727. Characterizing the Adeno-Associated Virus 1 Sialic Receptor Binding Site and Its Overlap with Antigenic Epitopes

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Adeno-associated viruses (AAVs) are being development as gene delivery vectors and have shown promise in several clinical trials. And an AAV1-based vector has been approved by the European Commission for the treatment of lipoprotein lipase deficiency disease. However, limitations in tissue transduction specificity as well as neutralization by pre-existing antibodies remain are two major obstacles. To overcome these problems, it is important to characterize functional capsid regions that dictate virus binding to cellular receptors during infection and the potential overlap(s) of these regions with capsid antigenic epitopes. In this study, the sialic acid (SIA) binding site on AAV1 was determined by solving the structure of the AAV1-SIA complex using X-ray crystallography. Residues that form the SIA binding pocket are identical between AAV1 and AAV6; hence, structurally predicted SIA binding sites were mutated on both serotypes to confirm their role in the SIA interaction. Binding and transduction assays with these mutants in CHO cell lines Pro5, Lec2, Lec8, and Lec1, which display different terminal glycans, confirmed the structurally mapped binding pocket. Furthermore, native dot blot showed that several of the AAV1 SIA binding mutants can escape from antibody recognition. This finding is consistent with the overlap between the SIA binding site and the previously mapped AAV1 epitopes. Significantly, a glycan binding mutant with slightly reduced transduction ability is able to escape from antibody recognition. This study identifies an overlap between receptor recognition and antibody reactivity and provides amino acid level information for rational capsid engineering of AAV vectors for improved therapeutic efficacy.

728. Tissue-Directed Transgene Engineering for AAV and Lentivector Gene Therapy Approaches

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Clinical gene therapy frequently is encumbered by low transgene product biosynthesis at predictably safe vector doses. It has been hypothesized that the presence of rare codons may regulate transgene product expression through depletion of the available cognate tRNA pool. Codon optimization is the prominent strategy utilized to overcome this hypothesized limitation and involves replacing rare, presumably translation rate-limiting, codons with the most frequent ones. Typical algorithms attempt to match the codon usage frequency of the transgene coding sequence to that of target organism's total mRNA pool, which has been shown to approximate the overall available tRNA concentrations. Upon closer examination, it appears that both codon frequency and tRNA content vary between tissue types. Therefore, we hypothesize that codon-optimization can be improved by tailoring to the codon-frequency of the most highly expressed genes present in target cell types. Our tissue-directed codon optimization algorithm utilizes novel codon usage indices generated from target cell gene expression data. As proof of concept, we developed tissue-codon optimized variants of coagulation factor VIII (FVIII) to be utilized in lenti- and adeno-associated viral (LV and AAV, respectively) vectors. These two vectors are the leading platforms for clinical gene therapy of hemophilia A. LV is utilized to target autologous hematopoietic cell types ex vivo while AAV is delivered intravenously to genetically-modify hepatocytes. However to date, development-stage LV and AAV gene therapy products for hemophilia A have been characterized by low-level FVIII transgene product biosynthesis. Initially, we designed human hepatocyte-, monocyte- and standard overall human-optimized FVIII constructs (LCO, MCO and HCO, respectively) to be compared to wild-type FVIII. Upon initial examination, expression of LCO-FVIII was shown to be 3-fold greater than either MCO- or wild-type FVIII from the human hepatoma cell line, HepG2, following transient transfection. In contrast, LCO-FVIII and MCO-FVIII expression was diminished 12 and 4-fold, respectively, compared to wild-type in the 'neutral' human embryonic kidney 293T cell line. Furthermore, following hydrodynamic injection of naked plasmid DNA into hemophilia A mice, LCO-FVIII exhibited a sustained 10-fold increase in FVIII expression relative to the HCO-FVIII comparator. In attempt to generate a lead candidate for clinical translation, we utilized several of our most promising vector components to construct a liver-optimized AAV8 vector consisting of LCO-ET3, our previously described high-expression bioengineered FVIII variant, transcriptionally driven by a novel 146bp liver-directed promoter and adjacent MVM intron. Following intravenous delivery into hemophilia A mice, vector doses of 1e12 and 1e11 vector genomes per kg achieved sustained, predictable curative plasma FVIII levels of 200% and 20% of normal human levels, respectively. These initial results support the utility of our novel approach of clinical tissue-directed transgene optimization.

Targeted Genome Editing: Methods and Technology

729. Inheritable Silencing of Endogenous Gene by Hit-and-Run Targeted Epigenetic Editing

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Gene silencing holds great promise for the treatment of several diseases and can be exploited to investigate gene function and activity of the regulatory genome. Here, we develop a novel modality of gene silencing that exploits epigenetics to achieve stable and highly efficient repression of target genes. To this end, we generated Artificial Transcriptional Repressors (ATRs), chimeric proteins containing a custom-made DNA binding domain fused to the effector domain of chromatin-modifying enzymes involved in silencing process of Endogenous RetroViruses (ERVs). By performing iterative rounds of selection in cells engineered to report for synergistic activity of candidate effector domains, we identified a combination of 3 domains (namely KRAB, DNMT3A and DNMT3L) that, when transiently co-assembled on the promoter of the reporter cassette, recreate a powerful embryonic-specific repressive complex capable of inducing full and long-term (>150 days) silencing of transgene expression in up to 90% of the cells. The ATR-induced silencing was cell type and locus independent, and resistant to metabolic activation of the cells. Importantly, these findings were holding true also for endogenous genes embedded in their natural chromatin context, as shown for the highly and ubiquitously expressed *B2M* gene. Here, transient co-delivery of TALE-based ATRs resulted in loss of surface expression of B2M and, consequently, of the MHC-I molecules in up to 80% of the cells. This phenotype was associated with a drastic switch in the epigenetic and transcriptional state of the constitutively active *B2M* promoter, which become highly decorated with *de novo*

DNA methylation and deprived of RNAP II. Importantly, silencing was sharply confined to the targeted gene and resistant to INF-γ, a potent natural activator of *B2M*. We further extended these studies by showing that our silencing approach is portable to the CRISPR/dCas9 DNA binding technology. In this setting, comparable levels of *B2M* silencing (up to 80%) were achieved using either pools or even individual sgRNAs coupled to dCas9-based ATRs. Yet, adoption of this technology allowed performing simultaneous, highly efficient multiplex gene silencing within the same cell, as shown for *B2M*, *IFNAR1* and *VEGFA*. Finally, we assessed resistance of the silenced gene to activity of potent artificial transcription activators and chromatin remodelers, and found that only targeted DNA demethylation was able to reawaken the silent gene. This allowed performing iterative cycles of silencing and reactivation of the same gene in the same cell population. Overall, these data provide the first demonstration of efficient and stable epigenetic silencing of endogenous genes upon transient delivery of ATRs. This was accomplished by repurposing the ERVs silencing machinery, which instructs self-sustaining repressive epigenetic states to the target gene. While silencing of *B2M* might be used to generate universally transplantable allogeneic cells, our hit-and-run strategy provides a powerful new alternative to conventional gene silencing for both basic and translational research.

730. Permanent Correction of Diverse Muscular Dystrophy Mutations in Human Cardiomyocytes by Myoediting

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Dilated cardiomyopathy (DCM) is one of the most common lethal features of Duchenne muscular dystrophy (DMD), caused by diverse mutations in the X-linked dystrophin gene (*DMD*). Without dystrophin, a large cytoskeletal protein, muscles degenerate, causing myopathy. Although several gene therapies have been tested, there is no curative treatment so far. In 2014, we first used CRISPR/Cas9-mediated genome editing (termed Myoediting) to correct the mutation in the germ line of *mdx* mice, the mouse model of DMD [Long *et al.* ***Science*** 345:1184-8]. Recently, we applied Myoediting to postnatal muscle tissues in mice by delivering gene editing components via a harmless adeno-associated virus. Cardiac and skeletal muscle showed progressive rescue of dystrophin protein [Long *et al.* ***Science*** doi: 10.1126/science.aad5725]. These studies paved the way for novel genome editing-based therapeutics in DMD. We have now advanced Myoediting to cells from human DMD patients by engineering the permanent skipping of mutant or out-of-frame exons in the genome of DMD patient-derived induced pluripotent stem cells (iPSCs). We have optimized Myoediting of DMD mutations using pools of sgRNAs to target the top 12 hot spot mutant exons. We targeted the conserved RNA splicing acceptor/donor sites of each exon. NHEJ-mediated indels efficiently abolished the splicing sites and skipped the mutated or out-of-frame exons. Based on the known DMD mutations, we established a publicly available online resource (**Duchenne Skipper Database**) for selecting the optimal target DMD sequences for Myoediting, which will rescue dystrophin function in 60-80% of DMD patients. We performed Myoediting on representative iPSC-derived cardiomyocytes from multiple patients with point, deletion or duplication mutations and efficiently restored dystrophin protein expression in cardiomyocytes. Rescued DMD cardiomyocyte shows enhanced function. Opportunities and obstacles in the path toward efficient and permanent elimination of the genetic cause of DMD and other DCM will be discussed.

731. High-Fidelity CRISPR-Cas9 Nucleases with No Detectable Genome-Wide Off-Target Effects

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CRISPR-Cas9 nucleases are widely used for genome editing but can induce unwanted off-target mutations at genomic locations that resemble the intended target. These so-called off-target effects can confound research applications and are important considerations for potential therapeutic use. Existing strategies for reducing genome-wide off-targets of the broadly used *Streptococcus pyogenes* Cas9 (SpCas9) have thus far proven to be imperfect by possessing only partial efficacy and/or other limitations that constrain their use. Here we describe a high-fidelity variant of SpCas9, called SpCas9-HF1, that contains alterations in the amino acid sequence designed to reduce non-specific contacts to the target strand DNA. SpCas9-HF1 retains on-target activities comparable to wild-type SpCas9 with >85% of the 37 single-guide RNAs (sgRNAs) tested in human cells. Strikingly, with eight different sgRNAs targeted to standard non-repetitive sequences in human cells, SpCas9-HF1 rendered all or nearly all off-target events imperceptible by genome-wide break capture and targeted sequencing methods. Even for atypical, repetitive target sites, the vast majority of off-targets induced by SpCas9-HF1 were not detected. With its exceptional precision, SpCas9-HF1 provides an important and easily employed alternative to wild-type SpCas9 that can eliminate off-target effects when using CRISPR-Cas9 for research and therapeutic applications. Our findings also suggest a general strategy for improving or optimizing the genome-wide specificities of other Cas9 orthologues and engineered variants.

732. CRISPR/Cas9 Mediates Highly Efficient Gene Editing in Long-Term Engrafting Human Hematopoietic Stem/Progenitor Cells

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Transplantation of genetically corrected autologous hematopoietic stem/progenitor cells (HSPCs) is an effective treatment for patients with inherited hematologic disease. Genome editing with CRISPR/Cas9 ribonucleoprotein (RNP) precisely modifies gene targets in human cells, including HSPCs. In order to translate this technology to a clinical setting, gene editing must be reproducible and efficient across multiple patient donors without compromising HSPC viability, multipotency, and long-term engraftment capability. To determine the reproducibility of Cas9 RNP mediated gene editing in HSPCs, human $CD34^+$ cells obtained from 15 different patient donors (cord blood n=12, mobilized peripheral blood [mPB] n=3) were electroporated with *S. pyogenes* Cas9 RNP targeting the β-hemoglobin (*HBB*) or *AAVS1* genetic locus. DNA sequence analysis of 15 separate experiments demonstrated that Cas9 supported up to 72% gene editing in cord blood $CD34^+$ cells and up to 61% gene editing in adult mPB $CD34^+$ cells (mean % editing by DNA sequencing: 57% ± 8%). Cas9 induced multiple modifications that comprise insertions and deletions at the *HBB* locus, and some of the lesions were repaired through an HDR repair mechanism that used the homologous sequences from the endogenous *HBD* gene as a template (Gene Conversion). Gene edited $CD34^+$ cells retained viability and hematopoietic colony forming cell (CFC) activity *ex vivo*, with no significant differences