OC 40.2 | Therapeutic Levels of FVIII Generated by CRISPR/Cas9-mediated *in vivo* Genome Editing in Hemophilia A Mice

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Background: Expression of Factor VIII (FVIII) from a FVIII cDNA that has been integrated into the genome of hepatocytes has the potential to provide a life-long cure for Hemophilia A (HemA).

Aims: Determine if the CRISPR/Cas9 nuclease system can promote non-homologous end joining (NHEJ) mediated insertion of a Factor VIII (FVIII) cDNA into intron 1 of the albumin gene of mice and thereby generate therapeutic levels of FVIII.

Methods: A human FVIII cDNA lacking the signal peptide and flanked by a splice acceptor and polyadenylation signal was packaged in AAV8. Streptococcus pyogenes Cas9 (spCas9) mRNA and a single guide RNA (sgRNA) targeting mouse albumin intron 1 were encapsulated in a lipid nanoparticle (LNP). Cohorts of 5 adult HemA mice or adult NOD *scid* gamma (NSG) mice were injected with 2e12 or 2e13 vg/kg respectively of this AAV8-FVIII donor and 2mg RNA/kg of the LNP. FVIII levels in the blood of HemA and NSG mice were measured with the Coatest® activity assay or a human FVIII specific capture-Coatest® assay, respectively. Droplet Digital PCR was used to quantify the frequency of integration of the FVIII gene in the forward orientation into albumin intron 1 in the liver.

Results: Mice injected with the AAV8-FVIII donor alone had no detectable FVIII in their blood. In HemA mice injected with both the AAV8-FVIII donor and the LNP, 30% of normal human FVIII levels were measured at 2 weeks. NSG mice injected with the AAV8-FVIII donor and LNP had 70% of normal human FVIII levels that were stable through the longest time point measured at 4 months. The frequency of FVIII cassette integration in albumin intron 1 was between 0.5% to 3% of the murine albumin alleles.

Conclusions: CRISPR/Cas9 mediated integration of a FVIII cDNA into albumin intron 1 at low frequency generated therapeutic levels of FVIII in mice.

OC 40.3 | Exon-Specific U1snRNA-Mediated Rescue of Splicing and Missense Changes in Hemophilia A

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Background: Splicing mutations account for 8-10% of Hemophilia A (HA)-causing defects, a highly underestimated proportion since even exonic variants, besides acting on protein biology, can affect splicing regulatory elements. In this context, splicing-rescuing approaches might represent innovative personalized therapies. Over years we demonstrated that engineered variants of the spliceosomal U1snRNA, named exon-specific U1snRNA (ExSpeU1), can correct multiple splicing mutations for therapeutic purposes.

Aims: To elucidate the molecular mechanisms underlying all HA-causing mutations on exon 19 and to test ExSpeU1s as a correction strategy.

Methods: *In vitro* expression of *F8* exon 19 minigenes to assess splicing pattern. Expression via lentiviral vectors (LV) of FVIII missense variants and evaluation of FVIII antigen (ELISA) and activity (chromogenic assays) levels.

Results: Highly variable degree of aberrant splicing was observed, ranging from complete exon 19 skipping for all changes at the 5' splice site (5'ss) to different proportions of exon 19 inclusion for exonic changes (p.Gly2000Ala, p.Arg2016Gly and p.Tyr2036Tyr). FVIII protein expression studies demonstrated that the p.Arg2016Gly change leads to reduced antigen and activity levels (8.3±1.6% and 10.7±1.0% of wild-type, respectively). Differently, the p.Gly2000Ala change did not affect FVIII antigen nor activity, indicating a major effect on splicing for this variant. Co-transfection experiments led to the identification of a single ExSpeU1, designed to minimize potential off-target effects, able to properly restore splicing. In particular, the ExSpeU1 was able to completely rescue (>90%) splicing variants (c.6115+3G>T, c.6115+4A>G and c.6115+6T>A) as well as exonic changes (p.Gly2000Ala and p.Tyr2036Tyr).

Conclusions: Overall, we provided insights into the molecular mechanisms underlying HA caused by all splicing and exonic changes in exon 19, strengthening the notion that also exonic mutations can impair splicing process by affecting splicing regulatory elements. Moreover, we provided evidence of the ability of a single ExSpeU1 in rescuing multiple HA-causing mutations, thus expanding the therapeutic potential of this approach.

OC 40.4 | CRISPR Activation on Coagulation F7 or F8 Promoters Potentiate Trascriptional Activity in the Normal and Mutated Gene Context

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Background: Engineered transcription factors (eTF) have been successfully exploited to modulate gene expression and represent potential therapeutic tools for human disorders. In this perspective, the emerging CRISPR activation (CRISPRa) technology gives great advantages compared to the first eTF, mostly based on Transcription-Activator-like Effectors (TALE). Coagulation factor disorders, in

which even modest protein level increase have a therapeutic impact, represent ideal models to test the CRISPRa.

Aims: To tailor the CRISPRa system on the promoters of coagulation F7/F8 genes and enhance transcription, and thus expression, in the normal and mutated conditions.

Methods: Creation of reporter gene (luciferase) constructs for F7 and F8 promoter. Transient transfection in hepatoma cells and evaluation of the luciferase activity. Evaluation of the FVIII expression by functional assays (FXa generation).

Results: Using reporter gene assays we identified a sgRNA able to trigger the activity of F7 promoter up to ~35-fold, either wild-type, or defective due to the disease-causing c.-61T>G mutation. The effect was higher than that of an engineered TALE-based effector targeting the same promoter region (~15-fold). The transcription increase was confirmed on the endogenous F7 gene, the dCas9-VPR/sgRNAF7.5 combination was more efficient (~6.5-fold) in promoting factor VII (FVII) protein secretion/activity than TALE-TF4 (~3.8-fold). The approach was translated on F8 promoter, whose reduced expression translates into Haemophilia A. Reporter gene assays identified sgRNAs that appreciably increased F8 promoter activity (sgRNAF8.1, ~8-fold; sgRNAF8.2, ~19-fold) with a synergistic effect (~38-fold) when combined.

Conclusions: Over this pioneer study we demonstrated that the CRISPR system can be addressed to increase the expression, or rescue disease-causing mutations, of different promoters, with potential intriguing implications for coagulopathies.

OC 40.5 | Adeno-associated Virus Vector Based on Serotype 3 Represents an Alternative Serotype for Hemophilia Gene Therapy

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Background: Hemophilia represents a disease of choice for gene therapy. Recently successful clinical studies have been conducted on hemophiliacs with the use of adeno-associated virus (AAV) vectors. These demonstrated that hemophilia could be cured by means of gene therapy. However, subsequent intravenous administrations of identical AAV vector serotypes is not possible. This is due to the fact that high-tier neutralizing antibodies against AAV capsid develop after the first administration.

Aims: We searched for an alternative serotype for the second round of gene therapy. We aimed at establishing a strategy for re-administration.

Methods: Various AAV serotypes were compared to express transgenes in hepatocyte cell lines, mouse, and macaque. The intra-portal injection of the vector in a macaque was used for the second administration.

Results: When AAV vectors were intravenously injected in mice, we obtained efficient and specific transgene expression by AAV8 vectors in the liver. AAV8 more efficiently transduced the murine hepatocyte cell line TLR3. While, AAV3 exhibited the strongest expression in the human hepatocellular carcinoma cell line HepG2. Intravenous injection of AAV8 and AAV3 vectors, but not AAV5, yielded FIX's therapeutic levels at a same vector dose in macaques. In addition, in a macaque AAV8 vector administration dramatically induced neutralizing antibodies against AAV8 (1:1,000). Subsequently, the levels of neutralizing antibody for AAV8 gradually diminished. However, it maintained a high-titer (1:100) in the course of a long term follow-up (5 years). Finally, we successfully re-administered the AAV3 vector in the macaque previously treated by AAV8 in combination with intra-portal injection.

Conclusions: AAV3-based vectors represent an alternative serotype for hemophilia gene therapy. We believe that the combination of different serotypes with intra-portal injection may become an attractive approach to re-administer AAV vectors.

OC 42.1 | PK-Guided Rurioctocog Alfa Pegol Prophylaxis in Patients with Severe Hemophilia A Targeting Two FVIII Trough Levels: Results From the Phase 3 PROPEL Study

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Background: Prophylaxis with extended half-life recombinant factor FVIII (FVIII) rurioctocog alfa pegol (BAX855/TAK-660) targeting FVIII troughs ≥1% has been shown to be effective and well-tolerated in patients with severe hemophilia A.

Aims: This prospective, randomized, open-label, multicenter study (NCT02585960) evaluated the safety and efficacy of pharmacokinetic (PK)-guided BAX855/TAK-660 prophylaxis targeting two different FVIII troughs.

Methods: This study enrolled previously treated patients 12-65 years of age, participating in previous BAX855/TAK-660 phase 3 studies (NCT01945593; NCT02210091; NCT01913405); or newly recruited, BAX855/TAK-660-naïve patients. Key inclusion criteria were: FVIII activity <