of mouse PR structure/function was achieved via targeted knockout of the human *GUCY2D(R838S)* sequence in a transgenic mouse model. Additionally, we show that a 'knock-out + complementation *in trans*' approach preserves PR structure/function. These results establish the feasibility of an AAV-CRISPR/Cas9-based therapy for the treatment of CORD6.

934. AAV-CRISPR/Cas9-Mediated Gene Knock-In Therapy to Rescue Photoreceptor Degeneration in the P23H Rhodopsin Mutant Mice

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Introduction: Photoreceptors play the most important role for vision as the primary light-sensing cells in the retina. In human retina, ~95% of the photoreceptors are rods, which are responsible for dark vision. Mutations affecting rod function and survival can lead to retinitis pigmentosa (RP), a common but untreatable inherited retinal degeneration disease that affects approximately 1.5 million people worldwide. RP is highly genetic heterogeneous, with more than 60 causative genes identified to date. A single RP causative gene *RHO,* which encodes a protein called rhodopsin, has more than 100 mutations. P23H is the most common mutation in autosomal dominant RP caused by a single point mutation c.68C>A (p.Pro23His) in the *RHO* gene. In this study, we aim to develop an AAV-CRISPR/Cas9 gene editing therapy to rescue rod degeneration in a P23H mice model by knocking in the T2A-wild type *Rho* coding sequence (T2A-wt *Rho*) at the exon2-intron2 site of the *Rho* gene using the published homology-independent targeted integration (HITI) method. If the T2A-*Rho* can be successfully inserted in-frame to the endogenous *Rho* ORF as designed, wt RHO protein will be expressed under the endogenous Rho promoter while the translation of full-length mutant RHO protein will be disrupted, eventually allowing normal rod function and survival. **Methods:** The SaCas9/gRNAs targeting the *Rho* gene were firstly screened in wt mouse embryonic fibroblasts (MEFs) analyzed by the Tracking of Indels by Decomposition (TIDE) method. One AAV vector expressing CMV-SaCas9 (AAV8-Cas9) together with another AAV vector carrying U6-gRNA, CMV-mCherry and HITI T2A-GFP donor (AAV8-mCherry/gRNA/HITI T2A-GFP) were subretinally delivered into neonatal CD-1 mouse eyes. The knock-in efficiency was quantified by GFP+/mCherry+ cells at 14 days post infection (dpi). Next, AAV8-Cas9 and AAV8-mCherry/gRNA/HITI T2A-*Rho* were subretinally delivered in neonatal P23H and wt mice to assess the therapeutic effects and safety of the vectors. The visual function of treated mice will be tested by electroretinography (ERG), which measures the electrical response of the retina to light stimulus, and by optomotor, which measures the visual acuity of mice. At last, mice will

be sacrificed, and the retina morphology and photoreceptor survival will be analyzed by immunohistology. **Results:** Our CRISPR/Cas9 system with donor design based on the HITI method can successfully knock in the T2A-wt-*Rho*/GFP into the *Rho* loci *in vivo*. The results showed that the knock-in efficiency of the GFP reporter was about 50% of the transduced photoreceptor cells by AAV delivery. GFP expression was only observed in rods, while other transduced retinal cell types remained GFP negative, as predicted by the activity of the endogenous *Rho* promoter. Sequencing results showed that the majority of the donor fragment was integrated into the *Rho* loci in-frame, in which case it will disrupt the translation of the full-length mutant Rho protein while allowing the expression of wt Rho protein. We are currently examining whether rod survival and visual function are significantly improved in the treated P23H mice. **Conclusions:** The AAV-CRISPR/Cas9-mediated gene knock-in therapy proves its feasibility and high efficiency to knock in an ORF at a gRNA targeting site. This method can be used not only on P23H mutant but also other *Rho* mutations. Such mutation-independent gene therapy can be applicable to other inherited diseases with high genetic heterogeneity.

935. Long-Term Rescue of Retinal Degeneration in Rho-P23H Knockin Mice via Dual AAV-Medicated Allele-Specific CRISPR-Cas9 Gene Editing

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Rhodopsin-P23H associated Retinitis Pigmentosa (RP) is the most common cause of autosomal dominant RP (adRP) in USA. This *Rho-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 recent publication, revealed that gene editing with a CRISPR-Cas9 variant and a truncated sgRNA could selectively inactive the *Rho-P23H* mutant allele, leading to a significant decrease of the P23H mutant transcript and protein, and preservation of photoreceptors in a *Rho-P23H* knock-in mouse model. In order to evaluate the long-term efficiency and allele-specificity of this method, we co-delivered the Cas9 nuclease and sgRNA via a dual-AAV approach. We utilized Anc80-pRK-SpCas9-VRQR and Anc80-pU6-sgRNA-pCMV-mCherry to deliver the CRISPR components into the retinas of *Rho-P23H* heterozygous mice at 14-18 days of age, and examined them 10 months post injection. Assessment of NHEJ-mediated editing efficiency by NGS analysis demonstrated that AAV-mediated Cas9/sgRNA gene editing in mouse retina could ablate the P23H mutant allele, with cleavage frequencies up to 26% in transduced cells. We observed that retinal morphology was significantly preserved in the treated regions of retinas, with two to five additional rows of outer nuclear layer (ONL) compared to retinas treated with sgRNA vector only or untreated retinas (in which only one row of cones remained after 10 months). We also examined the off-target effects of long-term expression of CRISPR/Cas components in the targeted cells of retinas. Collectively, our results demonstrate that our CRISPR-Cas9 dual AAV approach executes effective editing in retinas *in vivo*, and suggests that long-term preservation of photoreceptor degeneration can be achieved. Our study suggests that a prolonged therapeutic benefit of AAV-mediated allele-specific CRIPSR-Cas9 gene editing could be expected if it is applied to human *RHO-P23H* patients, and also more broadly for other inherited retinal diseases.

936. Novel AAV Capsids Display Expansive Transduction and Enhanced Potency in Subretinally Injected Mice

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Purpose: The majority of inherited retinal diseases (IRDs) are caused by mutations in photoreceptor (PR)- specific genes. While intravitreal injection (IVI) of AAV is a clinically attractive alternative to subretinal injection (SRI), development of AAVs capable widespread PR transduction via IVI has been elusive. Effective clinical application of SRI gene therapies will require vectors that transduce large areas of retina with low titer/volume, and efficiently transduce foveal cones, the cells responsible for acute, daylight vision. AAV44.9 is a recent isolate in the same clade as AAVrh.8. The purpose of this study was to evaluate AAV44.9 and related capsids for their 1) biophysical properties known to impact retinal transduction 2) transduction efficiencies in ocular cell lines, 3) retinal tropism and transduction efficiencies following IVI and SRI in normal mice, and 4) potency in a mouse model of retinal disease. **Methods:** Self-complementary smCBA mCherry constructs were packaged in AAV44.9, AAV44.9(E531D), AAV44.9(Y733F), AAV5, AAV8(Y733F) and AAVrh.8. Single stranded IRBP/GNAT2-GFP was packaged in AAV44.9 and AAV44.9(E531D). Single stranded constructs containing hGRK1 driving codon optimized *Gucy2e* were packaged in AAV44.9(E531D) and AAV8(Y733F). Heparin-binding profiles were determined using affinity chromatography. ARPE-19 and 661W cells were infected and mCherry expression quantified via flow cytometry. $2x10^9$ vg (high dose) or $2x10^8$ vg (low dose) in 1 ul was delivered via IVI or SRI to 4-5 weeks old Nrl-GFP and C57BL/6J mice. 4 weeks post-injection (p.i.), mCherry was visualized with fundoscopy. Retinas were dissociated, and flow-cytometry used to quantify cells positive for GFP (rods), mCherry (non-rod retinal neurons transduced by AAV), or both (rods transduced by AAV). Retinal sections were evaluated for mCherry, GFP and cone arrestin. *Gucy2e*$^{-/-}$*:Gucy2f*$^{-/-}$ (GCDKO) mice were SRI with either AAV44.9(E531D)-hGRK1-coGC1 or AAV8(Y733F)-hGRK1-coGC1. Retinal function will be assessed via electroretinogram (ERG) starting at 4 weeks p.i. and retinas will be immunostained for GC1 after sacrifice. **Results:** Transduction of ocular cell lines, or retina following IVI injection with AAV44.9 was inefficient. Consistent with the lack of IVI mediated transduction, AAV44.9 does not bind heparin. SRI led to extensive transduction

that was primarily restricted to PRs and RPE. High dose AAV44.9 and AAV44.9(E531D) transduced 61% and 82% of rods, respectively. Both outperformed AAV5 (40.8%) and AAV8(Y733F) (53.9%), and were comparable to AAVrh8 (85%). Low dose AAV44.9(E531D) (35%) outperformed AAVrh8 (21%). Addition of Y733F to AAV44.9 did not improve transduction efficiency. Cone transduction by AAV44.9 and AAV44.9(E531D) was confirmed with IRBP/GNAT2 containing vectors. Potency comparison between AAV44.9(E531D) and AAV8(Y733F) in SRI GCDKO mice is underway. **Conclusion:** AAV44.9 and AAV44.9(E531D) exhibit increased rod/cone/RPE transduction in SRI mice relative to benchmark vectors. The percentage of PRs transduced indicates substantial transduction outside of the injection bleb area. Our results support further testing of these capsids in primate retina to determine whether they will be useful for treating diseases where central retinal cones are the target, but detachment of the fovea is not preferred (i.e. Achromatopsia, Ushers syndrome), or to address IRDs that would benefit from an expansive treatment area (i.e. retinitis pigmentosa, choroideremia).

CAR T Cell Therapy II

937. Chimeric Antigen Receptors with a MyD88 and CD40 Endodomain Endow T Cells with Superior Antitumor Activity

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Background: Adoptive cell therapy with T-cells expressing chimeric antigen receptors (CARs) has not yet been nearly as successful for solid tumors as for leukemia. Lack of efficacy is most likely due to several factors, including suboptimal CAR signaling resulting in limited T-cell activation within the solid tumor microenvironment. The majority of CARs encode an endodomain that consists of CD3 zeta (z) and a canonical CD28 and/or 41BB costimulatory signaling domain. However, other molecules play important roles in T-cell costimulation, and the goal of this project was to evaluate the use of the TLR adaptor molecule MyD88 and the TNFR CD40 molecule as CAR endodomains. **Methods/Results:** We generated CARs with CD28.z, 41BB.z, or MyD88.CD40 (MC).z endodomains that recognize the solid tumor antigen EphA2. CD28-CAR, 41BB-CAR, and MC-CAR T-cells recognized and killed EphA2+ tumor cells (U373 and LM7 cells) in an antigen-dependent manner. In repeat stimulations assays with U373 or LM7 cells, the median-fold expansion of MC-CAR T cells was 87-fold, which was significantly greater ($p<0.05$) than for CD28-CAR (10-fold) or 41BB-CAR (4-fold) T cells. MC-CAR T-cells also had significantly greater cytolytic activity ($p<0.001$) than CD28-CAR or 41BB-CAR T-cells (MC-CAR T-cells: mean of 6 sequential target cell killing vs. 3 for CD28-CAR or 41BB-CAR T-cells). Improved effector function of MC-CAR T-cells was strictly antigen-dependent, since MC-CAR T-cells did not recognize or kill U373.koEphA2 cells. In vivo, MC-CAR T-cells had greater antitumor activity in the LM7 NSG mouse model resulting in improved overall survival at limiting CAR T-cell doses ($1x10^4$ or $1x10^5$ cells per mouse). Improved antitumor activity was mirrored by greater in vivo expansion and persistence of MC-CAR T-cells in comparison to CD28-CAR or 41BB-CAR T-cells. To gain additional mechanistic insights, RNA-seq analysis was performed on CD4- and CD8-sorted CD28-CAR, 41BB-CAR, and MC-CAR T-cells (n=3) prior to antigen-specific stimulation. T-cells transduced with a nonfunctional EphA2-CAR (delta-CAR) served as a control. Principle component analysis revealed clustering of i) CD28- and delta-CAR T-cells and ii) 41BB- and MC-CAR T cells. Genes upregulated in 41BB- and MC-CAR T-cells included genes involved in cell proliferation, mitosis, cell cycle progression, and DNA replication. In addition, 41BB- and MC-CAR T-cells expressed discrete sets of cytokines in comparison to CD28- and delta-CAR T-cells. Comparison of 41BB- and MC-CAR T-cells revealed a distinct gene signature for MC-CAR T cells with higher expression of genes within TLR and TNF pathways and lower expression of PD-1. **Conclusions:** We demonstrate here that MC-CARs endow T-cells with improved effector function in comparison to CD28- or 41BB-CARs. Mechanistic studies revealed that MC-CARs induce baseline T-cell activation, which does not result in T-cell exhaustion as judged by PD-1 expression. Thus, including MyD88 and CD40 signaling domains in CAR T-cells may improve current CAR T-cell therapy approaches for solid tumors.

938. Library Assembly and Selection of 4-1BB:zeta CAR Activation-Dependent Synthetic Promoters in Primary Human T Cells

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Synthetic functional outputs from anti-tumor CAR T cells can profoundly enhance their therapeutic potency, but often require stringent control for safety. Here, we describe the discovery process of CAR activation-regulated synthetic promoters by the rationale selection and random assembly of Transcription Factor Response Elements (TRE's) computationally identified within endogenous T cell activation-dependent promoters of the human genome. The resulting inducible synthetic promoter (iSynPro) library was transferred to lentivirus housing the GFP:ffluc reporter and transduced into CD19CAR(4-1BB:zeta)-expressing human $CD8^+$ T cells. Screening was performed by activating the resulting T cells with CD19+ simulator cells, selecting GFP positive T cells at defined intervals (24,48, and 72hrs after activation), then eliminating those T cells demonstrating constitutive reporter expression after antigen withdrawal. iSynPro sequences were identified by targeted NGS DNA seq from the isolated genomes of the sorted CAR T cells. Twenty-eight promoter sequences (mean size 207bp, range 85-338bp) selected from the most abundant ones were cloned in to individual LV's for further analysis. The majority of the iSynPro's were quiescent in proliferating T cells in the absence of target antigen, whereas transient (peak expression 24hrs after activation, return to baseline 7-days) could be induced *in vitro*

and *in vivo* upon CAR T cell encounter with target antigen expressing stimulator cells. Robust iSynPro transcriptional output could be re-induced over at least three cycles of antigen activation at promoter activity levels significantly higher than a control NFAT-RE promoter and greater OFF-state stringency. These iSynPro's are specific to CAR/TcR signals as transcription could not be elicited by costimulation alone or non-specific TLR activation. This work demonstrates that synthetic libraries of rationally selected TRE's can yield, upon cellular state specific screening, compact promoters having stringently regulated activation-dependent outputs amenable for therapeutics application in CAR T cells.

939. Hampering Tumor Glycosylation Improves the Therapeutic Index of CAR-T Cells Against Solid Malignancies

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Background: The adoptive transfer of CAR-T cells demonstrated impressive results against B-cell malignancies but still limited efficacy against solid tumors. 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, especially in richly glycosylated proteins. **Results:** To investigate if sugar chains may be sterically hulking for CAR-T cell targeting, we generated N-glycosylation-defective pancreatic tumor cell lines by knocking-out the expression of the glycosyltransferase Mgat5 using the CRISPR-Cas9 technology. As model antigens for CAR targeting, we focused on CD44v6 and CEA since they are both heavily glycosylated proteins over-expressed on a wide variety of solid tumors, including pancreatic adenocarcinomas. Strikingly, hampering N-glycosylation resulted in a dramatic increase of tumor targeting by both CD44v6 (4-fold, p<0,001) and CEA CAR-T cells (10-fold, p<0,001). This effect associated with improved CAR-T cell activation, suggesting a more proficient antigen engagement. These findings were further confirmed using the N-glycosylation inhibitor tunicamycin (CD44v6: 2,5-fold; CEA: 5-fold, p<0,01). 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 tumor cells to recognition by CAR-T cells, significantly increasing their elimination (CD44v6: 3-fold, p<0,01; CEA: 13-fold, p<0,001). Notably, 2DG alone proved to be ineffective, further suggesting a synergistic effect with CAR-T cells. Mechanistically, both Mgat5 knock-out and 2DG decreased PHA-L lectin staining on pancreatic tumor cells, showing that treatment effectively reduces N-glycan branching. Similar results were also confirmed by Western blot looking at the presence of de-glycosylated proteins on tumor cell surface after 2DG treatment. Next, we challenged the combined approach in a pancreatic adenocarcinoma xenograft mouse model. Accordingly with in vitro data, mice receiving CAR-T cells highly benefited from 2DG administration (5-fold less tumor at 7d, p<0,05), which conversely was unable to mediate any antitumor effect alone. Interestingly, improved antitumor activity was accompanied by a decrease in the frequency of CAR-T cells co-expressing exhaustion and senescence markers such as TIM-3, LAG-3, PD-1 and CD57 (SPICE software analysis, p=0,0105). Thanks to metabolic deregulation (Warburg effect), 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 induce protein de-glycosylation and to increase the elimination of healthy cells, such as keratinocytes. **Conclusions:** Our results indicate that i) the glycosylation status of tumor cells regulates the efficacy of CAR-T cells and ii) combining CAR-T cells with the de-glycosylation agent 2DG, which preferentially accumulates in tumor masses, may pave the way for a successful immunotherapy against solid tumors.

940. Augmenting CAR T Cell Mediated Anti-Tumor Efficacy through Genetic Modification to Secrete a Novel Cytokine IL-36γ

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Chimeric antigen receptor (CAR) T cell therapy has shown remarkable response in patients with B cell acute lymphoblastic leukemia. However, long-term follow-up studies suggest many patients who achieved complete response are still at risk of developing either antigen positive or antigen negative relapse, potentially due to the lack of persistent functional CAR T cells or the incapability of CAR T cells to overcome antigen escape. Therefore development of novel CAR design to overcome these limitations is in urgent need. IL-36γ is a novel cytokine belonging to IL-1 superfamily. It is abundantly secreted by epithelial cells, particularly in the skin and lung, in response to tissue damage or infection to initiate and amplify immune response locally. In mouse, IL-36 receptor has been found on both lymphoid and myeloid cells such as T cell and dendritic cells (DC), with the ability to enhance T cell proliferation and DC maturation, as well as promoting pro-inflammatory cytokine production. Tumor cells transduced to secrete IL-36γ have shown reduced progression and a transformed tumor microenvironment featured with enhanced type 1 lymphocyte infiltration. Given the potent immune modulating effects, we hypothesized that modifying CAR T cells to secrete this cytokine could act on both CAR T cells and endogenous immune cells to improve the overall immune response. We generated and validated a series of IL-36γ-secreting murine CAR constructs targeted to CD19. In a syngeneic tumor model without pre-conditioning, IL-36γ secreting CAR T cells exhibited superior anti-tumor activity and remarkable persistence, evidenced by the presence of B cell aplasia in treated animal for more than 40 weeks. Notably, the persistent CAR T cells are still functional at this moment and were able to protect mice from tumor rechallenge. Further analysis revealed that IL-36γ enhanced CAR T

cell function by promoting T cell expansion and cytokine production in vivo. IL-36-γ-secreting CAR T quickly expanded in the secondary lymphoid organs, resulting in a 10-fold higher cell number in the spleen and bone marrow compared to second generation CAR. The cytokine armored CAR T cells had a significant improved IFN-γ secretion both in vivo and upon ex vivo stimulation. Interestingly, the anti-tumor efficacy mediated by IL-36γ-secreting CAR depends on $CD8^+$, but not $CD4^+$ CAR T cells, as depletion of the former subset completely abrogated the survival benefit, while depletion of the latter subset had no effect on overall survival. In addition to the effects on CAR T cells, we observed that IL-36γ also significantly induced CD86 upregulation on dendritic cells and macrophages, and enhances the activation level on NK cells in the spleen, indicating IL-36γ plays pleiotropic roles in remodulating the immune environment. To extend our finding in a more clinical relevant setting, we examined the anti-tumor efficacy in the presence of lymphodepletion. In line with previous finding, IL-36γ-secreting CAR T cells outperform second-generation CAR at a low dose, leading to an improved survival and long-term B cell aplasia in treated animals. We are currently investigating the cellular mechanisms through which IL-36γ induced such profound functional enhancement on CAR T cells. Future work will involve evaluating this approach in multiple solid tumor models and examining the cytokine's effects within the tumor microenvironment to further understand the translational potential of this novel CAR design.

941. Novel Genomic Safe Harbors for Effective CAR T Cell Engineering

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Effective T cell engineering requires stable integration and dependable function of therapeutic transgenes. Current approaches utilize gamma-retroviral, lentiviral or transposon vectors, all of which afford semi-random integration. This results in variegated transgene expression and alterations in expression of endogenous genes near the integration site, possibly leading to oncogene activation. Both these major shortcomings can be overcome by site specific integration into designated genomic sites, which may either lie in gene loci or in genomic regions with no known function and remote from functionally important and conserved regions, defined as 'genomic safe harbor' sites (GSHs). Adoptive immunotherapy using chimeric antigen receptors (CARs) is one of the most promising new therapies to treat cancer and has shown remarkable results in treating refractory leukemias. Current T cell manufacturing procedures utilize gamma-retroviral or lentiviral vectors, resulting in variable and unpredictable CAR expression and hence variability in the potency of individual T cells. Previous research from our lab has established that the T cell receptor alpha (*TRAC*) locus supports optimal expression of a CAR to produce a highly uniform and potent T cell immunotherapy product. CAR cDNA delivery was achieved via targeted CRISPR-Cas9 cleavage and homologous recombination using an AAV6 donor template. Here, we sought to identify extragenic GSHs in T cells that could be used for potent CAR T cell immunotherapy approaches to express a CAR and/or any additional molecule, such as cytokines or scFv's that can further enhance the functionality and potency of the T cells. We previously defined theoretical criteria for the identification of potential GSHs. However, to achieve site specific transgene integration at such sites, it is imperative that these be amenable to efficient cleavage by endonucleases like CRISPR-Cas9. We hypothesized that cleavage efficiency by an endonuclease like Cas9, particularly at an extragenic safe harbor, is governed by the chromatin accessibility of the locus. We analyzed the chromatin accessibility of the human T cell genome through ATAC-seq and identified chromosomal regions that conform to the safe harbor criteria and possess measurable chromatin accessibility. The 6 highest accessible sites tested revealed high (>90%) cleavage efficiencies and also allowed for stable CAR cDNA integration and expression in human peripheral blood T cells. CAR expression levels, driven by the EF1α promoter, were measured within days following transfection and found to be elevated and comparable to those obtained at the *TRAC* locus. These edited T cells showed similar cytotoxic capacity as the *TRAC* CAR T cells in *in vitro* cytotoxicity assays, as well as similar proliferation ability upon antigenic stimulation. However, the level of CAR expression was observed to diminish over time. Hence, we decided to use chromatin insulator elements with barrier function to prevent heterochromatinization and thus preserve CAR expression and T cell function. Upon incorporation of chromatin insulator elements flanking the CAR cassette, our GSHs maintained their CAR expression level over multiple days in culture and exhibited equivalent cytotoxic potential as the *TRAC* CAR T cells in *in vitro* cytotoxicity assays and also a similar proliferation capacity over multiple weeks in culture in response to antigenic stimulation. Early data indicates that at least one of these sites is able to support tumor control in a similar manner as the *TRAC* CAR T cells *in vivo* in a mouse model of B-Cell Acute Lymphoblastic Leukemia. The approach that we describe here for identification of targetable GSH sites is useful for the identification of extragenic regions in which to safely and effectively insert therapeutic transgenes to achieve predictable and persisting gene expression.

942. Uni-Vect: Antigen-Inducible Immunomodulatory Molecule Expression from a Single Lentiviral Vector Platform to Enhance CAR T Cell Functions

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Compared to dramatic clinical results in treating hematological malignancies, CAR T therapy has thus far demonstrated limited success in the solid tumor setting. Various approaches have been employed to increase efficacy, including endowing engineered immune cells with ectopic expression of immunomodulatory molecules. An outstanding question is ***how to genetically integrate these effector molecules into CAR T cells in a clinically feasible manner***. To avoid systemic toxicities, tumor-localized expression is preferred. These approaches currently rely on two-component viral vector systems where a constitutive CAR and an inducible accessory molecule module are delivered with two separate viruses. This leads to a ***non-homogenous modification*** of the cellular

product which is only rectified using laborious and often clinically unfeasible selection procedures that ***limit translational potential***. To address these issues, we have developed ***optimized genetically integrated systems that combine autonomous antigen-induced production of an immunomodulatory molecule along with constitutive CAR expression in a single lentiviral vector system*** (**Uni-Vect; Figure 1a**). Using this system, coordinate NFAT-inducible and constitutive expression of the reporter genes occurs in primary T cells (**Figure 1b**). By introducing a CAR to the system, designer CAR T cells that respond specifically to target antigen, exclusively upregulate the inducible module (**Figure 1c**). Focusing on clinical translation, these systems comprise ***human-only genetic elements*** to avoid immunogenicity, have ***low basal leakage*** and ***high maximal activity*** of an inducible module while retaining clinically relevant ***virus titers***. Here we present implementations of the Uni-Vect system instilling enhanced efficacy or safety of CAR T cells. By combining antigen-driven secretion of an ***immunomodulatory cytokine IL-12*** with a clinically used CAR, ***enhanced antitumor activity*** of CAR T cells was achieved. To limit toxicities associated with adoptive CAR T cell therapy, we coupled clinically used CAR with ***in-situ secretion of a tocilizumab-based engineered antibody***. Currently, we are testing whether an autonomous and prompt delivery of the IL-6 receptor blocking antibody by therapeutic cells themselves might ***suppress cytokine release syndrome*** before it becomes clinically detectable. In conclusion, compared to two-component viral vector systems, the single Uni-Vect platform achieves homogenous modification of cell products and improves CAR T-cell manufacturing work-flows. The Uni-Vect system is broadly-applicable and easily amenable to construct and screen combinations of immune receptors and modulators of interest, while our featured implementations demonstrated enhanced CAR T cell functions.

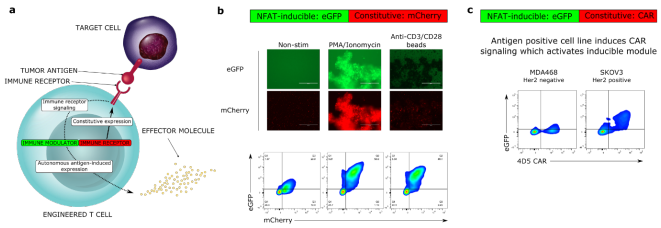

Figure 1: Concept and a proof-of-principle of Uni-Vect single lentiviral systems
(**a**) Upon recognition of cognate antigen, constitutively expressed immune receptor rewires endogenous NFAT signaling to the expression of immune modulator from the same lentiviral construct (**b**) Primary T cells were engineered with a prototype all-in-one lentivirus and stimulated chemically or with anti-CD3/CD28 beads (**c**) Signaling induced by Her2-targeting CAR activates the system only in co-culture with Her2 positive cancer cell line.

943. Improving CAR T Cell Therapy for Solid Tumors by Adopting a Dual-Targeting Binary Strategy

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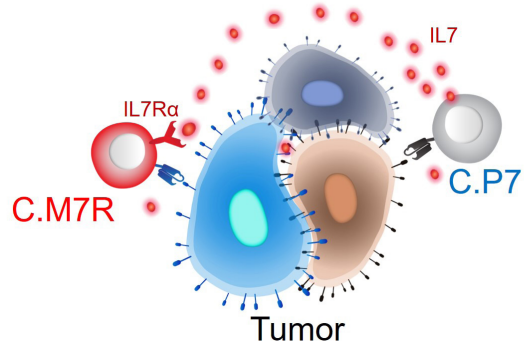
Introduction: Adoptive transfer of T cells modified to express chimeric antigen receptors (CARs) has proven highly effective for the treatment of certain hematological malignancies; however, the efficacy in solid tumors have been underwhelming. Among various mechanisms underlying the poor response rates are tumor associated antigen (TAA) heterogeneity, which can lead to immune escape when a single antigen is targeted and limited proliferation and persistence of the infused T cells at the tumor, which results in diminished anti-tumor activity. **Approach:** To address these barriers to the successful application of CAR T cells in solid tumors, we explored a dual antigen targeting strategy of co-administering two CAR T cell products with specificities against prostate stem cell antigen (PSCA) and Mucin-1 (MUC1), which are TAAs represented across a wide range of solid tumors including prostate, pancreatic, and breast cancers. Furthermore, to enhance T cell proliferation and persistence, we engineered the CAR-PSCA (C.P) T cells to secrete IL-7 - a homeostatic cytokine known to support T cell survival (C.P7), and CAR-MUC1 (C.M) cells to overexpress IL-7Rα (C.M7R) for maximum utilization of IL-7 produced by C.P7 T cells. We hypothesized that co-application of these two T cell products establishes a tumor-localized binary system (**Fig. 1**) with enhanced anti-tumor response and improved proliferation/persistence of T cells. **Results:** Using CAPAN1 pancreatic cancer model, which expresses both PSCA and MUC1, we confirmed the antigen specificity and tumor-killing capacity of C.P7 and C.M7R T cells in a ^{51}Cr-release assay. Next, we assessed IL-7 secretion by C.P7 cells upon antigen stimulation using ELISA which demonstrated significant IL-7 production (6132.9±583.6pg/ml) compared to CAR-only T cells (198.9±78.4pg/ml). To investigate if the IL-7 produced by C.P7 cells can promote T cell expansion, a mixed-lymphocyte stimulation assay was conducted using C.M7R cells, C.P7 cells, and irradiated CAPAN1 targets (cell ratio of 1:1:2, respectively). During the 7-day culture, compared to control (C.M7R+C.P) condition (3.45±0.11 vs. 0.33±0.03x10^6, day 7 vs. day 0), significant increase in cell numbers (8.90±0.64 vs. 0.32±0.05x10^6, day 7 vs. day 0, respectively) was observed, majority of which were C.M7R . We next assessed the anti-tumor activity and long-term persistence of T cells when challenged with FFLuc-labeled CAPAN1 tumors in single-targeted (C.M or C.P T cells alone), dual-targeted (C.M+C.P co-administration), or binary (C.M7R+C.P7 co-administration) settings. While all treatment conditions transiently controlled tumor growth compared to untreated control, only the binary approach resulted in robust and sustained anti-tumor activity indicated by substantial decrease in tumor luminescence. These observations were further confirmed by periodic flow cytometry which as indicated by a progressive decrease in the number of tumor cells and an increase in T cell numbers, exhibited improved anti-tumor activity and T cell persistence in the binary condition compared to single or dual-targeted methods. **Conclusions:** Our results demonstrate the feasibility of a binary system based CAR T cell therapy for solid tumors which simultaneously addresses tumor-antigen escape by dual-targeting and enhances T cell proliferation and persistence by the expression of a complementary cytokine-receptor pair.



944. Aborted Activation of CAR T Cells by Chronic Lymphocytic Leukemia Cells

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Background: Chronic Lymphocytic Leukemia (CLL) is a B cell malignancy that accounts for 25% of leukemia diagnoses in the developed world. While conventional therapies have some efficacy, patients ultimately progress to relapsed or refractory disease. CD19-targeting chimeric antigen receptor (CAR) T cell therapy has provided hope, but induces complete remission in only 26% of patients. This suboptimal response rate is believed to be due to T cell dysfunction and immune-suppression by CLL cells, the mechanisms of which are poorly understood. **Findings:** To understand the causes of this dysfunction we investigated the defects CLL cells induced in normal donor CD19-targeting CAR T cells. CAR T cells were repeatedly stimulated at 5-day intervals with either primary CD19+ CLL cells from 22 patients or a CD19-expressing artificial antigen-presenting cell (aAPC). Repeated stimulation of CAR T cells with aAPCs resulted in 5.36 ± .94 population doublings after three stimulations, whereas CLL cells only evoked 2.39 ± .92 population doublings. To investigate this proliferation defect, we used flow cytometry to assess expression of costimulatory and inhibitory molecules on the 22 CLL samples and correlated this with the ability of the cells to induce CAR T cell proliferation. We found no correlation between the ability to stimulate proliferation and the expression of any costimulatory or inhibitory marker. We also performed phenotyping, proliferation analysis, and cytokine analysis of stimulated CAR T cells. Consistent with the pseudoexhausted phenotype of T cells from CLL patients, T cells from our cultures expressed high levels of PD-1 and Eomes, low levels of LAG3 and TIM3, and had a higher proportion of $PD1^{+}Eomes^{+}$ cells compared to aAPC-stimulated cells. Furthermore, cytokine analysis showed that CLL-stimulated CAR T cells produced significantly lower levels of effector cytokines. To determine if CLL cells are immune-suppressive, we used a mixed co-culture assay where we stimulated CAR T cells with aAPCs and CLL cells mixed at varying ratios (*Fig. 1A*). Interestingly, all mixed cultures proliferated similarly to each other while cultures stimulated solely with CLL cells showed the proliferation defect (*Fig. 1B*). This indicates that CLL cells are not intrinsically suppressive, as suppression would have impaired proliferation in all CLL-containing cultures. Furthermore, we observed that CAR T cells form stable aggregates with aAPCs but not with CLL cells, suggesting that CLL cells do not sufficiently activate CAR T cells. We further hypothesized that CLL cells may induce changes in T cells that are stabilized by successive stimulations. To test this, we stimulated CAR T cells with CLL cells 1, 2, or 3 times and then stimulated with aAPCs instead. When CAR T cells were stimulated with CLL cells they failed to proliferate or produce cytokines. However, subsequent stimulation with aAPCs rescued proliferation and cytokine production. Rescue was independent of the number of times T cells were stimulated with CLL cells, suggesting that CLL-induced defects are reversible. **Conclusions:** Taken together, the above data suggest that CLL-induced CAR T cell defects are the result of insufficient activation and these defects can be rescued by providing a strong stimulus to the T cells.

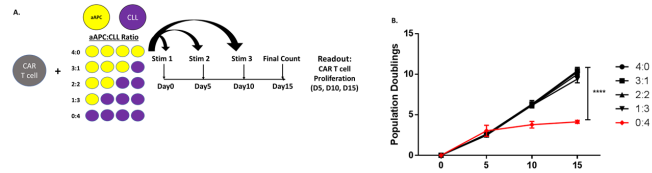

Figure 1. CLL cells do not suppress CAR T cell growth.
(A) Mixed co-culture assay experimental design.
(B) Replication of CAR T cells repeatedly stimulated with the mixed APC cultures indicated in *(A)* was measured. CLL cells fail to suppress CAR T cell growth when aAPCs are present, indicating that CLL cells are not suppressive. **** $P < .0001$, two-way analysis of variance, Tukey test.

AAV Vectors and Disease Targets III

945. Intra-Articular AAV9 α-Iduronidase Gene Therapy in the Canine Model of Mucopolysaccharidosis Type I Results in Rapid Synovial and Cartilage Iduronidase Expression, Clearance of Heparan Sulfate, and High Serum α-Iduronidase Levels

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Mucopolysaccharidosis type I (MPS I) is caused by the lack of a lysosomal enzyme, α-L-iduronidase (IDUA), in the degradation pathway of glycosaminoglycans (GAGs). Despite intravenous enzyme replacement and stem cell therapies, treated MPS I patients still struggle with significant skeletal and joint disease. This is thought to be in large part due to limited delivery of enzyme to joint cartilage and tissues. Previously, we showed that intra-articular enzyme replacement therapy with IDUA in MPS I dog joints reduced joint tissue inflammation and cleared synoviocyte and chondrocyte lysosomal storage. Since repeated intra-articular enzyme injections is not clinically feasible, we are studying the safety, efficacy, durability, and dose response of intra-articular AAV9-*IDUA* gene therapy in MPS I canine model and report our early preliminary findings. Baseline synovial and cartilage biopsies were obtained at 6 weeks from bilateral shoulder and stifle joints. At 10 weeks of age, left shoulder and stifle were treated with $5x10^{12}$ vector genomes (vg)/joint of AAV9-eGFP (control) whereas the right shoulder and stifle were treated with $5x10^{12}$ vg/joint of AAV9-canine *IDUA* (cIDUA). Serum, synovium, and cartilage were obtained two weeks' post-treatment; cIDUA and tissue glycosaminoglycan concentrations were quantified. Treatment was well-tolerated without pain or joint effusions. Baseline serum cIDUA level was below limit of detection (<0.3 units/mL); post-treatment serum cIDUA was 3.6 units/mL (mean heterozygote cIDUA 3.35 units/mL). Post-treatment control synovial cIDUA levels were undetectable, versus 43.5 and 153 units/mL in the

AAV9-cIDUA-treated joints. The difference between baseline and post-treatment synovial heparan sulfate in control joints was +6.7%, versus -96% in AAV9-cIDUA-treated joints. Heparan sulfate in cIDUA-treated joint cartilage was reduced 48%, compared with a 52% increase in control joints. One injection of intra-articular AAV9-cIDUA results in appreciable cIDUA enzyme levels in joint tissues and serum, effecting large reductions in synovial and cartilage heparan sulfate substrate storage. Tissue histopathology and gene expression analyses are ongoing; vector bio-distribution, dose response, durability of cIDUA expression, and long-term effects upon substrate concentration and joint morphology in additional animals are being evaluated.

946. Characterization of AAV5-FVIII-SQ Vector DNA in Human Blood by Real-Time and Droplet Digital™ PCR

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Residual vector DNA is detectable in human blood for several months after dosing with BMN 270 (valoctocogene roxaparvovec), an investigational AAV5 gene therapy for severe hemophilia A. To better characterize the distribution of vector DNA in different blood compartments, whole blood from subjects treated with BMN 270 was fractionated into plasma, peripheral blood mononuclear cells (PBMCs), and red blood cells (RBCs), or left unfractionated, and analyzed by a suite of PCR assays, including quantitative real-time PCR (qPCR) for the central site of the vector genome, Droplet Digital™ PCR (ddPCR) for ITR fusion events, and drop-phase ddPCR to analyze vector genome fragment length and contiguity. Two days after infusion, only about 0.1% of originally infused vector DNA could be detected in circulation. Though the fraction of the dose found in blood varied between subjects, the cellular location and progressive degradation followed a similar trajectory across all subjects. Cell-free vector DNA in plasma was a minor component (<10%) in the first two weeks after infusion compared to whole blood vector DNA, and was highly fragmented. Two weeks after infusion, total vector DNA in blood had decreased two orders of magnitude. During this time, most vector DNA was associated with cells and undergoing rapid fragmentation, consistent with degradation by phagocytic cells. From week 4 to week 16, when vector DNA in the plasma fraction fell below the limit of quantitation in most subjects, most vector DNA was associated with the RBC fraction, where there was limited processing, while the smaller portion of vector DNA in the PBMC fraction showed ITR fusions and preferential loss of smaller DNA fragments. In contrast, the RBC fraction during this period showed <1% ITR fusion, and increasing fragmentation of larger DNA structures over time. RBC-associated DNA decreased ~3-fold in quantity between week 4 and week 16, before rapid clearance between week 16 and 24, consistent with the lifespan of RBCs. As vector DNA in the RBC fraction largely disappeared, the whole blood vector DNA structure began to resemble the structural profile found in the PBMC fraction: largely full-length vector genomes and identifiable ITR fusions suggesting relative long-term stability of circularized structures in the nuclei of a small number of long-lived PBMCs. From week 28 to week 104 total vector DNA in blood continued to decrease. These observations demonstrate that vector DNA from an AAV5 gene therapy can be detected in several cellular fractions in blood, with different fractions dominating at different periods after dose administration. It also suggests differential processing of AAV vectors in those different types of circulating cells, with most initial cell-associated vector DNA undergoing more rapid fragmentation, RBC-associated vector DNA being more slowly degraded and ultimately cleared in a time frame consistent with RBC life span, and PBMC-associated vector DNA becoming more full-length and displaying ITR fusions by week 28, after which it very slowly decreases with an ~1 year half-life.

947. Safety and Efficacy of an Artificial miRNA Targeting Huntingtin in Cynomolgus Macaques

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Huntington's disease (HD) is a devastating, incurable neurodegenerative disease affecting up to 12 per 100,000 patients worldwide. HD patients have a single mutated copy of the Huntingtin (*Htt*) gene, containing an expanded CAG repeat. Reducing the *Htt* mRNA is expected to have significant therapeutic benefits. Studies in animal models with larger brains than mice including sheep and non-human primates are better for addressing distribution and safety of huntingtin lowering reagents relevant to human therapy. In a previous study we injected a self-complementary AAV9 vector with an artificial miRNA targeting the human *Htt* mRNA into striatum of transgenic HD sheep. These sheep express the human Htt with 73 CAG repeats. We found significant reduction of 50-80% in human *Htt* mRNA and protein without evidence of neuronal toxicity up to 6 months post-injection.[1] These studies tested an allele-specific approach that kept the normal sheep huntingtin intact. However, a reduction of both mutant and WT huntingtin will occur with a non-allele specific therapy in humans. To provide information about safety and tolerability of lowering WT huntingtin, we examined the effects of volume, dose, and formulation of AAV9-miR^{HTT} or a control AAV9 vector in 3-4 year-old *Cynomolgus macaques*. Three months after bilateral injection of AAV9-miR^{HTT} into the caudate and putamen, there was a marked reduction in the striatum in Htt mRNA and protein (up to 90% protein reduction in some striatal regions). In contrast, we observed no silencing in the cortex. No effect on levels of DARPP32, a marker of striatal neurons or GFAP, a marker of astrocytes was detected. In some animals the largest volumes of either the AAV control vector or the AAV9-miR^{HTT} caused some tissue damage at the injection site. These studies provide evidence that significant lowering of WT huntingtin in the striatum of non-human primate is effective and well tolerated up to three months after delivery.

1. Pfister, E. et al. Artificial miRNAs reduce human mutant Huntingtin throughout the striatum in a transgenic sheep model of Huntington's disease. Hum. Gene Ther. (2017). doi:10.1089/hum.2017.199

948. Evaluation of a Dual Function AAV3b Vector as a Therapy for Alpha-1 Antitrypsin Deficiency in Non-Human Primates

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Alpha 1-antitrypsin deficiency (α1-antitrypsin deficiency, AATD) is a genetic disorder that is caused by the defective and decreased production of alpha 1-antitrypsin (AAT). AAT is encoded by the *SerpinA1* gene and as the name implies it is part of the serine protease inhibitor family. AAT is the second most abundant serum proteins and has high anti-tryptic activity *in vitro* and more physiologically relevant high anti-neutrophil elastase activity *in vivo*. A common mutation known as the PiZ mutation leads to a toxic-gain of function in the liver where the protein polymerizes and accumulates causing inflammation that can result in liver failure, cirrhosis and/or liver cancer. The polymerization in the liver which is the main site of production leads to inefficient secretion and low levels of AAT in circulation leading to a loss of function in the lung due to a protease/antiprotease imbalance resulting from the unopposed neutrophil elastase activity which over time causes emphysema. In a previous study, we developed a silence and replace approach consisting of the delivery of an artificial miRNA (amiR) targeted to silence Z-AAT along with an amiR-resistance M-AAT cDNA in a single dual-function AAV vector.[1] This resulted in decreased Z-AAT accumulation in the liver and a restoration of amiR-resistant wild-type AAT (M-AAT) in circulation. To further evaluate the safety of this approach along with the transduction efficiency of AAV3b, we injected 4 Cynomolgus monkeys with a dual-function vector encoding an amiRNA targeting the cynomolgus AAT sequence followed by a miR-resistant wild-type cyno-AAT sequence. Since Cynomolgus monkeys do not have the PiZ mutation, the miR-resistant cyno-AAT cMyc-tagged to track transgene expression and to facilitate differentiating between AAV-derived and endogenous protein. Animals were injected intravenously with an AAV3b vector known to target preferentially the liver at a dose of 1e13 vg/kg. Our results showed that the vector has a narrow biodistribution and preferentially transduces the liver, with an average of 40 viral genomes/diploid genomes in each lobe and significantly lower distribution elsewhere. As observed in the mouse model, we observed miR-resistant cyno-AAT transgene expression in the serum over time and in the liver at Day 90 post-injection in the liver. The delivery was well tolerated with no adverse events and the immune response analysis has shown this approach does not lead to a detectable cellular immune response to the capsid or the transgene despite a systematic neutralizing antibody response to the AAV3b capsid.
1. Mueller, C, Tang, Q, Gruntman, A, Blomenkamp, K, Teckman, J, Song, L, et al. (2012). Sustained miRNA-mediated knockdown of mutant AAT with simultaneous augmentation of wild-type AAT has minimal effect on global liver miRNA profiles. Mol Ther 20: 590-600

949. Efficient Corneal Gene Delivery Following Subconjunctival Administrations of AAV Vectors

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The ideal application of a potential therapeutic gene to the eye would preserve the patients' blood ocular barriers while allowing efficient and targeted intraocular tissue transduction. One option for the deposition of AAV vectors is via the subconjunctival (SC) space. SC injections are simple, safe, and commonly used in the clinic. The purpose of this work is to determine the transduction efficiency of periorbital and ocular tissues following the SC administration of different AAV serotypes. Preclinical investigations include the volume/dose relationship, systemic biodistribution, the humoral capsid response and efficiency of vector re-administration. SC injections were performed in healthy WTB6 mice using a Hamilton syringe on a motorized pump and a 34G needle. Escalating doses of multiple serotypes of self-complementary (sc) CMV-GFP AAV were administered to the SC space in different injection volumes of one eye. Ocular exams included pachymetry, tear production, intraocular pressure, vector genome shedding, and slit lamp examination. At 8 weeks post-injection vector genome biodistribution and transduction efficiency were characterized using qPCR and RT-qPCR. Histological examinations included H&E and GFP immunofluorescence. SC injection of AAV vectors was well tolerated at lower injection volumes; corneal ulceration was noted in 25% of subjects at the highest volume. AAV transduction was serotype dependent in the eyelid, conjunctiva, cornea, periorbital tissue, and muscle. Transgene derived cDNA in the cornea was highest for AAV6 and AAV8, however, their cellular tropism was significantly different; AAV6 specifically transduced the endothelium layer while AAV8 was restricted to the stromal layer. Systemic vector genome biodistribution demonstrated that the tested AAV serotypes were predominantly found in extraocular and ocular tissues, however, capsid-specific antibodies were generated in all cases. SC injection of AAV vectors results in serotype-dependent gene delivery to multiple extraocular tissues that would be valid targets for a variety of diseases. Unlike other injection routes, administration of vectors to the SC space did not cause damage to mechanical ocular tissue. Considering applications to diseased, or compromised states, SC administration may offer a safer route for AAV gene therapy while maintaining efficient gene delivery.

950. Integration of rAAV in Human Hepatocytes

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A common and effective method for gene therapy employs Adeno-Associated Virus (AAV) vectors, wherein replication-deficient recombinant AAV are built encoding genes of interest for use in gene repair strategies. While AAV-mediated gene therapy has enjoyed significant success, concerns regarding unintended effects remain. The primary concern is inadvertent off-target genomic integration leading to oncogenesis. Indeed, wild-type AAV sequences have been found integrated in human hepatocellular carcinoma (HCC), and neonatal mice infected with AAV delivering a promoter to the *Rian* locus developed murine HCC. Rates of recombinant AAV (rAAV) integration in human tissues are thought to be extremely low. To assess the rates of rAAV integration in human tissues of interest, we used a human chimeric mouse model where human hepatocytes repopulate the mouse liver. We have developed a method to infect human hepatocytes *ex vivo* with rAAV prior to transplantation in mice, followed by expansion of the human hepatocytes *in vivo*. After full humanization, the mouse liver was perfused and human hepatocytes recovered, then re-transplanted into new cohorts of mice with subsequent repopulation of the liver with the transplanted cells. The serial transplantation and repopulation of human hepatocytes effectively removes all episomal rAAV expression, leaving only expression from rAAV that has integrated into the genome. We used rAAV encoding the florescent dTomato and GFP genes, and following transplantation quantitated human hepatocytes that have the integrated construct by flow cytometry. The rate of integration ranged from 0.5 to 3% and was stable over three serial transplantations. We found similar rates of integration when fully humanized mice received the rAAV (*in vivo* rather than *ex vivo* hepatocyte infection) followed by serial transplantation of human hepatocytes. In order to further characterize the rAAV integration in human hepatocytes, we performed capture-sequencing using biotinylated probes specific to the rAAV vector. This method enriches for genomic DNA containing integrated rAAV genome such that relatively rare integration events can be sequenced, thus allowing the identification of specific integration sites within the human genome. These results will provide insight regarding the integration frequency and oncogenesis risk for the rAAV gene therapy field.

951. AAV-Mediated Expression of Monoclonal Antibodies for the Prevention of Marburg Virus Infection

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Vectored monoclonal antibody (mAb) expression mediated by adeno-associated virus (AAV) gene delivery is able to generate protective and sustained concentrations of therapeutic mAbs in animal models for a variety of infectious diseases, including Ebola virus and HIV. Our rationally engineered AAV6 triple mutant capsid, termed AAV6.2FF, facilitates rapid and robust mAb expression following intramuscular administration. Previously, using AAV6.2FF-mediated expression of murine IgG2A mAbs 2G4 and 5D2 in mice, we demonstrated 100% protection against Ebola virus challenge. We have now re-engineered our expression platform to produce human IgG1 mAbs comprised of the heavy and light chain variable domains of MR191 and MR78, two potent antibodies against the Marburg virus glycoprotein. Intramuscular injection of mice with $6x10^{10}$ vector genomes (vg) of AAV6.2FF-MR78 and AAV6.2FF-MR191 resulted in serum concentrations of 100μg/mL and 300μg/mL of human IgG, respectively, for sustained periods of time (>32 weeks). Mice receiving $1x10^{11}$vg (high) and $1x10^{10}$vg (low) doses of AAV6.2FF-MR191 were 100% protected against lethal Marburg virus challenge. Serum human IgG concentrations immediately before challenge ranged from 24-137μg/mL for the low dose and 383-525μg/mL for the high dose group. Additionally, mice receiving AAV-mAb vectors were able to mount an equivalent humoral immune response against a non-lethal challenge of PR8 influenza A virus as their non-AAV treated counterparts, suggesting high concentrations of systemic mAbs do not interfere with the endogenous immune response. AAV-mediated antibody gene transfer is a viable method for prolonging the therapeutic effect of recombinant mAbs, and represents a potential "vaccine" strategy for those with compromised immune systems or in possible outbreak response scenarios.

952. Increased Volume Dramatically Enhances AAV Genome Persistence, Transduction Efficiency, and Spread Following Corneal Intrastromal Injection

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Corneal gene therapy using AAV vectors represents an attractive strategy for many genetic and acquired diseases affecting vision loss including lysosomal storage diseases, corneal vascularization, Fuchs dystrophy, and dry eye disease. However, AAV vector administration to the corneal stroma is not trivial and current injection regimens frequently result in inconsistent drug distribution and unintended endothelial perforation. Therefore, initially, needles of fixed length were generated for simple and consistent macroscopic drug administration to the corneal stroma and evaluated in viable corneas ex vivo. Porcine cadaver corneas were injected axially with increasing volumes of fluorescein using a purpose-designed, fixed-depth, precise corneal injection (PCI) needle. Color images, high frequency ultrasound (HFU), and fluorescent imaging were obtained post-injection to evaluate corneal thickness (CT), fluorescein distribution, and intensity. Additionally, eyes were injected using PCI needles of various lengths to determine depths of injection by HFU. Finally, corneas were injected with an AAV8 reporter at a fixed dose in escalating volumes. GFP fluorescence was evaluated by live imaging and histology, while vector

genomes and derived cDNA were quantitated by PCR. After injection with the PCI needle, CT increased significantly with a direct correlation of volume to area infiltrated ($p<0.0001$). Depth of injection was consistent and correlated to needle length. The fixed dose of AAV8-GFP in the lowest volume resulted in earlier GFP fluorescence in a smaller area while the higher volume demonstrated later-onset GFP over a broader corneal area and a 10-fold increase in genome abundance at day 8 post-injection. Additional experiments in rabbits investigating the dose/volume relationship of AAV vector transduction are ongoing and data will also be presented. Collectively, corneal injection using PCI needles provided simple and consistent drug distribution in the cornea which allowed recognition that a fixed dose of AAV vectors administered in a higher volume increased vector genome persistence and the intensity and distribution of transduction.

AAV Vectors and Disease Targets II

953. Pre-Clinical Gene Therapy in a Mouse Model for Charcot-Marie-Tooth Type 4J

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Charcot-Marie-Tooth Disease Type 4J (CMT4J) is an ultra-rare, autosomal recessively inherited peripheral neuropathy first described in 2007. CMT4J patients are typically compound heterozygotes carrying one unique null allele of FIG4 in combination with a partial loss of function missense FIG4 allele, typically I41T. FIG4 is a PIP2 phosphatase, and its loss leads to lysosomal abnormalities and "spongiform-like" vacuolization in neurons. Clinically, CMT4J is characterized by motor developmental delay, slow nerve conduction velocities, rapidly progressive paralysis, quadriplegia, resulting in respiratory complications and premature death. Later-onset patients have a more variable disease course, with some experiencing mild symptoms. There are currently no approved therapies for CMT4J patients. To explore gene therapy as a treatment approach, we conducted preclinical studies in the *pale tremor* mouse model, which lacks Fig4 expression. These preclinical studies used a codon optimized human FIG4 gene, delivered by AAV9. Administering this vector directly to the nervous system by either intracerebroventricular (ICV) in early neonates or later, after the onset of symptoms by intrathecal (IT) injection produced benefit, as indicated by a dramatic improvement in survival. Functional and histological measures were statistically improved, including gross motor assays of grip strength, rotarod, and peripheral nerve conduction. Histopathological outcomes in spinal cord and dorsal root ganglia, as well as other parts of the central nervous system also showed improvement. Mice treated early at postnatal day 1 and 4, were nearly indistinguishable from age matched wildtype controls. Mice treated later in their disease at postnatal day 7 and 11 also showed significant improvement. Importantly, these results indicate that the benefit is greatest the earlier the FIG4-AAV9 is delivered. Preclinical study results also indicate a clear dose-response of the treatment up to the maximum feasible dose in mice, justifying the maximum feasible dose to provide the most effective treatment. Importantly, there were no observable adverse effects, even when Fig4 was overexpressed in WT mice. Overall, our results predict a benefit of this treatment to pediatric subjects, with no obvious toxicity expected, and support the further exploration of clinical trials for CMT4J patients. Funding source, Center for Precision Genetics NIH U54 OD020351

954. Novel AAVHSCs Demonstrate Efficient Crossing of the Blood-Brain-Barrier and Potential in Gene Therapy for Metachromatic Leukodystrophy (MLD)

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Adeno-associated viruses (AAVs) have emerged as key viral-based delivery vehicles for gene therapy in the nervous system due to their stable transgene expression in post-mitotic cells, neuronal tropism, lower risk of insertional mutagenesis and diminished immune response. We have reported the identification of novel AAVs derived from human hematopoietic stem cells (AAVHSCs). These novel AAVHSCs map to AAV Clade F and have demonstrated to effectively cross the blood-brain-barrier (BBB) following intravenous (IV) delivery in non-human primates, thus creating the potential for therapeutic applications in treating human genetic diseases of the central nervous system (CNS). 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. Most commonly, MLD is 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) in the brain, spinal cord and peripheral organs, which become toxic. This excess in sulfatides leads to the destruction of myelin, a key protective layer of the nerve fibers. Herein, we are reporting preclinical gene therapy data in a murine model of MLD where a single intravenous dose of AAVHSC15 expressing human *ARSA* (*hARSA*) led to 30-115% of normal hARSA enzyme activity levels in the murine CNS, exceeding the established therapeutic target of 10-15%. Moreover, the AAVHSC15-hARSA expression patterns were detected in key biologically relevant regions of the brain (brainstem, cortex, cerebellum and white matter tracks) and spinal cord (gray matter and ascending white matter tracks of the posterior column) in both neuronal and glial cellular profiles. Based on these preclinical data, IND-enabling studies of CNS gene therapy development candidate HMI-202 have been initiated in MLD.

955. Whole Brain Delivery of an Instable MeCP2 Transgene Fully Protects from the Behavioral and Molecular Pathological Defects in Mouse Models of Rett Syndrome

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Neuroinfantile disorders with global impairment of brain functions represent a significant challenge for gene-based therapies. However, the recent introduction of the PHP.eB engineered capsid endowed with an unprecedented efficiency in crossing the blood-brain barrier after intravenous injection has provided an invaluable vehicle for gene transfer in the nervous system. Rett syndrome (RTT) is an incurable neurodevelopmental pathology caused by mutations in the gene methyl-CpG binding-protein 2 (MECP2). Given some inherent hurdles like the diffuse expression of MeCP2 in brain tissue and that its gene duplication leads to a severe neurological condition, the attempts to develop gene therapy strategies has been delayed in time and initiated only recently. We designed an instable MeCP2 (iMeCP2) transgene cassette which promotes physiological MeCP2 protein levels in transduced neural tissues by increasing RNA destabilization and inefficient protein translation of the viral MeCP2 gene. Intravenous injections of the PHP.eB-iMeCP2 virus in symptomatic male and female MeCP2 mutant mice resulted in a complete protection from disease progression with improved locomotor activity, coordination, lifespan and normalization of altered gene expression, epigenetic deficits and mTOR signaling. PHP.eB-iMeCP2 administration was safe in female MeCP2 mutant and wild-type animals also at very high viral doses with only a marginal alteration of MeCP2 protein levels throughout the brain. In contrast, we reported for the first time a strong immune response to the transgene in treated male MeCP2 mutant mice that was prevented under immunosuppressive regimen. Combining PHP.eB-mediated widespread brain delivery with physiological MeCP2 protein levels promoted by the iMeCP2 cassette defines a novel viral system with strong therapeutic efficacy and increased levels of safety holding important clinical implications for RTT.

956. Systemic Delivery of AAV9.LAMP2B for the Treatment of Danon Disease: Toxicology Studies in Mice and Cynomolgus Monkeys

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Danon disease (DD) is a rare and devastating X-linked inherited disorder that is characterized by severe cardiomyopathy (which is the cause of extensive early morbidity and mortality), skeletal myopathy and mild cognitive impairment. DD is caused by mutations in the gene encoding lysosomal-associated membrane protein 2 (*LAMP2*), an important mediator of autophagy. Although there are three distinct isoforms of the *LAMP2* gene (*LAMP2A, 2B, 2C*), *LAMP2B* has been identified as the essential isoform in DD pathogenesis. There is no definitive treatment for DD; current standard of care includes cardiac transplant, which is available selectively and not curative. There is a significant unmet need for novel and relatively safe treatments, such as gene therapy. In preparation for a clinical trial, we performed a GLP-toxicology study in mice to assess safety of systemically-delivered AAV9-mediated human LAMP2B (AAV9.LAMP2B) gene therapy. Male and female wildtype C57BL/6 mice were intravenously (IV) injected with AAV9.LAMP2B at 3×10^{13}, 1×10^{14}, and 3×10^{14} vg/kg and assessments were performed post-mortem on day 30 and day 91. Mice receiving the highest dose were also assessed at 6 months post-injection. Importantly, no treatment-related adverse signs were seen through the six-month timepoint; no significant toxicology or detrimental findings were noted for body weights, food consumption, ophthalmology, clinical pathology, and organ weights. Additionally, no test article-related gross or microscopic findings were noted at necropsy on study days 30, 91, and 180. The vector delivery resulted in increased neutralizing antibody (NAb) titers, and characteristic binding antibody response to AAV9 capsid, but no significant antibody response was noted to *LAMP2B* transgene, with no detectable toxicity or diminished efficiency at the day 91 timepoint. Differential biodistribution of vector genomes and transgene mRNA was detected in various tissues in a dose-dependent manner in mice receiving an IV vector injection versus PBS-control animals at the 30- and 91-day timepoints. mRNA levels were highest in heart and liver tissue followed by lower levels in skeletal muscle and brain. Transgene mRNA was also noted in gonads. To further examine safety and predictability of the drug product prior to initiation of clinical studies, we conducted a 102-day toxicity study in non-human primates (NHPs; cynomolgus monkeys) at the highest dose that was tested in the mouse toxicology study (3×10^{14} vg/kg). Intravenous administration of AAV9.LAMP2B was well tolerated in the NHPs and no significant physical, behavioral, hematologic, or biochemical abnormalities were observed at various timepoints up to the sacrifice day 102. No test article-related gross pathology or microscopic aberrations were detected. An increase in serum NAbs to AAV9 was observed in the AAV9.LAMP2B-treated NHPs as expected. Overall, no adverse events associated with IV administration of AAV9.

LAMP2B, including the highest evaluated dose of 3×10^{14} vg/kg, were observed in the mouse or cynomolgus monkey studies supporting the safety of systemically-delivered AAV9.LAMP2B gene therapy.

957. Assaying Patterns of rAAV Integration in Humanized Mice: Non-Random Integration Targets Genes, Especially Exons

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The controversy surrounding possible AAV genotoxicity highlights the need for comprehensive studies to determine rAAV integration profiles in human hepatocytes. Although post-treatment liver biopsy specimens represent the ideal samples for understanding possible genotoxic effects related to rAAV, the practical limitations of acquiring such samples in the setting of human gene therapy has necessitated the development of an alternative approach to study rAAV integration patterns. We have therefore used an FRG mouse model to gain insights into rAAV integrations and possible genotoxicity. Young adult *Fah*$^{-/-}$,*Rag*$^{-/-}$, *Il2rg*$^{-/-}$mice were repopulated with control human hepatocytes to create humanized mice with chimeric human/mouse livers. These mice were infected with either AAV8 (n=1) or AAV9 (n=3) CBA GFP reporters, allowing us to simultaneously explore *in vivo* rAAV integration into both the mouse and human genomes in the chimeric livers. We adapted an integration-capture technique to isolate and sequence rAAV integration sites (IS) and modified existing software to filter and map the reads to either the mouse or human genome. In order to assess the statistical significance of our findings, we created *in silico* simulated random IS to match each of the experimentally determined sites. For both mouse and human, we created 10,000 simulated datasets, each with the same number of mock IS as were in the experimental datasets. We analyzed the genomic characteristics of the mapped AAV integrations in human and mouse and calculated empirical *p*-values based on the simulated datasets.After combining all four liver sample results, we identified 1300 unique rAAV integrations in the human genome and 2827 in the mouse genome. In both the human and mouse datasets, we found higher than expected ($p < 0.0001$) numbers of integrations within +/- 5 kilobases (kb) of genes, consistent with previous observations. Of the 1300 human IS, 965 (74%) occurred within +/- 5 kb of a gene; 103 of those integrations were within exons, and almost half (n=48) of those integrations within the first exon (all with $p < 0.0001$). Integration in the mouse genome displayed a similar pattern, with 64% of IS mapping within +/- 5 kb of a gene, and more than one-third of exonic integrations occurring in exon 1 (all with $p < 0.0001$). A total of 82 of the genes with IS within +/- 5kb were found independently in both the human and mouse genomes. Looking separately at the four liver samples for common patterns of integration, we found that 22 mouse genes and 7 human genes had nearby IS in all four samples. With a few exceptions, we found similar numbers of integration events per chromosome when we compared the human and mouse datasets to the simulated data. However, we found fewer integrations than expected on human chromosomes 2, 3 and 13 and more integrations than expected on human chromosomes 22 and X (all with $p \leq 0.005$). Further examination of human chromosome X revealed a hotspot containing 93 integrations within 470 nucleotides. In addition, the number of *in situ* events were significantly higher in both datasets. In mouse, we found 304 instances of two integration events separated by a maximum distance of 50kb, and in human, a total of 88. In the 10,000 simulated datasets, the maximum number of times we observed two IS separated by 50kb, in a single dataset, was 185 and 49 in mouse and human, respectively. Taken together, these results indicate both a preference for rAAV integration in genes, especially exons, as well as a clustering tendency for integration site events, suggesting a non-random integration pattern for rAAV in human and mouse hepatocytes.

958. AAV Gene Therapy in a Canine Model of MPS1 Prevents and Reverses Corneal Blindness

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Mucopolysaccharidosis type 1 (MPS1) is a progressive lysosomal storage disease which can be lethal in the first decade of life. Cell therapy through bone marrow transplantation is able to prolong life by decades, however, non-lethal MPS1 manifestations significantly decrease the patient's quality of life. In particular, approximately 90% of MPS1 patients present corneal opacity with about 50% of cases leading to complete vision loss. There is currently no effective treatment for MPS1 corneal blindness, as corneal transplantation elicits a high incidence of rejection. To address the deficits of cell therapy for MPS1, an AAV corneal IDUA gene addition approach was investigated for treatment of MPS1 corneal blindness. After validation in MPS1 patient cells and human corneas ex vivo, efficacy was explored in MPS1 canines, which demonstrate corneal vision loss as well. Intrastromal AAV-IDUA injections were relatively difficult with variable drug dispersion, however, were well tolerated in presymptomatic and diseased corneas. In the AAV-IDUA treated corneas, clearing of storage was evident as early as 1-week post-injection the kinetics of which correlated to the vector dose. Extensive vascularization observed in advanced disease was also cleared, while corneas injected at early disease remained vascular free during the follow-up. In contrast, the contralateral corneas injected with a control vector continued to show disease progression with no sign of reversal or prevention of storage disease. In post-symptomatic corneas, AAV-IDUA administration resulted in clear corneas observed until the humane endpoint despite the occurrence of transient corneal edema observed in either the IDUA or control treated eye. Post-mortem analyses demonstrated reduced corneal thickness, corneal vascularization, high IDUA abundance, alpha smooth muscle actin staining, and H&E pathology grading was nearly completely corrected. In the second cohort of experiments in younger, largely presymptomatic, MPS1 canines AAV-IDUA vector injections also resulted in clear corneas even at the lowest dose of 1e9 viral genomes. Again, transient corneal edema was observed in a

subset of patients and a longer observation period demonstrated signs of storage disease returning in AAV-IDUA injected corneas that were previously corrected. Vector biodistribution studies detected genomes in particular peripheral organs however, the majority of subjects did not present capsid neutralizing antibodies following treatment. Collectively, the results demonstrate that AAV-IDUA corrects MPS1 corneal disease and although a transient edema was encountered in a subset of canines, it remains a likely possibility that prior hematopoietic stem cell transplantation, which tolerizes MPS1 patients to human IDUA, may eliminate these suspected immunological concerns resulting in complete therapy.

959. Towards AAV5-Mediated Gene Therapy for Hemophilia A with a Factor IX Variant that Functions Independently of FVIII

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Up to approximately 30% of patients with severe hemophilia A develop inhibitors that prevent efficacy of replacement therapies with infused blood coagulation factor VIII (FVIII) products. As a result, these patients are dependent on an on-demand therapy with bypassing agents such as activated prothrombin complex, activated recombinant factor VII, or a bispecific antibody linking FIX(a) to FX(a). Previously developed factor IX (FIX) variants demonstrating cofactor-independent activity are capable of promoting coagulation in the absence of FVIII both *in vitro* and *in vivo.* Experiments in hemophilic mice have shown that administration of DNA minicircles encoding these novel FIX variants resulted in FVIII-independent coagulation (Quade-Lyssy et al. *J. Thromb. Haemost.* 2014). However, the use of DNA minicircles by hydrodynamic injections is not suitable for human application due to the harsh procedure and transient expression of the transgene. Therefore, a powerful approach would be to investigate the feasibility of delivering these FIX variant transgenes using AAV5 vectors. Two FIX variants (FIX-FIAV and FIX-IDAV) were selected, in which four amino acid substitutions lead to their FVIII-independent function. Once activated, these variants most likely negate the requirement for FVIII by resembling the conformation of cofactor-mediated activated FIX (FIXa). *In vivo* assessments of the AAV vectors were performed in wild-type and hemophilic mice as well as in non-human primates. Administration of the AAV vectors to mice and non-human primates was well-tolerated and did not show possible induction of thrombogenicity following analysis of coagulation activation markers in plasma. Most importantly, the obtained transgene expression levels in the non-human primates will translate to therapeutic meaningful FVIII mimetic activity in patients. Our data support that AAV5 gene therapy development based on a FIX variant is feasible and indicate that therapeutic relevant FVIII mimetic activity could be achieved in hemophilia A patients using this approach.

960. Development of an AAV5-Based Gene Therapy for Fabry Disease

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Fabry disease is an X-linked hereditary metabolic disorder that is caused by a mutation in the alpha-galactosidase A (*GLA*) gene (Brady et al., 1967; Kint, 1970; Sweeley and Klionsky, 1963). The GLA enzyme is involved in degradation of glycolipids, specifically globotriaosylceramide (Gb3). In Fabry patients there is a continuous accumulation of Gb3 and its deacylated derivative globotriaosylsphingosine (lysoGb3) in cells and plasma, resulting in cell abnormalities and organ dysfunction particularly affecting heart and kidney (Aerts et al., 2008). The current treatment for Fabry disease is enzyme replacement therapy (ERT) that successfully improves kidney and heart pathology and the patients' quality of life. However, ERT also has disadvantages, including poor stability in blood, low incorporation efficiency into target organs, high costs and the production of anti-GLA antibodies by some patients. Moreover, it is estimated that 55-83% of the ERT-treated patients develop antibodies against GLA (Schiffmann et al., 2006; Wilcox et al., 2004). To prevent the adverse effect of the current ERT, the α-N-acetylgalactosaminidase (NAGA) protein showing high structural resemblance with GLA was modified. The NAGA substrate specificity was changed to exert GLA activity by introducing mutations in the active site (Tajima et al., 2009). This modified NAGA protein has been shown to cleave accumulated Gb3 in cultured fibroblasts from Fabry patients, as well as in Fabry diseased mice resulting in prevention of Gb3 storage in liver, kidney and heart (Tajima et al., 2009). We are currently developing a gene therapy for Fabry disease by expressing this modified NAGA from AAV5 vectors. Different strategies are being explored that either entail liver-targeted expression of the modified NAGA that upon expression in the liver will be secreted and taken up by the target cells into the lysosomes, or constitutive expression throughout multiple organs (depending on the tropism of the AAV5 vector together with the promoter for expression). Six AAV vectors expressing modified NAGA were developed with distinct design and two different promoters. *In vitro* all constructs were shown to result in GLA activity in transduced liver cells. Administration of the AAV vectors in C57Bl/6 mice resulted in 10 and 20-fold higher GLA activity in plasma and liver, respectively, compared to vehicle-treated mice. In addition, AAV5-modified NAGA administration to Fabry *GLA* knockout mice showed significant lowering of Gb3 and LysoGb3 in

plasma already at 2 weeks post-injection. Overall, the use of AAV gene therapy expressing modified NAGA with GLA-activity warrants further investigation as an attractive approach to treat Fabry patients.

Nuclease Mediated Genome Editing

961. Precise Gene Editing Preserves Hematopoietic Stem Cell Function Following Transient p53-Mediated DNA Damage Response

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Gene editing in Hematopoietic Stem/Progenitor cells (HSPC) holds the promise to provide a safe and effective cure for several blood-related disorders. Yet, despite its tremendous therapeutic potential and the continuous advances in perfecting gene editing platforms, little is known about the cellular responses triggered by programmable nucleases and viral vectors in *ex-vivo* edited HSPC and their functional consequences. Nuclease-dependent gene editing generates DNA double strand breaks (DSB) that trigger DNA Damage Responses (DDR) and may negatively affect the engraftment and long-term repopulation capacity of the edited HSPC. Similarly, the impact of AAV6 as preferred source of DNA template for Homology-Directed repair (HDR) in HSPC remains poorly investigated. Here, we induced site-specific breaks by state-of-the-art CRISPR/Cas9 and Zinc Finger nucleases with high and low specificity profiles and used AAV6 as vehicles for HDR donor DNA template, to study their impact on HSPC biology and functionality. We first characterized the DDR triggered by the two different editing platforms across 2 therapeutic relevant genomic loci (*IL2RG* and *AAVS1*) using molecular and cell proliferation analyses, showing that nuclease specificity and not the choice of platform is the major driver of DDR kinetics and extent. Then, in order to study the transcriptional changes induced by gene editing in an unbiased manner and interrogate individual cells within the heterogeneous stem/progenitor population, we performed single-cell RNA-Seq on treated CD90+ and CD90- primitive cell subpopulations (>15.000 cells). This analysis uncovered several extents of DDR activation that were consistent across different HSPC types/states, but none of the treatment induced a skewing in the distribution of HSPC clusters, suggesting that the transcriptional identity of the edited cells is preserved. P53 pathway activation and downregulation of cell-cycle progression resulted the predominant/almost-only detectable responses to the DNA DSB and were virtually harmless to HSPC when using highly specific nucleases transiently acting on the single/few intended target sites. Strikingly, the extent of the cellular responses substantially increased with lower specificity reagents or when employing AAV6 transduction for HDR-mediated editing. Specifically, nuclease-induced DNA DSB and AAV6 transduction converged to the p53 pathway and led to a more robust activation of its downstream effectors, up to the activation of pro-inflammatory programs and innate immune responses, partially affecting the yield of *in-vitro* clonogenic and *in-vivo* repopulating edited HSPC. Finally, we devised a strategy to counteract any potential adverse effect on HSPC function by delivering a mRNA encoding for a dominant-negative p53-peptide during the editing process. Despite the transient p53 inhibition obtained in our experimental condition was partial and detectable only within 24 hours post treatment, this strategy increased the efficiency of HDR (>40% in primitive CD90+ cells) and the output of edited HSPC with *in-vivo* repopulating capacity (2-fold over control), as measured by serial transplants into NSG mice. Notably, this incomplete and highly transient p53 dampening did not negatively affect the genome integrity of the treated HSPC, thus it did not detectably aggravate the genotoxicity risk of the gene editing procedure. Overall, these findings provide molecular evidence of the feasibility of seamless targeted gene editing in HSPC, giving confidence to its prospective translation in humans.

962. Ex Vivo Editing of Hematopoietic Stem Cells for Erythroid-Specific Expression of Therapeutic Proteins

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Genome editing can correct the underlying disease-causing mutation of virtually any genetic disease; however, insufficient expression from the endogenous promoters and the need of countless mutation-specific editing strategies represent major obstacles. Previous studies identified the human albumin gene as an ideal locus to achieve high liver-driven expression of integrated transgenes. Unfortunately, this approach is hampered by pre-existing immunity, as it relies on in vivo delivery of nucleases and donor DNA, and when the liver is compromised. 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 (HSPC). Specifically, by inserting transgenes under the control of the endogenous α or β-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 erythropoiesis. As a single erythroid progenitor can generate more than 10^6 erythroblasts, 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 evaluated the feasibility of the approach, we optimized its efficiency in HSPC and established its safety.First, using CRISPR/Cas9 we identified non-coding regions in α and β-globin genes, where the integration of a promoterless GFP cassette resulted in reporter expression in both K562 erythroleukemia and HUDEP2 erythroid progenitor cell lines. By replacing GFP with human transgenes, we demonstrated efficient expression of transmembrane proteins, secreted clotting factors and soluble lysosomal enzymes. Remarkably, all transgenes were expressed at high level (~100 fold more compared to hPGK promoter) and were

upregulated during erythroid differentiation (~100 fold). These results were further confirmed in single cell clones by extensive molecular analyses (PCR and digital droplet PCR, ddPCR).We then optimized nuclease and GFP transgene delivery for editing HSPC, reaching >50% of targeted events (ddPCR) using Cas9 ribonucleoprotein and an AAV6 donor DNA. As expected, GFP recapitulated hemoglobin expression patterns during erythroid differentiation and in colony-forming cell (CFC) assay. Notably, we did not observe any major alteration in erythroid markers expression or enucleation in edited cells. Additional experiments of targeted integration of therapeutic transgenes in HSPC and transplantation of edited HSPC in immunodeficient mice are ongoing. Finally, to address the safety of this approach, we studied both the effect of targeted integration on globin expression and potential nuclease off-targets. First, we confirmed that erythroblasts derived from edited HSPC maintained globin expression, although reduced compared to unmodified HSPC, validating our nuclease targets as safe harbors for targeted integration. Then, we verified that the selected CRISPR/gRNA are highly specific, with undetectable off-target events (1% 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, such as hemophilia and inherited metabolic disorders.

963. CRISPR/Cas9 Fusion to Dominant Negative 53BP1 Confers High Efficiency HDR-Based Genome Editing and Inhibits NHEJ Only at Cas9 Target Sites

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The CRISPR/Cas9 system allows for precise genome engineering by homology-directed repair (HDR) at a site-specific double strand break (DSB) when a donor template is provided. However, HDR is out-competed by the more efficient non-homologous end-joining (NHEJ) DNA repair pathway, which resolves the DSB with base insertions or deletions, resulting in gene knockout. To improve the efficiency of HDR, global cellular inhibition of NHEJ has been attempted, but this approach also inhibits the cell's ability to repair its naturally occurring DSBs, resulting in toxicity and potential mutagenesis. Here, we designed a Cas9 fused to a dominant negative (DN) form of human tumor suppressor p53-binding protein (53BP1) to promote HDR and reduce NHEJ specifically at the Cas9-induced DSB, thus leaving cellular NHEJ repair intact. Following a DSB, 53BP1 is one of the first proteins recruited in NHEJ-mediated repair, which recruits downstream effectors of NHEJ and blocks end-resection required for HDR to occur. We developed an optimal 53BP1 dominant negative construct, termed DN1S, where we removed domains that recruit NHEJ effectors and retained the minimal focus forming region (FFR) required for recruitment to DSB. Immunofluorescence confirmed that DN1S was recruited to DSB-induced DNA repair foci, displaced endogenous 53BP1 ($p<0.05$), prevented binding of NHEJ protein RIF1 ($p<0.0001$), and increased recruitment of HDR protein BRCA-1 ($p<0.001$). Next, we fused DN1S to Cas9 (Figure 1a) and used a FACS-based reporter system to assess DSB repair outcomes. We found that Cas9-DN1S increased HDR by 3-fold ($p<0.0001$) and decreased NHEJ by 2-fold ($p<0.0001$) over Cas9. Impaired NHEJ repair is toxic to cells and sensitizes them to low dose irradiation. We showed that global inhibition of NHEJ by NU7441, 53BP1 shRNA, or DN1S alone resulted in increased sensitization of cells to irradiation-mediated apoptosis, while Cas9-DN1S was similar to controls ($p<0.05$). Using the Cas9-DN1S model across different cell types and loci, using both SpCas9 and SaCas9, we found HDR to be significantly improved by ~2-fold ($p<0.01$-0.001), ranging from 22% to 82% HDR, and NHEJ to be significantly reduced by 2-4-fold ($p<0.01$). We next targeted a human CD18 CDS to the AAVS1 locus to correct EBV-immortalized B lymphocytes from a patient with leukocyte adhesion deficiency (LAD). Cas9-DN1S resulted in 45% CD18+ cells, with 57% of these cells with bi-allelic HDR. In contrast, with Cas9 alone, only 23% of cells were CD18+, with 6% bi-allelic HDR (Figure 1b,c). Furthermore, there was a significant reduction in NHEJ to 7% with Cas9-DN1S, versus 30% NHEJ with Cas9 alone. In conclusion, we have developed a CRISPR/Cas9-DN1S system that results in site-specific reduction of NHEJ and improvement in HDR at multiple loci using different Cas9 nucleases, resulting in overall improved efficacy and safety of Cas9 editing strategies that can be translated for disease correction.

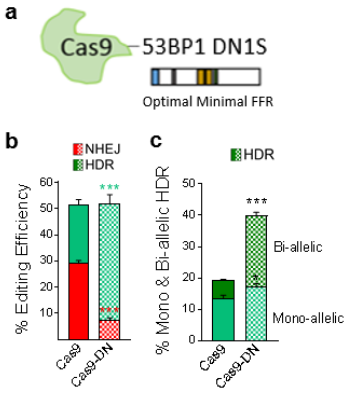

Fig. 1. Fusion of 53BP1 DN1S construct to Cas9 improves HDR-based gene editing.

a. Schematic of dominant negative 53BP1 protein fused to Cas9.
b. Quantification of HDR efficiency by FACS and NHEJ efficiency by TIDE assay of Cas9 or Cas9-DN1S in EBV immortalized B cells from a patient with LAD. Data are presented as mean ± SEM; n=3; *** $p<0.001$.
c. Relative frequencies of mono- and bi-allelic HDR frequency detected by MFI of CD18 staining by FACS and confirmed by PCR. Data are presented as mean ± SEM; n=3; * $p<0.05$, *** $p<0.001$.

964. Site Specific Knock-In Genome Editing in Human HSCs Using Baboon Envelope gp Pseudotypedviral Derived "Nanoblades" Loaded with Cas9/sgRNA Combined with Donor Encoding AAV-6

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Programmable nucleases have enabled rapid and accessible genome engineering in eukaryotic cells and living organisms. Here, we have designed "Nanoblades", a new technology that will deliver a genomic cleaving agent into cells. These are genetically modified Murine Leukemia Virus (MLV) or HIV derived virus like particle (VLP), in which the viral structural protein Gag has been fused to the Cas9. These VLPs are thus loaded with Cas9 protein together with the guide RNAs. Thus, nanoblades are devoid of any viral-derived genetic material. Highly efficient gene editing was obtained in cell lines, IPS cells and primary mouse and human cells (REF 1). However, their delivery into target cells can be technically challenging when working with primary immune cells. Here, we showed that nanoblades were remarkably efficient for entry into human T, B and hematopoietic stem cells thanks to their surface co-pseudotyping with baboon retroviral and VSVG envelope glycoproteins. We were able to induce efficient, transient and very rapidly genome-editing in human induced pluripotent stem cells reaching up to 70% in the empty spiracles homeobox 1(EMX1) and muscular dystrophy (MD) gene locus. A brief nanoblade incubation of primary human T and B cells resulted in 40% and 20% editing of the Wiskott-Aldrich syndrome(WAS) gene locus, while hematopoietic stem cells treated for 18 h with nanoblades allowed 30-40% gene editing in the WAS gene locus and up to 80% for the Myd88 genomic target. Moreover, for the HIV- and MLV-derived nanoblades no cell toxicity and low to undetectable off-target effects were demonstrated. Finally, we also treated hHSCs with nanoblades in combination with an AAV-6 donor encoding vector resulting in over 20% of stable expression cassette knock-in into the WAS gene locus. Currently, we are evaluating these gene modified HSCs for their long-term reconstitution of NOD/SCIDgammaC-/- mice. Summarizing, this new technology is simple to implement in any laboratory, shows high flexibility for different targets including primary immune cells of murine and human origin, is relatively inexpensive and therefore have important prospects for basic and clinical translation in the area of gene therapy. **Ref 1:** P.E. Mangeot, V. Risson, F. Fusil, A Marnef, E. Laurent, J. Blin, V. Mournetas, E. Massourid, T. J. M. Sohier, A. Corbin, F Aub, C. Pinset, L. Schaeffer, G. Legube, FL. Cosset, E.Verhoeyen, T. Ohlmann, E. P. Ricci. Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Nature communications, 2019 Jan 3;10(1):45.

965. CRISPR/Cas9-Engineered Transgenesis of Hematopoietic Stem Cells via NHEJ-Mediated Targeted Integration Retains Engraftment Potential

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The ability to edit the genome of long-term-engrafting hematopoietic stem cells (LT-HSCs) would provide a curative therapy for a wide range of hematological disorders. While mixed LT/ST-HSC (ST, short-term) pools edited via non-homologous end joining (NHEJ) yield engraftable transgenic cells, pools made transgenic via homology-dependent repair (HDR) do not. This observation is likely explained by the absence of DNA replication required for HDR in LT-HSCs. Quiescence renders LT-HSCs refractory to transgene insertion via HDR, with the transgene-positive population consisting overwhelmingly of actively dividing, short-term-engrafting HSCs. In contrast to HDR, NHEJ is a DNA repair mechanism active in non-dividing cells. We hypothesized that CRISPR/Cas9-mediated transgenesis of HSC pools via NHEJ-mediated targeted integration (NHEJ-TI) would allow modification of LT-HSCs and produce edited, long-term engrafting progeny. First, we investigated whether NHEJ-TI is a feasible method of genome editing in HSCs. Utilizing CRISPR/Cas9 to induce a DSB in the *AAVS1* (*PPP1R12C*) locus and a self-complementary rAAV6 (scAAV6) to deliver a GFP expression cassette dependent on the endogenous promoter, we optimized RNP nucleofection, scAAV6 infection, and HSC culture conditions for efficient NHEJ-TI in HSCs. Flow cytometry for eGFP confirmed transgene integration at frequencies ranging from 4-6%. Droplet digital PCR of the 5' junction of integrated transgene into the AAVS1 locus corroborated the flow cytometry results. The addition of the endogenous protospacer sequence flanking the donor transgene decreased integration of the AAV ITRs while increasing the integration rate of the donor sequence in the correct orientation. Next, we investigated whether the NHEJ-TI edited pool of mixed LT/ST-HSCs contained engraftable, edited cells by injection into irradiated NOD/SCID/IL2Rg$^{-/-}$ (NSG) mice. We compared the engraftment potential of transgenic HSCs cultured for either 2 days (2D) or 2 hours (2H) prior to editing. While the overall editing efficiency was higher in the 2D condition versus the 2H condition (GFP+ of hCD34+: 3.07%, 2H; 2.58%), the near absence of cell pre-stimulation in the 2H condition resulted in increased levels of overall engraftment, doubling the total engraftment level of edited hematopoietic cells at 16 weeks post-injection. Importantly, the percentage of edited cells among the engrafted human hematopoietic cell population remained essentially unchanged in the peripheral blood over the course of 16-weeks post-injection (GFP+ of hCD45+ population: 2D; 4.64±0.33%, 2H; 3.03±0.53%). Analysis of bone marrow-resident human CD34-positive cells at 16 weeks post-injection reveal the persistence of edited LT-HSCs (GFP+ of hCD34+ population: 2D; 4.43±0.49%, 2H; 2.65±0.80%). Our results show that NHEJ-TI is a feasible method of inserting transgenes into LT-HSCs whilst retaining their engraftment potential. To our knowledge, this is the first study to achieve successful NHEJ-mediated targeted integration in HSCs while retaining engraftment potential and transgenesis.

966. Genome Editing by Clade F AAV Homology Donors in the Presence and Absence of Site-Specific DNA Breaks

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Homology-dependent genome editing is commonly achieved using a 2-component system, whereby targeted nucleases such as CRISPR/Cas9 or ZFNs create a site-specific DNA break, which is then repaired using a homologous DNA donor template. Adeno-associated viral (AAV) vectors are frequently used as homology donors, with serotype AAV6 used for *ex vivo* editing of human hematopoietic cells, and serotypes 6, 8, and 9 used for *in vivo* applications in muscle and liver. A recent report suggested that AAV vectors containing capsids from certain clade F serotypes (AAV9 and 'AAVHSC' sequences that were isolated from human hematopoietic stem cells) could also mediate efficient editing in the absence of a targeted DNA break (Smith et al. 2018, PNAS), which would greatly simplify the genome editing process. To further examine this property, we packaged donor genomes with homology for the human CCR5 or AAVS1 loci into AAV vectors using capsids from AAV6, AAV9 or AAV9-G505R, which contains the reported amino acid sequence of AAVHSC13 and AAVHSC17 (Smith et al. 2014, Mol Ther). Vectors were generated with a typical 3-plasmid transfection system using AAV2 Rep and ITRs. The donor cassettes contained either an exogenous promoter (CCR5-PGK-GFP), so that initial gene expression would be independent of genome editing, or a promoterless construct similar to that used by Smith et al. (2018), whose expression would require insertion downstream of a promoter and splice donor (AAVS1-SA-2A-GFP). In K562 cells transduced with the CCR5-PGK-GFP series of vectors, all populations initially contained GFP+ cells, but rapidly lost GFP expression to be <0.5% GFP+ after 14 days. Only when CCR5-specific ZFNs were also provided, by mRNA electroporation, was stable GFP expression observed, at rates of 57.4% (AAV6), 11.8% (AAV9), and 6.4% (AAV9-G505R). The final gene editing rates strongly correlated with transduction rates (GFP expression) and vector copy number (by ddPCR) at day 2. Site-specific insertion was confirmed using a specific in-out PCR assay and detected only in the nuclease-treated samples. Similarly, for the promoterless AAVS1 homology donor, site-specific gene insertion and GFP expression were dependent on the co-delivery of a matched nuclease. Finally, we performed experiments in primary human CD34+ HSPCs, which were reported to support high rates of genome editing by clade F AAV donors in the absence of a nuclease (Smith et al., 2018). As we and others have reported, AAV9 does not transduce HSPCs well (~0.5% GFP+ at day 2 with the CCR5 donor), and GFP expression by day 10 had fallen to <0.1%, but could be stimulated by the matched CCR5 nuclease (0.67%). The G505R substitution further reduced HSPC transduction rates and did not support any detectable nuclease-independent editing. Similarly, the series of AAVS1-SA-2A-GFP vectors were completely dependent on a matched ZFN to achieve sustained GFP expression. In summary, efficient site-specific gene insertion in K562 cells and HSPCs only occurred in the presence of a matched targeted nuclease, including for clade F AAVs. Genome editing rates were also highly correlated with transduction efficiency, suggesting that the ability for AAV to serve as a homology donor is likely a generalizable phenomenon and that tropism for the target cells should be the most important consideration when choosing an AAV serotype for gene editing applications.

967. HDR-CRISPR: A Novel System to Promote Cas9-Mediated Homology Directed DNA Repair

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In the last decade, CRISPR-Cas9 has emerged as a powerful and easy-to-use system to perform genome engineering. It has been widely used to induce targeted DNA double-strand breaks (DSBs) at precise genomic locations with the aim of harnessing the error-prone non-homologous end-joining (NHEJ) DNA repair mechanism to induce targeted gene knockouts for multiple applications on the one hand. On the other hand, precise genome modifications based on homology-directed repair (HDR) remain a compelling application which might open new opportunities, in particular for the full exploitation of the therapeutic potential of CRISPR-Cas systems. HDR can repair DSBs in a scarless manner using the sequence of a properly designer exogenous DNA donor as a repair template. However, NHEJ predominates over HDR in mammalian cells, resulting in poor efficiency of precise genome editing, far below the desired clinically relevant frequencies. This has restricted clinical exploitations of the CRISPR-Cas systems to NHEJ-mediated gene knockout thus far. To increase the low rate of HDR-based gene editing, other strategies have been adopted, like the use of chemicals to synchronize the cells in the S/G2 cell cycle phase when the HDR pathway is most active or the use of compounds that inhibit NHEJ. While effective in some systems, both strategies might pose safety concerns if applied in clinically relevant cells. To address this issue, we aimed at increasing the local concentration of one - or more - factor(s) involved in HDR at the site of DSB to drive the cell to engage HDR rather than NHEJ for genome editing. Based on the known complex network of factors that drive HDR-mediated DSB repair, we identified eight candidates which are critical in DSB repair pathway choice. We generated 11 different Cas9-fusion proteins (referred to as HDR-CRISPRs) and investigated which of the two main DNA repair pathways was engaged by the cells 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 between the coding sequences of the two fluorescent proteins, NHEJ-mediated repair will result in Tag-RFP expression in about 30% of the repair events. If the break is resolved via HDR with an exogenous, corrective DNA template, the mVenus sequence is restored and the cells turn green. We extensively assayed single or double fusions of the selected HDR-related factors. Our best-performing HDR-CRISPR resulted in up to 3-fold increase in HDR-mediated repair of the induced DSB. Interestingly, in some instances, NHEJ-mediated repair was reduced in the presence of the DNA donor, suggesting that DNA repair pathway choice is influenced both by the availability of homologous DNA sequences and by the on target positioning of specific HDR factors. These results support our hypothesis that the availability of key HDR-promoting factors at the site of the DSB affects the DNA repair pathway choice exploited by the cell to repair the lesion. In conclusion, our novel HDR-CRISPR

technology directs the decision of how a DSB is repaired towards HDR without altering the physiology of the target cells. We envision that this strategy is readily translatable to clinically relevant systems, opening new opportunities for the future establishment of CRISPR-Cas-mediated precise genome editing for clinical applications.

968. Advances in Genome Editing Using Adeno-Associated Virus Delivery of a Compact, Hyper-Accurate Cas9 with a Dinucleotide PAM

Raed R. Ibraheim[1], Alireza Edraki[1], Aamir Mir[1], Ildar Gainetdinov[1], Yeonsoo Yoon[2], Chun-Qing Song[1], Wen Xue[1], Jaime A. Rivera-Pérez[2], Erik Sontheimer[1]

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The Cas9 ortholog from *Streptococcus pyogenes* (SpyCas9) has been widely used for genome editing. Several characteristics favor SpyCas9, including its efficiency and its minimally restrictive NGG PAM. Despite its utility, SpyCas9 is less than ideal for some clinical applications due to its large size that makes all-in-one (SpyCas9 protein + sgRNA) delivery by adeno-associated virus (AAV) difficult. Our group and others have identified compact Cas9s that are active in mammalian editing, including those from *N. meningitidis* strain 8013 (Nme1Cas9, PAM: N_4GAYW/N_4GYTT/N_4GTCT), *S. aureus* (SauCas9, PAM: N_2GRRT), *C. jejuni* (CjeCas9, PAM: N_4RYAC), *G. stearothermophilus* (GeoCas9, PAM: N_4CRAA/N_4GMAA) and *Streptococcus thermophilus* Cas9 (St1Cas9, PAM: N_2AGAAW). Despite their potential, their use is limited by the restrictive PAM requirements that lead to less abundant target sites. Here, we present our identification of a distinct, compact *N. meningitidis* Cas9 ortholog, Nme2Cas9, that (like SpyCas9) has only two required PAM nucleotides, allowing a broad targeting range. We show that this Cas9 has an N_4CC PAM and is an efficient nuclease in mammalian cell lines following delivery by plasmid transfection, lentivirus transduction, and ribonucleoprotein electroporation. Furthermore, our genome-wide data show that Nme2Cas9 is naturally hyper-accurate in mammalian cells and *in vivo*. The editing activity of this Cas9 can be switched off using anti-CRISPR proteins *in vitro* and in cells. Perhaps most importantly, Nme2Cas9 is compact (<3.25 kb, 1,082 amino acids), enabling delivery with its sgRNA in a single AAV vector for genome editing *in vivo*, including somatic editing in adult mouse liver as well as germ-line editing in pre-implanted zygotes. Our on-going work has further increased the utility of AAV-Nme2Cas9 through the truncation of the sgRNA scaffold sequence and AAV vector components. This streamlining has enabled us to build additional tools and capabilities into single vectors, such as dual sgRNAs for segmental deletions and multigene knock-outs. These and other applications reveal Nme2Cas9 as a valuable addition to the genome editing repertoire, including in clinical applications.

Gene Editing for Red Blood Cell Disorders

969. Editing Aberrant Splice Sites with Cas9 and Cas12a Efficiently Restores β-Globin Expression in β-Thalassemia

Kevin Luk[1], Shuqian Xu[2,3], Qiuming Yao[2,4], Anne H. Shen[2], Jing Zeng[2], Yuxuan Wu[2,5], Hong-Yuan Luo[6], Christian Brendel[2,7], Luca Pinello[4], David H. K. Chui[6], Scot A. Wolfe[1], Daniel E. Bauer[2]

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The thalassemias are compelling targets for therapeutic genome editing in part because monoallelic correction of a subset of hematopoietic stem cells (HSCs) would be sufficient for enduring disease amelioration. Prerequisite is the development of efficient repair strategies that are effective in HSCs. Therapeutic gene correction via homologous recombination, which requires co-delivery of an exogenous donor template, is cell-cycle dependent and competing with non-templated mutagenic repair; this strategy remains challenging in quiescent HSCs. Here we demonstrate that non-homologous end joining mediated allelic disruption of aberrant splice sites, one of the major classes of thalassemia mutations, is a robust approach to restore gene function. The IVS1-110G>A and IVS2-654C>T mutations are among the most common mutations in Mediterranean and East Asian populations, respectively. Each of these mutations creates an aberrant intronic splice site that disrupts normal splicing of β-globin. We targeted the IVS1-110G>A mutation using SpCas9 ribonucleoprotein (RNP) and the IVS2-654C>T mutation by LbCas12a/Cpf1 RNP in primary CD34+ hematopoietic stem and progenitor cells (HSPCs) from nine β-thalassemia patients. These results are to our knowledge the first demonstration of Cas12a RNP editing in primary HSPCs. Both Cas9 and Cas12a nucleases achieved high efficiency of therapeutic edits (94.5% indel frequency at IVS1-110G>A alleles and 77.0% indel frequency at IVS2-654C>T alleles) (Figure 1). Erythroid progeny of edited HSPCs showed reversal of aberrant splicing, restoration of β-globin and adult hemoglobin (HbA, $\alpha_2\beta_2$) expression (Figure 1), and normalization of terminal erythroid maturation efficiency. Clonal analysis of the erythroid progeny revealed that even a +1(A) insertion adjacent to the IVS1-110G>A mutation restored β-globin splicing to a normal pattern. The indels produced by Cas12a in HSPCs were exclusively deletions spanning the mutation and aberrant consensus splice donor site. Similar indel frequencies were observed in the HSC-enriched CD34+ CD38- CD90+ CD45RA- population as in CD34+ CD38+ progenitors. The high rate of indels in HSPCs and the penetrance of aberrant splice site disruption indicate the robustness of this therapeutic strategy. This approach, only requiring monoallelic *HBB* repair, could alleviate a substantial fraction of transfusion-dependent β-thalassemias with currently available gene editing

technology. More, these results demonstrate the exquisite precision of Cas12a RNP editing in HSPCs, with sensitivity to even single base mismatches in the crRNA sequence.

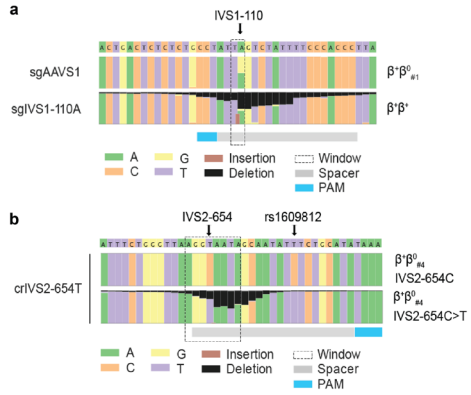

Figure 1. Nucleotide quilts showing indels and substitutions at each position around the mutation a) SpCas9 RNP treatment targeting IVS1-110. $\beta^+\beta^0_{\#1}$ with sgAAVS1 shown as a representative example of an unedited IVS1-110G>A heterozygous donor and $\beta^+\beta^+$ with sgIVS1-110A as representative example of an edited IVS1-110G>A donor. b) LbCas12a RNP treatment targeting IVS2-654. $\beta^+\beta^0_{\#4}$ shown as a donor in which the IVS2-654C/rs1609812-C and IVS2-654C>T/rs1609812-T alleles could be distinguished based on the adjacent heterozygous SNP rs1609812.

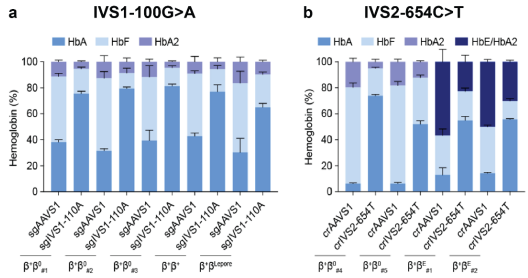

Figure 2. Hemoglobin HPLC shows increase in the hemoglobin A (HbA) fraction after therapeutic editing. a) SpCas9 RNP treatment targeting IVS1-110. b) LbCas12a RNP treatment targeting IVS2-654. AAVS1 is a control target site.

970. Towards The Clinical Translation of Gene Correction in Hematopoietic Stem Cells for Sickle Cell Disease Treatment

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Sickle cell disease (SCD) is one of the most common monogenic diseases, affecting millions of people worldwide, which results from a single point mutation in the beta-globin gene. The ideal curative strategy for SCD is *ex vivo* beta-globin gene correction of patient-derived hematopoietic stem and progenitor cells (HSPCs) followed by autologous hematopoietic stem cell transplantation and reconstitution of a healthy red blood cell compartment. We developed a genome editing platform that utilizes the CRISPR/Cas9 nuclease system coupled with AAV6 to induce homologous recombination (HR) directed-correction of the SCD-causing mutation in HSPCs. Here, we present the preclinical research and key scale-up processes enabling the clinical translation of a first-in-human gene correction strategy. We isolated CD34$^+$HSPCs from fresh plerixafor-mobilized apheresis products from healthy donors and SCD patients and conducted several key translational experiments, including the following: 1) Optimization of manufacturing to mediate high frequency HR in HSPCs, 2) Xenotransplantion in NSG mice to demonstrate engraftment and multilineage reconstitution of targeted cells. Of note, at week-16 post-transplantation, bone-marrow residing human lymphoid (B and T/NK cells) and myeloid progeny as well as HSPCs showed an allelic HR frequency greater than the hypothesized therapeutic threshold of 20%, 3) Scale-up of AAV6 production process together with a large-scale culture and electroporation systems to prove feasibility of clinical-scale manufacturing. We quantified the allelic modification frequency in liquid culture and the allelic distribution in edited HSPCs derived-colony forming units (CFUs). Notably, we achieved up to 60% allele correction of the sickle cell mutation in large-scale process. Moreover, a mean average of 56% of edited HSPC derived-CFUs carried a mono/bi-allelic HR event, potentially indicating high frequency of functional correction in the myeloerythroid lineage,4) HBB edited HSPCs and donor matched untreated cells were phenotyped, characterized, karyotyped and then injected in the NSG animal model to perform a long-term safety tumorigenicity/toxicology study. No evidence of genotoxicity and tumorigenicity were reported as a result of the modified HSPC cell product. Taken together, we will present the whole safety and efficacy profile as well as the plan to file an IND to initiate a phase I/II clinical trial testing the strategy in SCD patients.

971. Correction of B-Thalassemia Phenotype by CRISPR/CAS9 Editing of the Human A-Globin Locus

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Adult hemoglobin consists of two pairs of globin subunits (α2β2), 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 of the current clinical approaches aim to increase β and β-like globin chains. In particular, HBB addition strategies are quite successful for β$^+$-thal, where the residual endogenous HBB expression contributes to the clinical benefit, but still insufficient for β^0-thal (no residual HBB expression). Alternatively, globin balance can be restored reducing

HBA production 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. We designed and tested several gRNAs targeting non-coding regions of HBA and we selected one candidate with a high cleavage efficiency (83.1±7.0% InDels) that resulted in an average of one deletion per cell in an erythroid progenitor cell line (HUDEP2). To evaluate the therapeutic effect of our strategy we generated an HBB-KO clone that recapitulates β^0-thal phenotype (HUDEP2β^0-thal). In these cells, we demonstrated significant reduction of α-globin precipitates (by HPLC) upon deletion of 1 or 2 HBA genes and concomitant amelioration of the α β-globin balance, which correlated with HBA deletion efficiency (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). However, as expected for β^0-thal, edited cells showed lower hemoglobin levels compared to wild type cells. For this reason, we combined the HBA deletion with the knock-in of β^{AS3}-globin transgene under the control of the HBA promoter. Strikingly, we succeeded in expressing high level of HBB protein in HUDEP2β^0-thal with one integration per cell (β^{AS3}=81.5±13.8 % of β-like globins in edited HUDEP2β^0-thal vs β=85.6±4.1 % in wt HUDEP2) and in restoring adult hemoglobin production to therapeutic levels (~40% of wt hemoglobin level). In primary human hematopoietic stem progenitor cells (HSPC) we achieved similar editing efficiency in terms of HBA deletion and HBB integration. Long-term engraftment studies of edited HSPC in immunodeficient mice and targeted integration experiments on HSPC from β-thal patients are currently underway. Finally, to address the safety of this approach, we verified that the selected CRISPR/gRNA is highly specific, with undetectable off-target events (1% threshold) as assessed by PCR analyses of in silico predicted off-targets and unbiased genome wide screening (IDLV capture). 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.

972. Zinc Finger Nuclease-Mediated Disruption of the BCL11A Erythroid Enhancer in Human Hematopoietic Stem and Progenitor Cells Results in Enriched Bialleleic Editing with Highly Replicable and Precise On-Target Small Indels and Allele-Additive Increases in Fetal Hemoglobin

Hui Ling[1], Samuel Lessard[1], Benjamin Vieira[1], Kevin Moran[1], Pauline Rimmele[1], Yi-Dong Lin[1], Vu Hong[1], Andreas Reik[2], Denny Dang[2], Kenneth Huttner[1], Tim Harris[1], Alexandra Hicks[1]

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High fetal hemoglobin (HbF) levels are associated with asymptomatic sickle cell disease (SCD) and less severe beta-thalassemia. We are developing a novel gene-edited cell therapy for these diseases using autologous hematopoietic stem and progenitor cells (HSPCs) that have been genetically modified with zinc finger nucleases (ZFNs) to reactivate HbF expression (Phase 1/2a clinical trials NCT03653247 and NCT03432364). The ZFNs target the GATA1 binding motif (GATAA) within an intronic erythroid-specific enhancer (ESE) of *BCL11A*, the gene encoding a major transcriptional regulator of the human fetal-to-adult hemoglobin switch. Recently, we have reported reproducible editing of the *BCL11A* ESE and reactivation of HbF expression in plerixafor-mobilized HSPCs obtained from healthy human donors. For more detailed characterization, we performed single-cell erythroid differentiation using *BCL11A*-edited (57%-78% indel) plerixafor-mobilized HSPCs from 4 healthy donors. Individual cells were sorted and cultured in erythroid differentiation medium. Genomic DNA and protein lysate were collected at day 14 and 20, respectively. In total, we successfully genotyped 954 single-cell derived clones by next-generation sequencing. The distribution was highly skewed towards biallelic-edited cells ($P<1x10^{-146}$) representing >90% of total edited clones, suggesting that nuclease-expressing cells are likely edited at both alleles. We measured HbF protein levels by UPLC and found that each edited allele contributed additively to an increase of γ/(γ+β) ratio of 15% (defined as β in a linear regression model, $P=2x10^{-78}$). To understand the impact of indels that did not disrupt the GATA1 binding motif, we separated the effect of HbF induction by total editing and GATAA-disruption. Editing was associated with HbF independently of GATAA-disruption (β=3%, $P=0.003$), suggesting that non-GATAA editing patterns (e.g. those disrupting the adjacent TAL1 binding site) also contribute to HbF induction. However, GATAA-disruption yielded an additional 14% increase in HbF per allele (β=14%, $P=8x10^{-49}$). Overall, our data revealed that >90% of edited cells were biallelic, and these biallelic edits add a 27-38% increase of γ/(γ+β) ratio to the basal level. We observed that biallelic-edited homozygotes (same indel pattern at both alleles) were more frequent than expected (151 vs 15; $P<0.00001$). It is possible that these clones harbor larger deletions at the target site, which encompass the primer sites used for the amplicon and thus are not captured by sequencing. Using an amplicon design that encompasses both the target site and an informative heterozygous SNP allowing allele discrimination in 2 donors, we confirmed that 12 out of 40 (30%) clones were actual homozygotes. For clones containing uncharacterized alleles, we amplified and sequenced by Nextera XT a 12-kb region centered on the target site. We resolved all the indel patterns, with most deletions being less than 500 bp, and only one allele being a deletion of 1.4 kb. None of these deletions is expected to disrupt the *BCL11A* coding region (>26 kb away). These data demonstrate that ZFN editing of the *BCL11A* ESE in human HSPCs results in enriched biallelic editing with precise on-target small indels, and allele-additive increases in fetal hemoglobin. These data support the potential efficacy and specificity of ZFN-edited HSPCs as a novel cell therapy for patients with hemoglobinopathies.

973. Lentivirus-Mediated Expression of the RNA-Binding Protein IGF2BP1 Reverses Fetal-To-Adult Hemoglobin Production in Culture-Differentiated Erythroid Cells from Patients with Severe Hemoglobin Disorders

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Sickle cell disease (SCD) and β-thalassemia result from structural abnormality or deficient synthesis of the β-globin chain of adult hemoglobin (HbA, $\alpha_2\beta_2$). Disease symptoms develop after birth when hemoglobin switches from the fetal (HbF, $\alpha_2\gamma_2$) to adult type. Continued expression of HbF can reduce or prevent complications of both disorders making the identification of factors that control fetal γ-globin expression of great therapeutic interest. Here we focus on insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), which we found to be highly expressed in culture-differentiated erythroid cells from CD34⁺ cells of fetal liver (42±19% of internal control) versus adult bone marrow (0.01±0.01%). We constructed lentiviral vectors where an erythroid-specific α-Spectrin promoter was used to express IGF2BP1 fused to puromycin or ZsGreen by the 2A peptide. An erythroid culture model was used to assay for hemoglobin production after gene delivery into CD34⁺ cells obtained from mobilized peripheral blood of five healthy adult donors or steady-state bone marrow from two patients with β-thalassemia and one with SCD. For healthy donors, flow cytometry and western blot demonstrated that vector-mediated expression of IGF2BP1 expression was within the physiological range of fetal liver-derived erythroid cells. Adult erythroid cells expressing IGF2BP1 had a 7-fold increase in the relative level of γ-globin mRNA to total β-like globin transcripts (γ/γ+β=8.8±2.3 control; 61.1±10.7 IGF2BP1, p=0.004). Hemoglobin electrophoresis and HPLC showed a robust increase in HbF for erythroid cells with IGF2BP1 expression (control: 7±4%; IGF2BP1: 43±10%; p=0.01). IGF2BP1 not only restored a high level of HbF, but caused the individual $^{A}\gamma$- and $^{G}\gamma$-globin chains to be synthesized in the ratio characteristic of fetal development. We also determined if IGF2BP1 expression could ameliorate the chain imbalance in β-thalassemia or suppress expression of sickle β-globin by reversing the γ-to-β switch (Figure 1). Compared to healthy donors, differentiated β-thalassemia cells demonstrated a higher baseline level of γ-globin to total β-like globin mRNA (56%). Still, IGF2BP1 expression shifted this ratio to a mean level of 94%. The resulting level of HbF increased by 50% compared with controls. A small amount of HbA (20%-25%) was detected in β-thalassemia controls, which is consistent with the patients having a β^+/β^0 or β^+/β^+ genotype. IGF2BP1 expression suppressed both HbA and HbA_2 indicating reversal of the γ-to-β switch. The impact of IGF2BP1 was more pronounced for cultured cells from the SCD patient where γ-globin mRNA was more than 90% of total (γ/γ+β) causing SCD cells with IGF2BP1 expression to almost exclusively produce HbF. We conclude that expression of IGF2BP1 is sufficient to potently reverse developmental fetal-to-adult hemoglobin switching and maximize HbF production in patients with severe β-thalassemia or SCD.

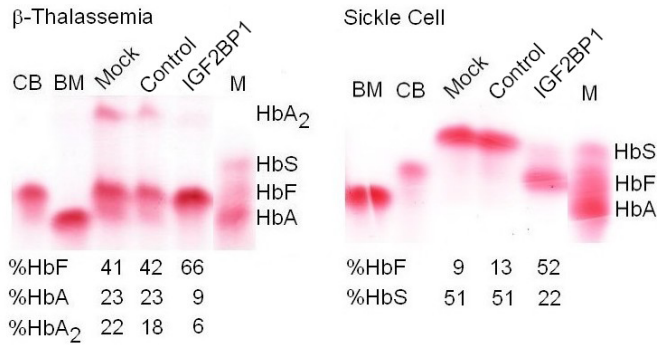

Figure 1. Hemoglobin gels for erythroblasts derived from CD34⁺ cells of patients with β-thalassemia (Left) or SCD (Right). Controls were from umbilical cord blood (CB) or healthy adult bone marrow (BM). Percentage of each hemoglobin tetramer as determined by HPLC analysis is reported below each lane.

974. Stable and Therapeutically Relevant Long-Term Engraftment of CRISPR/Cas9-Edited HSCs for HbF Reactivation in NHPs

Olivier Humbert[1], Stefan Radtke[1], Ray Carrillo[1], Anai M. Perez[1], Sowmya Reddy[1], Christopher Lux[2], Sowmya Pattabhi[2], Lauren E. Schefter[1], Olivier Negre[3], Ciaran M. Lee[4], Gang Bao[4], Jennifer E. Adair[1], Christopher W. Peterson[1], David J. Rawlings[2,5], Andrew M. Scharenberg[2,5,6], Hans-Peter Kiem[1,5]

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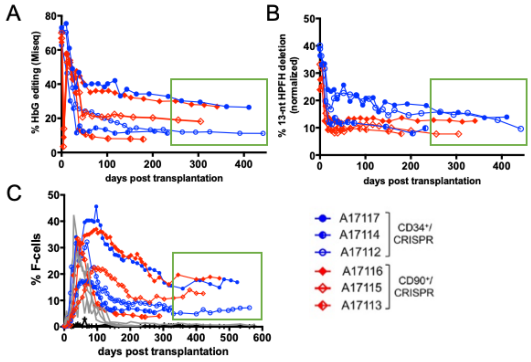
Reactivation of fetal hemoglobin (HbF) is being pursued as a treatment for hemoglobinopathies. Here, we evaluated the therapeutic potential of hematopoietic stem and progenitor cells (HSPCs) gene-editing to recapitulate a deletion causing hereditary persistence of fetal hemoglobin (HPFH). Six nonhuman primates (NHPs) were transplanted with either CD34+ HSPCs or a stem cell enriched CD34+CD90+CD45RA- subset that was treated by CRISPR/Cas9 ribonucleoprotein electroporation. All animals were monitored for >1 year for engraftment of edited cells and peripheral HbF production. Results were highly comparable between the two experimental cohorts, with *in vivo* editing levels stabilizing at up to 27% of total nucleated blood cells (**Fig.A**), of which 7-13% consisted of the 13-nucleotide (nt) HPFH deletion (**Fig.B**). Deletion patterns were stable in the periphery and matched those measured in 6-month bone marrow. HbF reactivation strongly correlated with *in vivo*editing, consisting of up to 18% circulating F-cells for >1 year (**Fig.C**). Safety of our approach was demonstrated by rapid reconstitution of all blood cell lineages with counts that remained within normal range during the entire course of the study. Specificity of the nuclease was queried by deep sequencing of 35 predicted off-target sites pre- and post-transplantation, which revealed no signal above background. A potential limitation of this approach is the presence of the CRISPR/Cas9 target sequence in the promoters of both *HBG1*

and *HBG2*, which results in a deletion spanning over 4kbp upon simultaneous cleavage of both sites. This deletion was detected in the infusion product at approximately 27% but frequency gradually decreased post-transplant, and was undetectable by 300 days. In conclusion, the levels of in vivo gene editing described here using bulk CD34+ or the CD34+CD90+CD45RA- subpopulation should be within a therapeutically relevant range for a number of genetic diseases. The conservation of the CD34+CD90+CD45RA- phenotype and the *HBG* CRISPR/Cas9 gRNA target site between NHP and human, combined with the use of a pre-clinical large animal model for stem cell gene therapy and transplantation, should permit the direct translation of this approach to patients.



A) *HBG* editing efficiency measured in peripheral total nucleated cells from transplanted animals. **B)** Levels of 13nt-HPFH deletion in the same animals as in (A) after normalization of all deletion frequencies to 100%. **C)** F-cells frequencies in transplanted animals as compared to historical transplant controls (grey) and one untransplanted control (black). Green rectangles focus on stabilization of editing or F-cell levels.

975. Efficient Genome Editing of the PKLR Locus in Human Long-Term Hematopoietic Stem Cells Using Specific CRISPR/CAS9 RNP and AAV6-Delivery of Donor Templates to Treat Pyruvate Kinase Deficiency

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Pyruvate kinase deficiency (PKD) is an autosomal recessive disorder caused by mutations in the *PKLR* gene that lead to a reduction of the activity of the erythroid pyruvate kinase (RPK) protein. This disease is associated with reticulocytosis, splenomegaly and iron overload, and may be life-threatening in severely affected patients. In selected and severe cases, allogeneic Hematopoietic Stem Cell Transplantation (HSCT) has been shown to correct the disorder; however this is associated with extensive toxicity and not considered a standard therapy in PKD. Autologous HSCT of genetically corrected cells will offer a durable and curative therapeutic option. Over the last years, gene editing has emerged as a promising gene therapy approach for blood cell disorders. The clinical application of gene editing to correct some genetic hematopoietic diseases is supported by the high level of correction got in Hematopoietic Stem and Progenitor Cells (HSPCs). With that in mind, we conducted a gene editing approach to correct PKD in human HSPCs. We developed a knock-in gene editing strategy at the genomic starting site of the *PKLR* gene by combining RNP electroporation and adeno-associated viral vector (AAV6) carrying donor sequences. Specific gRNAs generating up to 60% indels at the RPK starting site in human Cord Blood $CD34^+$ ($CB\text{-}CD34^+$) were designed. Two different AAV6 constructions were produced to deliver either a TurboGFP expression cassette or a promotor-less therapeutic codon optimized RPK cDNA (coRPK), flanked by specific homologous arms. Specific donor integration and stable expression of turboGFP and of coRPK driven by *PKLR* endogenous promoter was detected in edited human K562 erythroleukemia cells. Furthermore, up to 40% specific integration and stable expression of both donors were detected in colony forming units (CFUs) generated from gene edited $CB\text{-}CD34^+$cells, in the absence of toxicity related to the procedure. Moreover, these gene edited $CB\text{-}CD34^+$ cells engrafted efficiently in both primary and secondary NSG mice, demonstrating that gene editing of Long-Term HSCs has been achieved. These results demonstrate the feasibility of editing the *PKLR* locus in HSPCs at efficiencies that could be clinically applicable to treat PKD. Gene editing experiments using PKD patient derived $CD34^+$ cells are now being conducted to further prove the clinical relevance of this approach to correct PKD.

976. Gene Correction of Beta-Thalassemia Ex Vivo and In Vivo Mediated by PNA Nanoparticles

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Blood disorders caused by a single mutation such as β-thalassemia are attractive targets for gene editing and efforts to correct hereditary genomic mutations have advanced with the improvement of genome engineering technologies. Programmable RNA-guided Cas9 endonucleases enable efficient editing, but also exhibit high off-target effects. Moreover, *in vivo* delivery of CRISPR/Cas9 remains challenging. These drawbacks have motivated the development of non-nuclease-based platforms, such as peptide nucleic acids (PNAs). PNAs designed to bind to sites in genomic DNA and form PNA/DNA/PNA triplexes can initiate an endogenous DNA repair response mediated by nucleotide excision repair and homology-dependent repair pathways. Our recent work has demonstrated a safe and efficient method for site-specific gene editing to correct disease-causing mutations *in vivo* via intravenous (IV) administration of biodegradable polymeric nanoparticles (NPs) loaded with PNAs and single-stranded donor DNA. For example, we have mediated significant gene editing of the β-thalassemia-associated IVS2-654 mutation in the bone marrow (BM), leading to functional improvement with extremely low off-target effects using polymeric

poly(lactic-co-glycolic acid) (PLGA) NPs in both adult animals and fetuses (1,2). Here we demonstrate that PNA-mediated gene editing can be significantly enhanced using a new class of polymeric vehicles consisting of members from a family of mildly cationic poly(amine-co-ester) (PACE) degradable polymers (3) that are designed for nucleic acid delivery and result in robust encapsulation of PNA/DNA editing agents as well as safe and effective delivery both *ex vivo* and *in vivo*. We first studied the uptake and biodistribution of PLGA and PACE NPs encapsulating a fluorescent dye *ex vivo* and *in vivo* by intravenous administration and found that NPs formed from certain compositions of PACE are significantly taken up in target BM cells as well as other organs affected by β-thalassemia, such as the spleen. We next encapsulated PNA and DNA editing agents targeting the β-globin locus in PACE NPs with PNA/DNA cargo encapsulation efficiencies up to 90%. We further demonstrated elevated site-specific editing levels using these new formulations using flow cytometry or droplet digital PCR (ddPCR), which resulted in up to ~9% gene correction of the β-globin locus in BM and mouse embryonic fibroblasts isolated from a GFP reporter or disease mouse model of β-thalassemia compared to ~1.5% previously reported with PLGA NPs that were administered at twice the dose. Subsequently, we sought to determine if treatment with PNA/DNA PACE NPs targeting the β-globin locus can be used to correct the underlying genetic mutation and improve the disease phenotype in a β-thalassemic mouse model harboring the IVS2-654 splicing mutation. *In vivo* treatment by IV injection of PNA/DNA-loaded PACE NPs at half the PLGA NP dose previously used (1) resulted in sustained blood hemoglobin elevation into the wild-type range, reduction of splenomegaly associated with extramedullary hematopoiesis, and improved splenic architecture. Importantly, PACE NPs did not result in toxicity or immunogenicity after repeated IV administration. On the whole, our findings demonstrate the promise, safety, and efficacy of PNA-mediated genome editing technology combined with effective delivery vehicles for the treatment of β-thalassemia. Further, we have established a site-specific gene correction approach applicable to numerous monogenetic disorders.

References (1) Bahal R, et al. *Nat Commun*. 2016. (2) Ricciardi A, et al. *Nat Commun*. 2018. (3) Kauffman A and Piotrowski-Daspit A, et al. *Biomacromolecules*. 2018.

Engineered Cell Therapies

977. Mesenchymal Stem Cell-Mediated Delivery of Artificial Transcription Factors for Neurologic Disease

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The advent of nuclease-deficient DNA binding domains (DBDs) such as zinc fingers and CRISPR/dCas9 for tunable gene expression have revolutionized approaches towards treating rare neurologic disorders. An effective delivery system that can traffic these proteins to target tissue remains a key barrier for translating gene modifying DBDs into the clinic. Cell-based delivery methods, such as mesenchymal stem cells (MSC), are an attractive delivery vehicle for DBDs due to their transient nature and ability to create favorable microenvironments through the release of trophic factors. Along with their strong clinical safety profile, MSCs can be readily reprogrammed to secrete large proteins and are not limited in their transgene packaging size unlike adeno-associated viral approaches. To evaluate this system, we have engineered bone-marrow derived MSCs to act as in-vivo biofactories that secrete DBDs into the extracellular space whereby the DBDs are uptaken by neighboring cells, and shuttled into the nucleus to alter gene expression. Previously, our group has shown activation of the paternally imprinted *Ube3a* gene in the brains of the E6-AP adult Angelman Syndrome mouse following i.p. injection of a recombinant zinc finger DBD protein (S1K) targeted towards the *Snurf/Snrpn* promoter - effectively silencing expression of the antisense transcript and activating paternal *Ube3a*. MSC-DBD proof-of-concept studies were evaluated with lentiviral-engineered bone marrow-derived MSCs that secrete S1K to activate the paternally silent *Ube3a-YFP* gene in the E6-AP:YFP reporter mouse. Administration of MSC-S1K conditioned media to E15.5 E6-AP:YFP primary neurons demonstrated significant activation of YFP fused *Ube3a* as compared to a scramble zinc finger MSC (MSC-SCR) and non-transduced MSC (MSC-NT) 48-hrs post treatment. We then bilaterally transplanted 250,000 MSCs (MSC-S1K, MSC-SCR, or MSC-NT) into 8-week old E6-AP:YFP mice. We observed significant activation of silent *Ube3a* via IHC and western blotting for YFP expression in the hippocampus, cerebellum, and cortex as compared to MSC-SR6 and MSC-NT treatment groups 3- and 6 weeks post-transplantation. Additionally, we performed longitudinal assessments of YFP reactivation in mice transplanted with MSCs via the cisterna magna compared to intracranial injected controls to evaluate non-invasive delivery approaches. The approach of using MSCs to deliver DBDs throughout the brain provides a broad application for the treatment of other neurologic disorders. Presently, we report the first-of-its-kind use of MSCs as a delivery platform for gene modifiers in neurologic disease - expanding the therapeutic potential of both systems for genetic diseases.

978. Reprogramming CAR-T Cells to Metabolize Lactate as a Fuel in Oxygen-Deprived and Nutrient-Deficient Environments

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Chimeric antigen receptors (CARs) provide a promising approach to redirect T cell specificity against tumor cells. CARs contain built in co-stimulatory domains derived from the signaling co-receptors 4-1BB or CD28. Previously, we showed how 4-1BB vs CD28 signaling gives rise to CAR-T cells with distinct metabolic features; providing mechanistic insight into why 4-1BBz outperform 28z CAR-Ts in blood-based malignancies. Importantly, our findings show that CAR-T cell metabolism is not fixed and is dynamically regulated by emphasizing distinct signaling and metabolic pathways. We reason that CAR-T cell metabolism can

be reprogrammed to enhance durability in solid tumor models. The metabolic nature of the solid tumor environment can also influence CAR-T proliferation and function. Tumor cells outcompete T cells for glucose, glutamine, and fatty acids thereby impairing CAR-T proliferation and effector function. As proliferating tumor cells undergo Warburg metabolism, they release lactate into their microenvironment. We recognized that CAR-T cell function could be enhanced by introducing non-native metabolic enzymes with novel capacities that enable them to oxidize lactate as a fuel source in solid tumors. Ideally, in the process of oxidizing lactate to support CAR-T expansion, CAR-T cells will consume O2 further starving cancer cells in the tumor. These dual goals of supplying CAR-T cells with an ability to oxidize lactate and depleting the tumor of O2 can be achieved by expressing a single exogenous enzyme, NADH Oxidase (NOX). As seen in Fig.1, NOX shifts the pyruvate: lactate equilibrium towards lactate oxidation providing theoretical support for a model where CAR-T cells can be reprogrammed to metabolize lactate in a hostile TME. Using 13C metabolic tracers, we show that vectorial flux from lactate to pyruvate provides an important fuel to support TCA cycle metabolism in CAR-T cells. We demonstrate that NOX promotes increased steady state oxygen consumption in CAR-T cells; increasing the contribution of oxidative metabolism to ATP production, proliferation, and differentiation (Fig.2). Collectively, these data suggest that NOX may sustain increased oxidative function in CAR-T cells traversing hostile TMEs. These preliminary results are promising as it's well established that 28z signaling gives rise to glycolytic, effector cells with enhanced cytolytic activity in solid tumors than their 4- 1BBz counterparts. However, 28z CAR-T are short-lived and reprogramming their metabolism to extend durability through improved oxidative function may increase their potency in select xenograft models.

Fig 1. A model illustrating how NADPH oxidase (NOX) shifts the pyruvate:lactate equilibrium towards lactate uptake and oxidation in CAR-T cells.

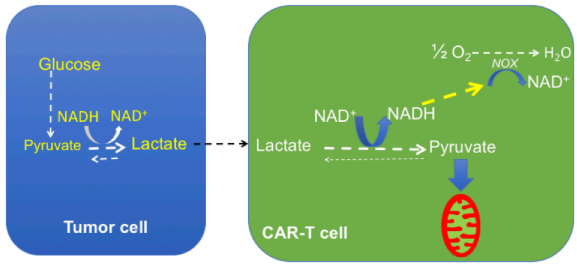
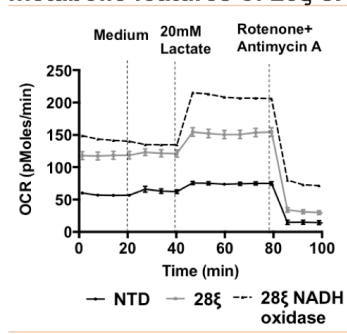


Fig. 2 NADH oxidase enhances the oxidative metabolic features of 28ξ CAR-T cells



Primary human T cells were co-infected with either a control construct or NADH oxidase, in conjunction with anti-mesothelin 28ξCAR. These cells were propagated for 10 days and their metabolic function

979. A Novel Aryl Hydrocarbon Receptor Antagonist Expands Adult Human Hematopoietic Stem Cells from Mobilized Peripheral Blood and Bone Marrow and Increases the Dose of CRISPR/Cas9 Gene-Edited NSG-Repopulating Cells

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Gene-modified human hematopoietic stem cell (HSC) transplant is a promising approach to treat a variety of inherited genetic diseases. Achieving sufficient doses of gene-modified HSCs is crucial in the success of these therapies. Expansion of gene-modified HSCs *ex vivo* may circumvent this challenge. Several small molecule approaches have been reported to expand cord blood (CB)-derived HSCs *ex vivo,* but their ability to expand gene-modified, adult HSCs remains unknown. Work by our group comparing molecules for CB expansion has shown that aryl hydrocarbon receptor (AHR) antagonism is the most effective method of expanding human NSG-engrafting cells. In a clinical trial of patients who received an AHR antagonist-expanded CB CD34+ cell product, MGTA-456, time to neutrophil recovery occurred more than 10 days faster than historical and concurrent patients transplanted with unmanipulated CB at the same institution (Wagner et al, Cell Stem Cell, 2016). Based on the encouraging clinical results, we identified a novel AHR antagonist (E478) and evaluated its ability to increase the number of gene-modified, NSG-engrafting human HSCs from mobilized peripheral blood (mPB) and bone marrow (BM). Culture of CB CD34+ cells with E478 led to a 6-fold increase in CD34+ number over vehicle-cultured cells and a 2.3-fold increase in engraftment in NSG mice. To evaluate the ability of E478 to expand gene-edited cells, CD34+ cells from mPB and BM were cultured with vehicle or E478 and edited with CRISPR/Cas9 targeting beta-2

microglobulin. In mPB CD34+ cells, after 7 days of expansion the culture had 3.4-fold more CD34+CD90+ cells, the cell type responsible for engraftment (Radtke et al, STM, 2017), than the vehicle-treated cells. Upon transplantation into NSG mice, the E478-expanded cells provided a 4-fold increase in engraftment compared to vehicle-treated cells. Importantly, the 85% editing rates of the expanded cells were maintained *in vivo*. BM CD34+ cells gave similar results: E478 resulted in a 4.4-fold increase in CD34+CD90+ cell numbers relative to vehicle control and a 5-fold increase in engraftment of cells with preservation of editing rates. Notably, when compared to cells cultured for just 2 days, which is considered the minimal manipulation time for editing success, E478-treated cells led to significantly improved engraftment while maintaining editing rates of 80%. Gene correction approaches rely on cytokines to promote HSC cycling, but this is accompanied by differentiation and subsequent loss of durable engraftment. To counter this, we explored E478's ability to increase HSC number, preserve HSC function, and maintain active cell cycling required for efficient HDR. We observed that mPB CD34+ cells in cytokine culture largely exit quiescence after 3-4 days. To determine optimal cell culture conditions that enhance both number and frequency of gene-corrected cells, we cultured mPB CD34+ cells for 2-4 days in the presence or absence of E478, prior to electroporation (EP) with CRISPR reagents targeting the beta-globin gene, and cultured cells for 2 or 4 days post-EP. Compared to a 2-day pre-stimulation culture with a 2-day post-EP culture, a 4-day pre- and post-EP culture in the presence of E478 provided nearly 2-fold higher correction rate and 7-fold higher numbers of CD34+CD90+cells. We have identified a novel AHR antagonist, E478, that can expand gene-modified, adult human HSC. In addition, we have shown that E478 allows for prolonged culture conditions in the presence of cytokines to provide higher gene correction rates while also increasing total CD34+CD90+ cell dose. This approach could improve autologous gene therapy and gene editing via *ex vivo* HSC expansion.

980. Can't Live without "U": Genetic Engineering of UMPS to Create Auxotrophy in Human Cells

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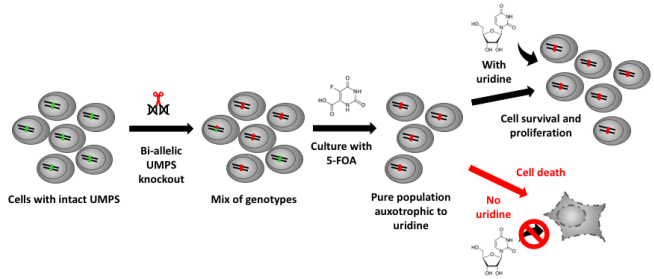
Background: Human cells are increasingly being used in a wide range of applications for immunotherapies and regenerative medicine but are associated with inherent risks. Auxotrophy is an attractive concept that has not been explored as containment strategy for human cell therapies. We hypothesized that disruption of a gene involved in metabolism through genome editing could create dependence of cellular survival towards an externally supplied compound. We identified *UMPS*, a key gene of pyrimidine de-novo synthesis, as an attractive target, the disruption of which would lead to auxotrophy towards uridine and additionally allow for selection using 5-FOA, a compound which is converted to cytotoxic 5-FU only if *UMPS* is intact. **Methods:** We used Cas9 RNP/rAAV6-mediated targeted integration of cassettes expressing selection markers to disrupt both alleles of the *UMPS* gene in human cell lines, pluripotent cells and primary T cells. We evaluated auxotrophy in vitro by culturing the cells in the presence of different uridine concentrations, and tested the sensitivity or resistance of *UMPS*$^{-/-}$ and wildtype cells towards 5-FOA in co-culture assays. Additionally, auxotrophy was evaluated in vivo in a xenograft model in NSG mice that were fed either standard diet or a diet enriched with uridine triacetate (UTA), a prodrug of uridine. The specificity of the endonuclease was confirmed by prediction algorithms and targeted next-generation sequencing of putative off-target sites. **Results**: We show that human cell lines with bi-allelic disruption of the *UMPS* gene proliferate in the presence of uridine but die within days after withdrawal. Culture with 5-FOA leads to depletion of cells with intact *UMPS* while cells with complete knockout continue to proliferate. We confirmed uridine auxotrophy and 5-FOA resistance in primary human T cells and in human pluripotent cells with knockout of both *UMPS* alleles. *UMPS*$^{-/-}$ cells transplanted in a xenograft mouse model proliferated when the mice were fed UTA while withdrawal of the uridine source inhibited cell growth. The off-target analysis of the genome editing approach found no detectable InDel activity at sites other that the on-target site. **Conclusion:** We use genetic engineering to create auxotrophy towards a non-toxic substance in human cells and target UMPS as a key gene involved in an indispensable metabolic pathway, which will prevent the cells from developing escape mechanisms. The ability to use an external compound to influence proliferation and survival of human cells and the possibility to deplete residual non-auxotrophic cells enable the development of this approach for a range of applications where a pure population of externally controllable cells is necessary. Genetically engineered auxotrophy provides an addition and alternative to current safety mechanisms for cell therapies that offers several potential advantages.



981. Directing Skeletal Myogenic Progenitor Cell Lineage Specification with CRISPR/Cas9-Based Transcriptional Activators

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Human pluripotent stem cells (hPSCs) are a promising source for cell therapies, disease modeling, and drug discovery for neuromuscular disease. Directed differentiation of hPSCs into skeletal muscle cells has been demonstrated by exogenous cDNA overexpression of myogenic transcription factors such as Pax7 and MyoD. Advances in genome engineering technologies have established the CRISPR/Cas9 system as a platform for engineering programmable transcriptional regulators capable of targeted activation of endogenous genes. Nuclease-null

dCas9 can be fused to effector domains such as the transactivation domain VP64, to potently activate genes in their natural chromosomal context. In contrast to ectopic expression of transgenes, activation of endogenous genes can facilitate chromatin remodeling and induction of autonomously maintained gene networks. Targeting endogenous genes can also capture the full complexity of transcript isoforms, mRNA localization, and other effects of non-coding regulatory elements. Here, we use ^{VP64}dCas9^{VP64} to activate the endogenous myogenic transcription factor Pax7 in hPSCs to direct differentiation into skeletal muscle progenitors. We hypothesize that activation of Pax7 will induce the myoprogenitor gene expression program, as it plays a key role in specification and regulation of muscle progenitors. hESCs and hiPSCs were transduced with lentiviruses encoding tetracycline-inducible ^{VP64}dCas9^{VP64}-2A-mCherry and a gRNA targeting the Pax7 promoter. Control samples were transduced with lentivirus encoding tetracycline-inducible Pax7 cDNA-2A-mCherry. After initial CHIR99021 mediated mesodermal differentiation, transduced cells were sorted based on mCherry expression. Induction of ^{VP64}dCas9^{VP64} and gRNA expression led to increased transcript levels of multiple endogenous Pax7 mRNA isoforms by >1,000-fold. In contrast, Pax7 cDNA overexpression led to high levels of total Pax7 mRNA but only 2-fold activation of transcription from the endogenous gene. Pax7 protein expression was detected 12 days post-transduction by immunofluorescence staining, indicating robust gene activation. These cells also express human satellite cell surface markers CD56 and CD29. Importantly, activation of endogenous Pax7 led to higher expansion potential over multiple passages, averaging 270-fold increase in cell number at 14 days after sorting, compared to 178-fold for Pax7 cDNA overexpression. Furthermore, Pax7 expression was sustained over 5 passages in >90% of cells when activated from its endogenous locus, compared to 25% of cells when Pax7 was exogenously expressed from the lentiviral vector, presumably as a result of silencing of the lentiviral promoter. When ^{VP64}dCas9^{VP64} expression is discontinued by withdrawal of doxycycline for one week, myogenic progenitor cells differentiate into myotubes expressing MyoD, MyoG, and myosin heavy chain. Interestingly, immunofluorescence staining showed that a significant number of cells (~40%) retain Pax7 expression despite lacking ^{VP64}dCas9^{VP64}, suggesting that endogenous gene activation results in persistent transcriptional reprogramming. When transplanted into NSG immunodeficient mice, these cells engrafted into host muscle fibers as evidenced by human spectrin expression. Ongoing studies are aimed at assessing differential chromatin accessibility and transcriptome profiles between myogenic progenitor cells derived from endogenous activation of Pax7, exogenous Pax7 overexpression, and freshly isolated human satellite cells. These studies introduce a novel method for specification and expansion of myogenic progenitors from hPSCs by deterministic editing of transcriptional regulation with new genome engineering tools, which can enable new possibilities for disease modeling and cell therapy in disorders of skeletal muscle regeneration.

982. Sustained Engraftment and Protein Secretion using Gene-Edited Human B Cells in Humanized Mouse Models

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Protein or peptide drugs are a growing class of therapeutics that now constitute ~10% of the pharmaceutical market. While protein drugs have great promise to specifically target signaling pathways and target cell types, they also have drawbacks that include a repeated dosing requirement, poor solubility and requirement for mammalian-specific post-translational modifications. Recently, we developed a cell-based method to deliver protein drugs. To do this, we coupled CRISPR/Cas9-based nucleases with adeno-associated virus for delivery of donor homology templates to candidate safe-harbor loci in B cells. The result was a durable population of antibody-secreting cells (ASC) that produced high levels of exogenous proteins. A subset of these cells resembled classically defined long-lived plasma B cells (CD38hiCD138+), whereas other durable ASCs exhibited phenotypes (CD38+CD138-) not previously associated with longevity. Here, we present an extensive characterization of the survival and function of adoptively transferred, engineered ASCs *in vivo*. We show that engineered B cells can home to and engraft in the bone marrow of recipient, immune deficient, NOD/SCID/γc-null (NSG) mice and stably produce human antibody for greater than 1 year. ASCs engineered to express firefly luciferase primarily migrated to the bone marrow, the endogenous location of human long-lived plasma cells. Upon provisioning NSG mice with key human cytokines (IL6, and/or BAFF) known to promote survival of long-lived plasma cells, we observed substantial increases in antibody production and durability of engineered B cell grafts: serum IgG titers at 1-year post engraftment exceeded 10 ug/mL. Each cytokine exerted distinct effects on the phenotypes of engrafted cells in NSG recipient mice. BAFF preferentially promoted survival of class-switched, CD138^{+} plasma cells. In contrast, IL6 promoted surface IgM-expressing CD38^{+}CD138^{-} ASCs, although CD138+ plasma cells were also durably observed with increased numbers *in vivo* in the presence of IL6. Finally, quantification of human ASC in the murine bone marrow and spleen indicate that sustained engraftment with as few as 20,000 engineered cells/recipient was sufficient to maintain IgG titers of 10 ug/mL, levels that could be of therapeutic valuable if achieved using expression of candidate mAb reagents. Together, these studies show that engineered human B cells have the capacity to engraft long-term and function normally *in vivo,* strongly supporting further studies using this novel cell therapy platform for long-term delivery of protein drugs.

983. UM171 Efficiently Expands Genetically Modified Haematopoietic Stem Cells from Mobilized Blood and Transduces Preferentially the Erythroid Lineage

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Objectives: *Ex-vivo*, lentiviral vector (LV) hematopoietic stem cell (HSC) gene therapy has shown strong therapeutic potential. However, the challenge of obtaining high numbers of engraftable HSCs under culture and transduction conditions, generates the need for further process optimization. Recently, high throughput screening of chemical libraries has identified a number of small molecules (SMs) that improve the expansion, retain the self-renewal and inhibit the differentiation of unmanipulated cord blood-derived HSCs. Nevertheless, the capacity of SMs to expand genetically modified, adult HSCs remains largely unexplored. In the present study, we sought to investigate whether SMs can sufficiently expand genetically modified CD34+ cells derived from mobilized peripheral blood (mPB), the main source of HSCs for gene therapy. **Methods:** LY2228820 (LY), pyrimidoindole derivative UM171 and Stem Reginin 1 (SR1), either alone or in combination (X3), were used to test the expansion of mPB-CD34+ cells transduced with an LV-GFP vector. SMs were added in serum-free and cytokine-supplemented culture, either from the initiation of transduction (5-day culture) or after its completion (7-day culture). The expansion of immunophenotypically primitive (by flow cytometry) HSCs was evaluated. The effect of SMs on transduction, clonogenicity (CFU-GM/BFU-E), erythroid and myeloid differentiation were also assessed. **Results:** The 7-day culture resulted in higher expansion of total CD34+ cells over the 5-day culture, without however, quantitative differences among tested SMs and the control (DMSO). In total CD34+cells, SMs had no effect on GFP expression or colony formation. Importantly however, HSCs bearing a primitive phenotype (% CD34+CD38-, CD34+CD38-CD90+, CD34+CD45RA-CD133+) were expanded up to 5-fold (p=0.0002) and expressed higher GFP levels over control (p<0.0001) when cultured with the UM171 or X3. In single erythroid and myeloid methylcellulose colonies, UM171 and X3 produced overall higher VCN (0.88±0.11, 0.97±0.11, respectively) over DMSO (0.4±0.07, p=0.0002) and particularly in colonies of erythroid origin (*BFU-E*: DMSO: 0.91±0.13, UM171: 2.25±0.28, X3: 2.58±0.33, p=0.01, *CFU-GM*: DMSO: 1.049±0.14, UM171: 0.950±0.16, X3: 0.918±0.13, *p:* not significant). In erythroid liquid cell differentiation cultures, UM171 and X3 delayed erythroid differentiation (p≤0.0001), while enhanced GFP expression in CD235a$^+$ cells (p=0.0014). In contrast, myeloid differentiation was not affected by SMs and GFP expression in CD33$^+$ cells was similar among all groups. The X3 did not provide any additive or synergistic effect over UM171 alone in all the above parameters. **Conclusions:** We here provide evidence for significant expansion of primitive, genetically modified mPB HSCs. Should these data be solidified by xenograft studies, the incorporation of UM171 in the manufacturing process may advance the *ex-vivo*, HSC genetic engineering and improve the overall outcome of gene therapy, especially for hemoglobinopathies.

984. Generation of High-Purity Human iPS Cell-Derived Hepatocyte like Cells Using CRISPR-Cas9 System

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Human iPS cell-derived hepatocyte-like cells are expected to be utilized in pharmaceutical research such as drug screening. However, the purity of high-functioning hepatocyte-like cells is not high enough. In particular, the percentage of cytochrome P450 3A4 (CYP3A4), which is a representative hepatic drug-metabolizing enzyme, positive cells is quite low (approximately 20%). To address this problem, we generated high-purity and high-functioning human iPS cell-derived hepatocyte-like cells for pharmaceutical research. Therefore, we tried to establish the CYP3A4-NeoR-EGFP transgenic reporter human iPS cell line (CYP3A4-NeoR iPS cells) using genome editing technology. However, in spite of use of CRISPR-Cas9, the efficiency of genetic engineering of human iPS cells in transcriptionally inactive genes is extremely low, unlike that in transcriptionally active genes. To enhance the homologous recombination efficiency in human iPS cells, we performed screenings of accessorial genes and compounds. We found that RAD51 overexpression and valproic acid treatment could enhance biallelic-targeting efficiency in human iPS cells regardless of the transcriptional activity of the targeted locus (ref. 1). By using RAD51 and valproic acid, we succeeded in establishing CYP3A4-NeoR iPS cell line (ref. 2). The CYP3A4-NeoR-EGFP iPS cells were differentiated into hepatocyte-like cells according to our efficient hepatocyte differentiation protocol established previously (ref. 3). Then, the hepatocyte-like cells were treated with Neomycin to concentrate the hepatocyte-like cells which strongly express CYP3A4. After the Neomycin treatment, the percentage of CYP3A4-positive cells was higher than 80%. The gene expression levels of various drug-metabolizing enzymes, transporters, and hepatic transcription factors were significantly enhanced by Neomycin treatment. In addition, the CYP1A2, 2C19, 2D6, and 3A4 activities and biliary excretion capacities were also significantly increased by Neomycin treatment. We also confirmed that the detection sensitivity of drug-inducing hepatotoxicity was enhanced by Neomycin treatment. We succeeded in obtaining human iPS cell-derived hepatocyte-like cells that highly express CYP3A4 at high purity. We believe that our high-purity and high-functioning hepatocyte-like cells could be used to evaluate the risk of drug candidates more accurately than ever before.

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Immunotherapy II

985. Depletion of CD45RA-Positive Cells Removes an Inhibitory Component that Potentiates the Reactivation of EBV-Specific T-Cells from Lymphoma Patients

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~40 % of Hodgkin and Non-Hodgkin Lymphomas (HL/NHL) carry the EBV genome and express viral type-2 latency proteins (T2-Ags) that provide targets for immunotherapy. Autologous EBV-specific T cells (EBVSTs) targeting T2-Ags (T2-EBVSTs) can be produced from EBV+ lymphoma patients and have produced complete tumor responses in up to 50% of patients. However, broader application of EBVST therapy is limited by challenges associated with manufacturing failure. EBVSTs are difficult to reactivate and expand in-vivo from most lymphoma patients, likely because they circulate with low frequency and are rendered anergic by their tumors. In our current clinical trial, we were unable to generate EBVSTs from 25% of the patients procured - manufacturing failures were associated with lack of cell expansion and proliferation, and of the lines successfully expanded, EBVSTs demonstrated lack of T2-Ag-specificity and often contained a high frequency of NK cells. Of those passing release criteria, 22% patient lines recognized only a single T2-Ag with most lines recognizing only two T2-Ags, increasing the risk of tumor-antigen escape. Finally, even in successfully manufactured EBVSTs, the frequency of T2-Ags specific T-cells in final products was low. Our goal is to improve and increase the success rate of EBVSTs manufacture and their clinical efficacy. After resolution of viral infections, virus-specific T-cells (VSTs) enter the memory compartment, with the majority residing in the CD45RA-negative $T_{CM}$and T_{EM}fractions, while the CD45RA positive population includes naïve T-cells, suppressive Regulatory-T cells (T-regs), NK-cells and the majority of B-cells. ***We hypothesize that removal of the irrelevant CD45RA+ cells from PBMCs prior to viral-antigen specific stimulation would enable successful generation of antigen-specific VSTs*** by eliminating a competing naïve T-cell, and NK cells and reducing T-regs capable of inhibiting the outgrowth of antigen-specific T-cells. We therefore investigated the effects of selective depletion of CD45RA-positive cells from PBMCs prior to stimulation with EBV T2-Ags. This approach produced ~one log greater expansion and decreased the frequency of NK cells, which have dominated some of the final products in our current clinical trial. The specificity for viral antigens in the final product increased by ~5-10 fold as measured by γ-IFN release in response to T2-Ags stimulation and superior cytotoxicity against limiting dilutions of EBV+ target cells, and the number of viral antigens recognized also increased in both healthy donors and patients. Most importantly, this approach restored responsiveness to antigen stimulation in most unresponsive patients enabling the reactivation and expansion of EBVSTs from patients that were manufacturing failures previously using whole PBMCs. This advantage extends to generation of VSTs with enhanced antigen specificity to VZV and Adenoviral antigens, suggesting that we have identified a general approach for improving the activity and availability of VSTs to treat both viral-infection and virus-associated malignancy. EBVSTs generated from CD45RA-depleted PBMCs demonstrated superior metastatic EBV+ tumor burden control and rapid tumor clearance when adoptively transferred to an EBV+ murine xenograft model. This approach is in the process of being translated to generation of VSTs for use in multiple clinical trials. In future, we aim to elucidate the mechanisms underlying the inhibitory effects of the CD45RA positive population in the reactivation and expansion of EBVSTs.

986. Targeting Autophagy Enhances the Adoptive Immunotherapy of Glioblastoma with Multi-Functional Genetically-Engineered NK Cells

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Background: Despite aggressive treatments and care, the median life expectancy for GBM patients is only around 15 months, highlighting the need for new therapeutic approaches. Natural killer (NK) cells, innate cytotoxic effectors, are showing potential in immunotherapy of tumors including GBM. However, it is hard for NK cells to cross the blood brain barrier and infiltrate into hypoxic GBM cores. NK cells were one of the least numerous immune cell populations infiltrating the GBM [1]. Moreover, the generation and accumulation of immunosuppressive substances such as adenosine in the tumor microenvironment (TME) can inhibit NK cell function such as through the reduction of NKG2D expression [2]. **Methods:** We developed an innovative approach to improve immunotherapy of GBM via 1) generation of multi-functional CAR-NK cells which consist of a cleavable single chain antibody targeting CD73 alongside dual chimeric antigen receptors directed against GD2 and NKG2D ligands and 2) targeting the key autophagy gene *BECN1* in GBM cells to drive NK cells infiltration. **Results:** We have designed and synthesized a multi-functional CAR construct that expresses a CD73 scFv which is cleavable by GBM-associated proteases, and a dual CAR redirected against ligands for NKG2D and GBM-associated GD2 receptors (**Fig. 1A** and **B**). We have generated genetically modified NK-92 cells through a simple, efficient and biocompatible non-viral mRNA transfection method. Transfected NK-92 cells show significantly higher expression of NKG2D, GD2 scFv and CD73 scFv, respectively (**Fig. 1C-F**). We identified that the protease-sensitive linker could be cleaved by treatment with uPA to release the expressed CD73 scFv. Furthermore, CAR-NK-92 cells showed a significantly higher *in vitro* killing ability towards GBM43 targets, patient-derived GBM cells with both high CD73 and GD2 expression. (**Fig. 1G**). In order to improve homing of NK cells to GBM, we showed that targeting autophagy with chloroquine (CQ) can inhibit the proliferation of GBM43 as well as induce high secretion of the chemokine CCL5 (**Fig. 1H** and **I**). We also generated *BECN1*$^{-}$ GBM43 cells through *BECN1* short hairpin (shRNA) lentiviral particles to amplify the recruitment of NK cells to GBM bed. **Conclusions:** We have built multifunctional NK cells that show improved *in vitro* cytotoxicity against GBM cells through increased resistance to

the immunosuppressive TME via adenosinergic CD73 blockade and the ability to specifically target GBM cells via dual CARs. We have also recorded enhanced chemokine (CCL5) secretion from GBM cells by targeting the autophagy pathway. Based on these results, in order to achieve combined therapeutic effects, we are currently characterizing the effect of higher CCL5 secretion on the recruitment of NK cells into local GBM sites and evaluating these multi-functional NK cells pre-clinically. Taken together, this novel approach will provide a promising platform for the treatment of GBM with reprogrammed NK cells.

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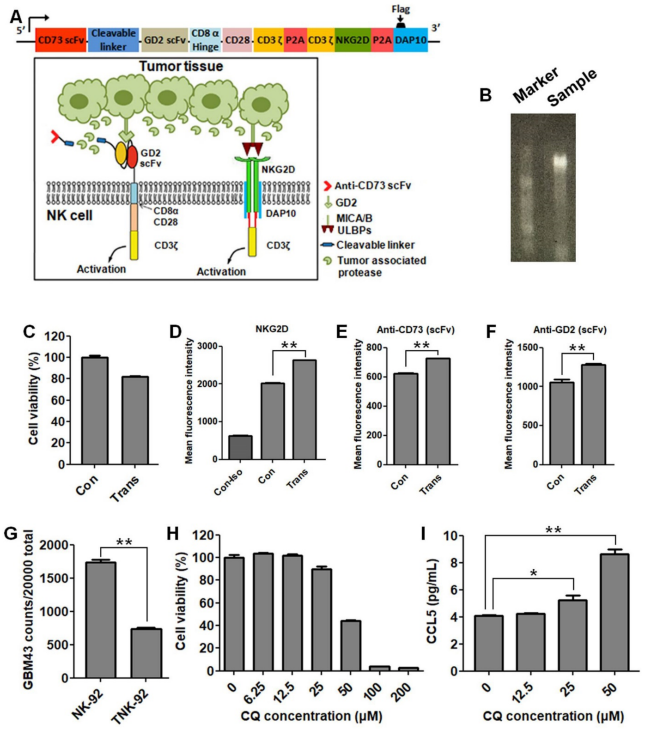

Fig. 1. (**A**) Multi-functional, responsive CAR constructs; (**B**) Agarose gel electrophoresis images of CAR mRNA; (**C**) NK-92 cell viability after 48 hours post-transfection; (**D-F**) Expression of NKG2D, anti-CD73 scFv and anti-GD2 scFv on transfected CAR-NK cells, respectively; (**G**) Killing efficiency against GBM43 after co-incubation with transfected NK-92 at a E:T ratio of 10:1 for 16 hours; (**H** and **I**) Effects of CQ on GBM43 cell viability and CCL5 secretion after 24 hours treatment. Data are presented as the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

987. Next Generation T-Cell Therapy for CMV Infection Post Allogeneic SCT

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Background: Cytomegalovirus (CMV) infection occurs in 60-85% of CMV seropositive patients and in 20-40% of seronegative patients with seropositive grafts post-allogeneic stem cell transplantation (ASCT). This is largely a consequence of quantitative and qualitative deficiencies in T cell function. Untreated, 50%, will develop CMV disease and even with conventional therapy, up to 10% will develop life-threatening complications. Despite advances, antiviral drug treatments are often unsatisfactory due to toxicity, viral resistance, high costs and prolonged hospitalisation. Attempts to hasten restoration of CMV immunity by adoptive transfer of CMV-specific T cells have demonstrated the potential to control CMV infection with minimal side-effects in ASCT patients. However, patients at highest risk for CMV infection and related complications are those with graft-versus-host-disease (GvHD) requiring immunosuppression with glucocorticoids, which reduce both the number and function of CMV T-cells, rendering such patients ineligible for CMV T-cell immunotherapy trials. **Aim:** We hypothesised that donor-derived, high purity, selected CMV T cells could be genome-edited using CRISPR-Cas9 to be resistant to glucocorticoids by 'knock out' (KO) of the glucocorticoid receptor (GR) yet retain viral-reactivity and cytotoxic capability. We describe preclinical development leading to large scale engineering runs to Good Manufacturing Practice (cGMP) compliance for use in a clinical study. **Methods:** We evaluated a number of targets within the GR gene locus, and validated guides to target exon 2, scaling all steps of the cell manufacture process to GMP-readiness. The proposed therapeutic comprises HLA-multimer-selected, high purity CMV+ donor T-cells, genome-edited using CRISPR/Cas9 to knock out the GR. Cells are stimulated and expanded in vitro to numbers relevant for clinical use. Testing includes: (1) Efficiency of GR KO in T-cells, determined by mRNA transcript levels and targeted deep-sequencing; (2) Functionality of GR KO T-cells by cytokine release, cytotoxicity and proliferation assays and (3) Immunophenotypic profiling for maturation and exhaustion using selected markers. **Results:** (1) We show that HLA-multimer technology permits selection of CMV-specific T cells to a median of 92% purity (range 88-97%). In reactivity assays, selected T-cells show antigen-specific cytolytic activity against target cells in vitro. (2) Using our guides, we report efficient CRISPR/Cas9 mediated knockout of the GR in primary T cells to a median of 60% efficiency (range 45-85%). (3) In contrast to control T-cells, genome-edited GR knockout T-cells demonstrate resistance to glucocorticoid-mediated T-cell death in vitro. Furthermore, cytolytic activity of GR knockout CMV T-cells is preserved despite concurrent glucocorticoid exposure, illustrating the desired functionality for clinical application. (4) To achieve the T-cell numbers required for patient use, we optimised a scaled-up activation/expansion protocol with preservation of central memory T-cell elements. **Conclusion:** We report a novel immunotherapeutic strategy for the treatment of CMV infection following ASCT in patients with GvHD requiring glucocorticoids, a population normally excluded from CMV T-cell therapy trials. We have additionally developed a cGMP-ready manufacture process for use in a Phase I clinical study.

988. Engineered Type-1 Regulatory T Cells for Treatment of Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplant Recipients

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Type 1 regulatory cells (Tr1) are a promising therapy for the prevention of Graft-versus-Host-Disease (GvHD) in allogeneic hematopoietic stem cell transplantation (aHSCT) due to their ability to promote immunological tolerance. Tr1 cells can also exert a direct graft-versus-leukemia (GvL) effect by killing myeloid cells, demonstrating a potential additional therapeutic synergistic effect of Tr1 when used in concert with aHSCT to treat myeloid leukemia patients. Due to the scarcity of endogenous Tr1 cells, a number of different *in vitro* protocols have been developed to produce sufficient numbers of Tr1 cells for use as a cell therapy. However, subsequent characterization shows only 10-30% purity of Tr1 cells in the final product, with the remainder either not contributing to or actively hindering the suppressive potential of the product. To address this, our group has developed a protocol to efficiently produce Tr1 cells for use as a cell therapy by lentiviral transduction of the human *IL10* into CD4+ T cells, which we have termed LV-10. Successfully transduced IL-10 expressing LV-10 cells are isolated using the clinical grade selection marker ΔNGFR and expanded using irradiated allogeneic feeder cell mixtures. LV-10 cells constitutively express moderate levels of IL-10 that can be increased 5-10 fold upon TCR stimulation. LV-10 cells also mimic the classic Tr1 cytokine profile by downregulating expression of IL-4 and increase expression of IFN-γ compared to control cells transduced only with green fluorescent protein (LV-GFP). *In vitro*, LV-10 cells kill myeloid cells through a perforin/granzyme B-mediated mechanism and can also suppress the proliferation of responder CD4+ T cells through secretion of IL-10 and TGF-β1. We are currently working on identifying additional features of the LV-10 cells that will help us understand their function. We compared the transcriptomes of LV-10 cells against LV-GFP, which revealed that resting LV-10 cells specifically overexpress *IL22* at baseline. By ELISA, we validated that LV-10 cells secreted high levels of IL-22 (10240 pg/mL) compared with LV-GFP (214.7 pg/mL) after TCR stimulation. This suggests that LV-10 cells, when administered as an add-on cell therapy to AML patients treated with aHSCT, could also have a role in tissue repair via their secretion of IL-22. Since IL-10 and IL-22 are rarely co-expressed by endogenous T cell subsets yet are regulated by a common *STAT3* signaling pathway, we are also initiating studies to investigate genetic and epigenetic regulation of these genes in the LV-10 system. We are currently validating additional novel biomarkers identified by the transcriptomic data that could be used to define stages of Tr1 phenotype development in LV-10 cells. This insight will be used to accelerate Tr1 polarization in vitro, which currently requires several expansion cycles. We are also initiating *in vivo* experiments to test the safety of LV-10 cells and determine whether LV-10 shows differences in efficacy against autologous or third-party responder T cells in a xeno-GvHD model.

989. Potentiating Engineered T Cells with Durable Epigenetic Repression of Immune Checkpoint Genes

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Reactivation of T cell function via blockade of immune checkpoint receptors including CTLA4 and PD-1 has revolutionized the treatment of cancer. However, efficacy is observed only in tumors that are particularly immunogenic, and systemic use has potency-limiting toxicities. Another transformative technology, engineered T cell therapies (CAR-T, TCR-T), has the potential to address the lack of existing tumor-reactive T cells. The potency of CAR-Ts and TCR-Ts can also be enhanced by antagonizing T cell exhaustion mediated by immune checkpoint genes. We therefore sought to develop a strategy for checkpoint gene repression that was limited to engineered T cell products but was safer than gene knock-out. Repression of gene expression via targeted delivery of epigenetic regulatory factors is well-described, though difficult to implement with both high potency and high specificity. Additionally, in order to be effective in the setting of an autologous graft, checkpoint gene inhibition must endure through the critical ~14-28d post-infusion period. Here we report the development of a highly potent, single-gene specific epigenetic repressor of PD-1 that is capable of sustained epigenetic repression of gene expression following transient introduction and clearance of an epi-repressor protein. Using genomic footprinting, we identified a unique chromatin structural feature that could be precisely targeted by an epi-repressor comprising Transcription Activator-Like Effector (TALE) DNA binding domains coupled to a KRAB repressor variant and achieve potent gene control in primary engineered T cells. Remarkably, durable repression is variable and tunable at single-nucleotide resolution, requiring base-by-base addressing to identify optimal repressors. We show further that it is possible to engineer single-gene-specific repressors, and that specificity is likewise under nucleotide-level precision control. Using this approach, we have engineered a suite of potent and specific reagents targeting immune checkpoint genes (e.g. PD-1, LAG3, TIM3, and CTLA4) and other clinically relevant T cell targets that can be applied as single effectors or in combination to achieve control of the immune checkpoint system and key aspects of T cell function and differentiation. Because they are non-disruptive to genome integrity, these novel effectors can be combined with current pipelines for generating CAR-T and TCR-T cell products to produce enhanced cell therapies without incurring the genotoxic consequences of double-stranded DNA breaks.

990. Differentiating T-Cells from hiPSCs to Create Off-The-Shelf SPEAR T-Cell Therapies

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Harnessing the power of T-cells to fight cancer has generated many different experimental treatment options for patients. The majority of these therapies use the patients' own T-cells that must be expanded *ex vivo* to generate large numbers of cells, which can recognize tumor either via enrichment of endogenous tumor-infiltrating lymphocytes (TILs) or by the introduction of an engineered receptor. Using patient cells as starting material introduces inherent variability into any manufacturing process, as the functionality of these cells can be affected by health, age, and prior treatment regimens. Individualized manufacturing also leads to a complex chain of identity and a lag time in patient treatment while the personal cell product is being manufactured. Human induced pluripotent stem cells (hiPSCs) offer an alternative source of T-cells that can be used to treat patients by enabling pre-manufacturing and freezing of T-cell product stock, thus shortening the lead time between patient screening and treatment. hiPSCs have the ability to divide indefinitely in an undifferentiated state and are amenable to genetic editing to produce stem cell banks that can be characterized for clinical use. hiPSCs can be directed to differentiate through multiple precursor stages towards a mature T-cell phenotype over a prolonged culture period *in vitro.* We directed differentiation of cells from a pluripotent state ($SSEA4^+OCT4^+TRA\text{-}160^+$) through various intermediate stages: $CD34^+CD45^+$ hematopoietic progenitor cells (HPC), pro/preT $CD7^+CD5^+$cells, $CD4^+CD8^+$ double positive cells towards $CD3^+CD8^+TCR^+$ single positive T-cells. TCR^+ cells generated by this process show many similarities to mature T-cells from peripheral blood. Single cells were isolated and assessed by PCR using a panel of 96 genes to discern lineage and differentiation stage. Unedited hiPSCs can express either αβ or γδ TCR after differentiation; both subsets show multiple effector functions and release cytokines (IFNγ and TNFα) and lytic granules (Granzyme A and B, CD107a) when stimulated. Lentiviral transduction of progenitors with SPEAR (specific peptide enhanced affinity receptors) promotes surface expression of CD3 and SPEAR on the differentiated cells, which show antigen-specific activation. These TCR^+ cells show functional characteristics that make them ideal to produce allogeneic, off-the-shelf T-cell therapies for oncology, where consistent batches can be manufactured to treat multiple patients. This approach, when combined with genetic engineering to express SPEAR TCRs, thus enhancing tumor recognition and preventing GVHD through endogenous TCR, could accelerate the next generation of SPEAR T-cell therapies.

991. Nascent Transcripts of Target-Activated CD19-CAR-T Cells Reveal Early Activation of the C-REL and NFκB Pathway

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Background: Chimeric antigen receptor (CAR) T cells are modified genetically enabling activation of their killing machinery as well as cell proliferation and survival programs upon target cell recognition. The need to be able to better control these cells calls for in-depth understanding of CAR functionality. Here we have used global run-on (GRO) sequencing, which measures the activity of RNA polymerase II-driven transcription, to reveal early signs of CAR-derived signaling and to identify the key initiating transcription factors. **Methods:** CD19-targeted second-generation CAR T cells carrying the CD28 and CD3z signaling domains, derived from healthy donors were briefly stimulated in a co-culture with CD19-positive NALM6 leukemia cells, or with adherent NIH3T3 mouse embryonic fibroblasts expressing a truncated CD19 antigen. Formation of immune synapses and nuclear translocation of NFκB were evaluated using an imaging flow cytometer. To analyze immediate changes in gene transcription, activated CAR T cells were recovered and nuclei were isolated and nascent RNAs extended by running nuclear run-on assays (NRO) to produce highly enriched cDNA libraries, which were then sequenced by next-generation sequencing. The results were confirmed with Taqman quantitative PCR and nuclear translocation assays. **Results:** CD19-targeted CAR T-cells formed actin-positive immune synapses upon target cell binding. The expression of a total of 136 nascent transcripts was significantly changed in activated CD19-CAR-T cells compared to controls without activation. The most significantly activated pathway on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was the cytokine-cytokine receptor interaction pathway (04060). The *IL-2* gene showed by far the highest transcriptional activation. Several genes linking to the nuclear factor (NF) κB signaling pathway were highly represented e.g. *c-REL, IFNγ, NFKB1 (p50), NFATC1, TNFRSF9 (4-1BB), NR4A3,* and *SEMA7A*. At the protein level, NFκB1 was translocated into the nuclei of CAR T-cells upon target cell recognition, indicating functional activation of this pathway. **Discussion:** C-REL and NFκB are transcription factor of the NFκB family that are important regulators of function and differentiation of activated T cells. Many c-REL target genes are involved in lymphoid cell growth and survival. Interestingly, c-REL also participates in a priming mechanism involving IκB factor that permits a T cell to respond more rapidly and efficiently. Thus, agents that modulate c-REL activity might have beneficial effects on CAR-T cell regulation *in vivo*. This study provides unique information about the early transcriptional effects of CAR-driven signaling and the methodology could be applied to characterize patient-derived CAR-T cells by matching distinctive transcriptional signatures to known clinical outcomes.

992. AdoptCell®-NK: A New Class NK Cells Manufactured in Accordance with GMP/GCTP that Can Eliminate the Solid Tumors

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Background: Cancer immunotherapy has been established as a new therapeutic category since the recent success of immune checkpoint inhibitors and a type of adoptive immunotherapy, namely chimeric antigen receptor-modified T cells (CAR-T). Although CAR-T demonstrated impressive clinical results, serious adverse effects (cytokine storm and on-target off-tumor toxicity) and undefined efficacy on solid tumors are important issues to be solved. We've developed a cutting-edge, simple, and feeder-free method to generate highly activated and expanded human NK cells from peripheral blood (Saito S, et al. *Hum Gene Ther*2013), and have been conducting further investigation why our new type of NK cells, named as GAIA-102, are so effective to kill malignant cells. **Materials and Methods:** Cryopreserved PBMCs purchased from HemaCare Corporation were processed by using LOVO and CliniMACS®Prodigy (automated/closed systems). $CD3^+$and $CD34^+$cells were depleted by CliniMACS®beads, and the cells were cultured at a concentration of 1 x 10^6cells/ml with high concentration of hIL-2 and 5% UltraGRO®for 14 days in our original closed system. Then, we confirmed the expression of surface markers, CD107a mobilization and cell-mediated cytotoxicity against various tumor cells and normal cells with or without monoclonal antibody drugs *in vitro*and antitumor effects against SKOV3 *in vivo*. **Results and Discussion:** Importantly, we've found that our GAIA-102 exhibited $CD3^-$/$CD56^{bright}$/$CD57^-$immature phenotype that could kill various tumor cells efficiently from various origins, including Raji cells that was highly resistant to NK cell killing. More importantly, massive accumulation, retention, infiltration and sphere destruction by GAIA-102 were affected neither by myeloid-derived suppressor cells nor regulatory T-lymphocytes. GAIA-102 was also effective *in vivo*to murine model of peritoneal dissemination of human ovarian cancer; thus, these findings indicate that GAIA-102 has a potential to be an 'upward compatible' modality over CAR-T strategy, and would be a new and promising candidate for adoptive immunotherapy against solid tumors. We now just started GMP/GCTP production of this new and powerful NK cells and first-in-human clinical trials in use of GAIA-102 will be initiated on 2019.

Use of New Technologies for Hepatic Therapy

993. Efficient and Long-Term Correction of Liver Metabolic Diseases by Coupling AAV-Mediated Promoterless Gene Targeting to SaCas9 Nuclease

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AAV-mediated gene replacement therapy in the liver has demonstrated efficacy in adult patients, but faces potential limitations associated with episomal DNA loss during hepatocyte proliferation, a major concern when considering paediatric disorders. Gene targeting is a promising approach, overcoming these issues by means of the permanent modification of the genome. We successfully performed a gene targeting strategy, named GeneRide, without nucleases in Crigler-Najjar mice, rescuing neonatal lethality by inserting a promoterless *UGT1A1* cDNA just upstream of the albumin stop codon, such that both proteins are produced from a chimeric mRNA. To further increase the recombination rate and, thus, therapeutic efficacy, we took advantage of the CRISPR/*Sa*Cas9 platform.To set up the strategy, we first i.v. injected neonatal WT mice with two AAVs, serotype 8: one expressing the *Sa*Cas9, under the control of a liver-specific promoter, and the sgRNA, and one containing the albumin homology regions plus a promoterless cDNA (eGFP, or human coagulation factor IX, hFIX). Targeting efficiency increased up to ~100-fold compared to the group without nuclease, reaching ~24% of eGFP-positive hepatocytes and ~200% of normal hFIX levels, respectively. Next, we applied the strategy to our Crigler-Najjar mouse model by i.v. transducing neonatal P2 mutant mice with two AAV8 vectors carrying the SaCas9 and the sgRNA (high or low dose, 6.0E10 and 2.0E11 vg/mouse, respectively), and the donor sequences with the hUGT1A1 cDNA (2.0E11 vg/mouse). All treated mice were rescued from neonatal lethality, with normal plasma bilirubin levels at 10 months after administration. Protein levels were ~6-fold higher than in WT liver, and immunofluorescence analysis showed ~5% of hUGT1A1-positive hepatocytes with a 90-fold increase in recombination rate. Liver histologyand inflammatory markers were normal and no off-targets were detected in predicted sites. Molecular analysis of the on-target site showed that most gaps were shorter than seven nucleotides, with no changes in plasma albumin levels, supporting the safety of the approach.In conclusion, we demonstrated enhanced homologous recombination when the GeneRide strategy was coupled to the CRISPR/*Sa*Cas9 platform. The improved efficacy and safety supports the potential of the developed approach when considering clinical application.

994. Gene Editing Approach to Disrupt Hydroxyacid Oxidase 1 for the Treatment of Primary Hyperoxaluria Type 1

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Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive disorder that leads to kidney and liver failure. PH1 is caused by a mutation in the alanine glyoxylate aminotransferase (AGXT) gene, which encodes a key metabolic enzyme responsible for converting glyoxylate to glycine in the liver. The inability to metabolize glyoxylate leads to the metabolic overproduction of oxalate, which yields insoluble calcium oxalate crystals and the accumulation of these crystals leads to progressive organ failure. Here, we used a novel, minimally disruptive, gene editing approach to disrupt the mechanism of action of hydroxyacid oxidase 1 (HAO1), an upstream enzyme in the metabolic pathway for glyoxylate. Successful gene editing and disruption HAO1 is expected to result in increased levels of glycolate, a harmless intermediate of the glycine metabolic pathway, thereby preventing the formation of calcium oxalate crystals. Targeting of the HAO1 gene was initially characterized in vitro with ARCUS nucleases expressed from mRNA in multiple cell lines. Digital droplet PCR (ddPCR) analysis of cellular genomic DNA (gDNA) showed a significant level of editing in the HAO1 target site across cell lines. In vivo analysis of HAO1 targeting was evaluated in both wild type and AGXT knockout (KO) mice, a mouse model of PH1. We intravenously administered both strains of mice with an AAV vector expressing the ARCUS HAO1 nuclease. In C57BL6 mice we observed >50% editing by amplicon-seq analysis and found increased glycolate serum levels in treated mice compared to untreated controls by mass spectrometry. We observed >30% editing of HAO1 in AGXT KO mice, which correlated with a dose-depending increase in serum glycolate levels. At the highest dose tested, there was a 79% increase in urine glycolate levels and a concomitant decrease in urine oxalate levels of 75%. In vivo targeting was also evaluated in non-human primates injected with AAV expressing the ARCUS HAO1 nuclease. gDNA was isolated from liver biopsies and editing was measured using amplicon-seq. Our results indicate that ARCUS nucleases are effective at targeting HAO1 in both mice and NHP with high level editing of the HAO1 gene. Increased glycolate levels in serum indicate a significant effect on HAO1 mediated glycolate to glyoxylate pathway suggesting that this approach could be effective for the treatment of PH1.

995. Nuclease-Free and Promoter-Less Aavhsc-Mediated Genome Editing In Vivo Corrects the Disease Phenotype in a Mouse Model of Phenylketonuria

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A group of Clade F adeno-associated viruses isolated from human CD34+ hematopoietic stem cells (AAVHSCs) mediate homologous recombination (HR)-based and nuclease-free genome editing that is efficient and precise. Here we describe the utility of AAVHSCs for *in vivo* gene insertion at two genomic loci and in two murine models: phenylketonuria (PKU) and humanized liver. Single-stranded DNA in AAVHSC vectors containing a promoter-less luciferase cassette flanked by sequences homologous to mouse F8 gene (AAVHSC-mF8-Luc) to drive HR integration to the mF8 locus and express off the endogenous promoter were prepared. A single intravenous (IV) administration of AAVHSC15-mF8-Luc into albino C57BL/6J mice resulted in liver-specific luciferase expression that was dependent on dose the of AAVHSC15-mF8-Luc administered. Genome editing assessed by droplet digital PCR was linear across three doses, reaching a maximum of 20% of alleles edited at the highest dose tested, and was linearly correlated with total body luciferase expression. Luciferase expression within tissues was dependent on the presence of functional transgene splice acceptor and ribosomal skipping sequences (SA2A) as removal of SA2A sequences reduced luciferase expression greater than 95%. Durable editing of the hepatic F8 locus and luciferase expression was seen for 470 days (end of study). AAVHSC-mediated gene insertion to correct a disease phenotype was assessed in Pah^{enu2} mice, the standard PKU murine model. AAVHSC15 single-stranded genome editing vector containing a promoter-less cDNA encoding human *PAH* gene flanked by sequences homologous to the murine *Pah* gene (AAVHSC15-mPAH) was prepared. A single IV dose of AAVHSC15-mPAH produced a significant reduction in serum Phe to below the targeted therapeutic level in humans ($\leq$360 umol/L) one week post-dosing and was sustained to the end of the study at eight weeks. Elevations in serum levels of tyrosine (Tyr), a byproduct of Phe metabolism required for production of neurotransmitters, were noted resulting in normalization of the serum Phe/Tyr ratio. Changes in coat color from brown to black were observed in all treated animals, indicating reconstitution of the Phe-Tyr-melanin metabolic pathway. Molecular characterization of liver DNA from treated animals treated displayed on average 6.4 ±1.9% of *Pah* alleles successfully edited (range 3-9%), resulting in expression of human-specific *PAH* mRNA. Next-generation sequence analysis across the integration site revealed no insertion or deletion mutations. No reduction of serum Phe was observed in Pah^{enu2}mice using an AAVHSC15 genome editing vector with homology arms targeting the orthologous region of human *PAH* (AAVHSC15-hPAH), supporting selectivity of editing across species. In FRG^{-}knockout mice repopulated with human hepatocytes, animals treated with a single IV dose of AAVHSC15-hPAH showed 5-6% of *PAH* alleles edited in human hepatocytes with no measurable editing

in mouse hepatocytes at sixweeks post-dosing. Genome editing in human hepatocytes was associated with increases in human specific *PAH* mRNA to levels observed in normal human liver. Thus at two distinct genomic loci, these *in vivo*data show AAVHSCs can mediate promoter-less, nuclease-free and precise gene integration at efficiencies that reach therapeutic levels, demonstrating this platform can be employed for precise *in vivo* gene editing in the liver and correction of disease phenotype.

996. Reduction of Transthyretin Expression by AAV Gene Delivery of a Novel Endonuclease in Mice

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Transthyretin amyloidosis (ATTR) is a progressive and fatal disease caused by the accumulation of transthyretin (TTR) amyloid fibrils in tissues, which disrupts organ function. ATTR can occur as either an inherited or age-related amyloidosis with the two primary manifestations of hereditary ATTR being polyneuropathy and cardiomyopathy. In ATTR, the tetrameric TTR protein dissociates in the blood and, subsequently, the misfolded monomers aggregate into amyloid fibrils. As TTR is primarily synthesized in the liver, liver transplantation can cure patients with familial ATTR. Additional treatment approaches include TTR stabilizers and the recently approved RNA interference therapeutic, Patisiran. However, these treatments require regular readministration. A gene editing approach could provide an effective one-time treatment. Here, we evaluated a meganuclease-based gene editing approach to reduce TTR levels. The meganuclease TTR5-6 targets a 22 bp sequence that is conserved in mice, rhesus macaques, and humans. We evaluated the ability of the nuclease to edit both the mouse genomic TTR sequence and the V30M mutant version of the human TTR gene expressed as a transgene by an adeno-associated viral (AAV) vector. The V30M mutation in TTR is the most common mutation associated with polyneuropathy in ATTR patients. We intravenously administered immunodeficient mice with an AAV8 vector expressing human TTR containing the V30M mutation in the sequence. Two weeks later, we readministered these mice with one of three doses of an AAV8 vector expressing the TTR5-6 meganuclease. Following intravenous administration of $3x10^{11}$ genome copies (GC)/kg, $3x10^{12}$ GC/kg, or $3x10^{13}$ GC/kg of the meganuclease vector, we observed insertions and deletions (indel) percentages of 10%, 52%, and 59%, respectively, in the mouse TTR gene with corresponding reductions to 92%, 36%, and 21% of normal mouse TTR levels. In the same mice, we evaluated the ability of the TTR5-6 nuclease to edit the episomal human TTR sequence. We found that 5%, 50%, and 56% of the indels in the human TTR sequence provided by the AAV vector correlated with reductions in human TTR levels of 50%, 67%, and 99% following administration of $3x10^{11}$ GC/kg, $3x10^{12}$ GC/kg, or $3x10^{13}$ GC/kg of the meganuclease vector, respectively. In conclusion, the significant reduction in serum TTR levels following genomic editing of the TTR gene indicates that this approach could be effective for the treatment of ATTR.

997. Exploiting the Regenerative Capacity of Liver for Nuclease-Free Genome Editing

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Genome editing techniques based on DNA homologous recombination (HR) have been extensively exploited for gene therapy approaches, but also to study fundamental biological mechanisms in animal models and cell lines. However, the majority of these strategies rely on the utilization of nucleases in order to increase the rate of HR and genomic integration. Adeno-associated viruses (AAV) have shown to naturally induce a certain rate of HR, and our lab has optimized a promoterless nuclease-free approach (AAV-HR) for genome editing by exploiting the induction of HR upon AAV transduction (Barzel et al., Nature, 2015). Specifically, the human factor IX (FIX) gene was flanked by murine *albumin* homology arms in order to integrate, in a non-disruptive way, the FIX into the *albumin* locus. Thus, the chimeric mRNA made by the host transcriptional machinery will produce the albumin protein and the human FIX. This technology was named GeneRide™ and it has been successfully tested by various laboratories in animal models for the treatment of hemophilia B and liver metabolic disease such as the methylmalonic acidemia, Crigler-Najjar and ZZ alpha 1-antitrypsin deficiency. Nevertheless, the percentage of edited cells, upon AAV-HR liver gene transfer, has been shown to be between 0.4 and 1% of total hepatocytes. In order to improve the rate of HR, we recently performed a high throughput screening in vitro to identify possible factors that may enhance the HR. The Fanconi Anemia complementation group M (FANCM), RecQ Mediated Genome Instability 1 (RMI1), and Bloom Syndrome RecQ Like Helicase (BLM) came up as the genes that may impair the process of HR. Thus, in vitro experiments in which we inhibited these genes showed up to a 18-fold improvement in nuclease-free AAV-HR. Liver has the unique capacity to regenerate in disease processes that lead to a hepatic loss or after surgical resection (partial hepatectomy [PHx]). Several events take place during liver regeneration and each of these events are precisely tuned throughout this process. We found that FANCM and RMI1 were downregulated 24 and 36 hours after PHx in mice, and this is concomitant with the previously established peak of DNA synthesis. To test this hypothesis, we have compared AAV GeneRide™ vectors ($3x10^{11}$vg/mouse) designed to express human FIX only after undergoing homologous recombination with the murine *albumin* locus in mice after PHx in order to assess whether the HR was possibly increased. Mice injected with vector 24 and 36 hours after surgery, displayed about 2x higher levels of circulating hFIX compared to non-hepatectomized animals. Since FANCM complex was reported to be involved in mechanisms of DNA repair and HR, we are currently investigating whether different proteins in the DNA mechanism repair/synthesis might affect the HR during regeneration or some other factors such as chromatin-related complexes or cell cycle proteins may be involved. Finally, we are pursuing additional studies in mouse models of liver disease which cause pathological liver regeneration.

998. Genome Editing of the Liver for Treatment of Alpha-1 Antitrypsin Deficiency Using Homology-Independent Targeted Integration (HITI)

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Homology directed repair (HDR) is often selected for correction of genomic mutations for therapeutic benefit due to its favourable repair fidelity, but activity of the main repair pathway, non-homologous end joining (NHEJ) is several-fold higher in most systems. Using NHEJ, Homology-Independent Targeted Integration (HITI) allows for precise integration of a chosen sequence by cutting both the genome and donor sequence. This approach is applicable for diseases such as Alpha-1 Antitrypsin (AAT) deficiency in which genome editing could allow for expression of functional AAT whilst simultaneously reducing expression of the highly prevalent, toxic PI*Z form of the gene. Here, we assess the feasibility of HITI genome editing for AAT deficiency in liver cell lines and human liver organoids. A fluorescent reporter 293T cell line containing an mCherry cDNA flanked by two defined murine saCas9 gRNA target sites provided a platform to test four permutations of HITI dependent genomic integration. Each permutation could support a different therapeutic application, using mCherry as a proxy for the target mutant gene. One permutation proved optimal for the treatment of the PI*Z mutant version of AAT. In this permutation, the integration event is targeted to the 5` UTR region of AAT and the integrated cDNA (reporter or therapeutic) is followed by a transcription blocker to prevent expression of the downstream PI*Z allele. Transfection of Huh-7 liver carcinoma cells with a 1:2 molar ratio of SaCas9/HITI Donor plasmid was effective, both in expressing the integrated cDNA of mNeonGreen from the AAT promoter as well as in preventing endogenous AAT expression in ~12% of total cells despite only ~30% transfection efficiency (n=4). A ddPCR copy number variation assay at the 5`-integration junction confirmed this observation. Sequencing showed that HITI integration was almost seamless (~90% in 78 clones from 2 different loci) with any mutations limited to 1 or 2bp indels, implying that NHEJ is less error-prone than expected. Analysis of a pilot capture-seq experiment proved that the majority of the integration events occurred at the intended integration site. This proof-of-concept in a cell line is currently being translated to more relevant models, including human and murine liver organoids. Human liver samples were obtained, with consent, from liver resection surgery and healthy tissue, as assessed by a pathologist, was processed into liver organoids. Organoids were transfected with plasmids or transduced with recombinant adeno-associated virus (rAAV) as a single cell suspension to allow access to the basolateral membrane of the hepatocytes. Transduction of mouse organoids with rAAV of various serotypes showed that rAAV capsid 6.2 was the most efficient with 45% and 39% of organoids EGFP-positive 2 days post-transduction (n=2 independent experiments). Human liver organoids cultured as immature, actively dividing cells were differentiated into mature liver organoids as evidenced by AAT expression, providing a human liver model for ongoing genome editing studies. We conclude that HITI is capable of efficiently integrating a DNA sequence into a targeted location in the genome in a variety of therapeutically applicable permutations, one of which can provide potential correction of AAT deficiency regardless of the patients' mutation. Human liver organoids provide a physiologically and genetically relevant *in vitro* model of the human liver, acting as an appropriate stepping stone to *in vivo* studies in mouse disease models.

999. Hemophilia a Cured in Mice by Crispr-Based In Vivo Genome Editing of Human Factor FVIII

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Hemophilia A is a monogenic disease with a blood clotting factor VIII (FVIII) deficiency caused by a F8 gene mutation. Current and emerging treatments for hemophilia A are, respectively, replacement therapies via recombinant FVIII protein injection and gene therapies via adeno-associated virus (AAV)-delivered B-domain deleted F8 (BDD-F8) transgene in an episome. Both approaches are costly and short-lived. Here we describe a CRISPR/Cas9-based in vivo genome editing method enabling permanent chromosomal integration of a modified human BDD-F8 at the albumin (Alb) locus in liver cells. Using this approach in hemophilic mice, BDD-F8 was expressed in liver cells as functional human FVIII, leading to increased plasma levels of FVIII and restoration of blood clotting properties. These effects were sustained for at least 7 months with no detectable liver toxicity or off-target effects in genomic regions where CRISPR-mediated non-specific cleavage was predicted. The same approach was conducted in non-human primates (NHP) and humanized liver mouse model (FRG mice). No liver toxicity was detected following viral administration. Liver infectivity and genome editing efficiency (indel %) were determined by ddPCR assays. Based on these findings, our genome editing approach, through site-specific human BDD-F8 integration, may offer an efficacious, long-term and safe treatment for patients with hemophilia A. Further studies in NHP and humanized liver FRG mice are ongoing to evaluated in vivo gene insertion efficiency and characterize BDD-F8 expression and secretion.

1000. CRISPR/Cas9-Mediated Gene Knockout to Address Primary Hyperoxaluria

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Primary hyperoxaluria (PH) is a rare, autosomal recessive genetic disease caused by mutations in one of three genes (*AGXT*, *GRHPR* and *HOGA1*), giving rise to PH types 1, 2, and 3, respectively. In PH, glyoxylate breakdown is blocked, and accumulated glyoxylate is converted to oxalate, which in turn complexes with calcium to form insoluble deposits in the kidney and other organs, leading to renal failure and systemic oxalosis. Currently, the treatment of late-stage disease is limited to sequential or combined liver-kidney

transplantation. Alternatively, these diseases could be addressed by gene therapy, genetic correction of the mutant gene, or metabolic engineering to avoid glyoxylate formation and allow the accumulation of a safely excreted metabolite. Here we report the effects of CRISPR/Cas9-mediated knockout of *Ldha* and *Hao1*, two genes involved in oxalate formation from glyoxylate. Knockout of *Hao1* is expected to cause the accumulation of glycolate rather than oxalate, which does not complex with calcium and is readily excreted. Guide RNAs spanning the murine genes were screened for editing activity, and selected gRNAs were formulated with *Spy* Cas9-encoding mRNA in lipid nanoparticles (LNPs). These LNPs were tested in both wild-type and PH1 disease model ($Agxt^{-/-}$) mice. We observed significant editing of both *Hao1* and *Ldha* in wild-type mice, correlating with a dose-dependent increase in the level of serum glycolate with editing of *Hao1*, while in the disease model we saw a reduction of urinary oxalate levels by 57% and 63% with editing of *Hao1* and *Ldha*, respectively. Oxalate reduction was sustained throughout the 15-week long observational phase. The data suggest that editing of genes in the glyoxylate detoxification pathway using a nonviral delivery approach constitutes a potential one-time treatment option for genetic forms of hyperoxaluria.