protein in *E. coli*, where issues with protein yield, proper folding, lack of post-translational modification may ultimately reduce activity. The use of recombinant protein is further complicated by a lack of efficient and consistent delivery methods into target cells of interest. Improved methods, combining mammalian based protein production and efficient packaging into particles into one step can address these shortcomings. Here we report cellular delivery of DNA modifying proteins using VSV-G induced microvesicles (Gesicles). Gesicles are produced by co-overexpression of the spike glycoprotein of VSV-G with a protein of interest (POI), within a mammalian packaging cell. This leads to production of Gesicles containing active amounts of the POI expressed in mammalian cells. Based on this principle, we have developed a method for actively packaging genome modifying proteins into the Gesicles via ligand dependent dimerization. This approach allowed us package a POI containing a nuclear localization signal (NLS) efficiently into this particle. Analysis of the physical properties of these Gesicles demonstrated that they are highly stable over multiple freeze-thaw cycles, are consistent in size, and demonstrate minimal aggregation. Functionally, these Gesicles could efficiently deliver genome modifying proteins to a variety of cells, ultimately leading to genomic alterations. This effect could be demonstrated in over a dozen different cell lines; in all cases, cells maintained high viability and the results closely mimicked those obtained with viral transduction. Taken together, this work suggests that Gesicles can be considered a novel and universal tool for genome modification, providing a direct, rapid, and transient method for delivering active genome modifying proteins to target cells.

123. Helper-Dependent Ad5/35 Vectors for ZFN Mediated Gene Editing in Hematopoietic Stem Cells

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A homozygous [Delta]32 deletion in the CCR5 gene, found in about 1% of Caucasians, confers a natural resistance to HIV-1. In a recent pivotal study it was shown that transplantation of hematopoietic stem/progenitor cells (HSCs) from a donor who was homozygous for CCR5 [Delta]32 in a patient with acute myeloid leukemia and HIV-1 infection resulted in long-term control of HIV. This finding supports the development of gene therapy approaches to eliminate CCR5 in HIV target cells, including approaches based on CCR5-knockout by zinc finger nucleases (ZFNs). Recent trials involved the *ex vivo* transduction of patient CD4+ T-cells with a CCR5-ZFN expressing adenovirus Ad5/35 vectors. More recent attempts have focused on CCR5 gene knock-out in hematopoietic stem cells (HSCs). Because HSCs are the source for all blood cell lineages, CCR5 knock-out would protect not only CD4 cells but also all remaining lymphoid and myeloid cell types that are potential targets for HIV infection. We generated a helper-dependent, capsid-modified HD-Ad5/35 vector for CCR5-ZFN expression in HSCs from mobilized adult donors. The production of these vectors required that ZFN expression in HD-Ad5/35 producer 293-Cre cells was suppressed. To do this, we developed a miRNA-based system for regulation of gene expression based on miRNA expression profiling of 293-Cre and CD34+ cells. We demonstrated that, after in vitro CD34+ cell transduction, the HD-Ad5/35.ZFNmiR vector conferred *ccr5* knock-out in primitive HSC (i.e. long-term culture initiating cells and NOD/SCID repopulating cells). Upon transplantation of in vitro transduced CD34+ cells into irradiated NOG mice, the *ccr5* gene disruption frequency achieved in engrafted HSCs found in the bone marrow was about 12%. However, we also found in transplantation studies that the HD-Ad5/35.ZFNmiR vector decreased CD34+ engraftment rates 7-10-fold compared to HD-Ad5/35-GFP (control) transduced cells. A possible interpretation of these data is that sustained high-level ZFN expression afforded by the HD-Ad5/35 vector platform might be detrimental to the HSC, particularly if the cells do not actively divide allowing the extended persistence of HD-Ad5/35 vector genome. This deficit has not been observed with previous first generation recombinant adenoviral vector delivery of ZFNs to CD34+ HSCs (Li et al. Mol Ther 2013), thus the source of the engraftment inhibition is unclear. However, we plan to investigate and develop methods for maximal ccr5 knock-out efficiency in HCSs for future HIV challenge experiments.

124. Cas9-Mediated Genome Editing in Hematopoietic Stem/Progenitor Cells

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Transplantation of genetically modified autologous hematopoietic stem/progenitor cells (HSPCs) has proven to be an effective clinical treatment for patients with hematologic disease. Genome editing with the CRISPR/Cas9 platform has been shown to precisely alter endogenous gene targets in multiple human cell lines and animal models. Here, we compared Cas9-induced gene modification in primary human HSPCs, including mobilized peripheral blood and steady state bone marrow $CD34^+$ cells. Co-delivery of *Streptococcus pyogenes* or *Staphylococcus aureus* Cas9 paired with locus-specific guide RNAs induced targeted gene editing in $CD34^+$ cells and hematopoietic progeny, as determined by T7E1 assay and DNA sequence analysis. Multiple gene targets and delivery strategies were evaluated in these primary human $CD34^+$ cells. Importantly, hematopoietic progeny differentiated from Cas9 gene-edited HSPCs maintained ex vivo hematopoietic colony forming potential. This study provides further evidence of Cas9-mediated genome editing in clinically relevant primary cell populations.

125. Engineered Nucleases-Mediated In Situ Correction of a Genetic Defect By Homologous Recombination Into the Native Locus

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Engineered nucleases specific for genomic targets are extensively used to generate DSBs that increase the rate and efficiency of homologous recombination (HR). We seek to determine the efficacy of nucleases in a clinical relevant genetic defect.

The genetic defect we are addressing as model to test the nucleases-mediated genome editing technology is the junctional epidermolysis bullosa (JEB), a family of severe skin adhesion disorders due to autosomal recessive mutations in the LAMB3 gene coding for the laminin-332 heterotrimer, a key component of the dermal-epidermal

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junction. Recently, we provided proof of principle that ZFN-mediated, AAVS1-targeted GFP addition can be achieved in human keratinocytes and in long-term repopulating epithelial stem cells in a validated preclinical model of xenotransplantation of human skin equivalents on immunodeficient mice.

This project aims at the demonstration of a successful in situ correction of the LAMB3 gene in primary keratinocytes from Herlitz JEB patients. Recently TALEN-based gene correction for dystrophic EB has been reported. Similarly, we have developed a genome editing approach for JEB. In particular we have designed TALENs specific for the second intron of LAMB3 gene and a HR cassette including a splicible LAMB3 cDNA (from exon 3 to the end of the gene). In particular immortalized JEB keratinocytes were transfected with TALEN mRNAs and infected with an IDLV vector carrying the HR cassette. The in situ gene correction has been evaluated by site-specific PCR and knock-in expression of the corrected LAMB3 gene on bulk population. We then assessed targeting efficiency and specificity by extensive molecular analyses of single-cell clones isolated by limiting dilution from the TALENs/IDLV-treated immortalized JEB population. We isolated 256 clones and expanded 69 of them. Sixteen out of 69 clones showed an in vitro adhesion advantage, hosted the HR cassette correctly integrated into the predetermined locus, expressed the corrected LAMB3 gene and produced the laminin-332 protein. In parallel, CRISPR-Cas9 nuclease has been designed on the same locus to compare the transduction efficiency and cleavage activity and to translate the knock-in targeting platform to primary JEB keratinocytes.

126. Non-Viral Gene Therapy By Liver-Directed Hydrodynamic Delivery of *Sleeping Beauty* Transposons to Treat Hemophilia and Mucopolysaccharidoses in Dogs

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The goal of gene therapy is to achieve sustained expression of a transgene encoding an enzyme deficient in patients. Others and we have reported effectiveness of the *Sleeping Beauty* (SB) transposon system for gene therapy of both hemophilia B and mucopolysaccharidoses (MPS) types I and VII in adult mice. Although more than 99% of transgene expression comes from the liver following hydrodynamic delivery, restoration of deficient enzyme activity in other organs can be achieved through metabolic cross-correction whereby IDUA enters the circulation and is distributed to other tissues. However, the efficacy of hydrodynamic delivery to treat animals larger than mice has been discouraging. We have tested the use of balloon-catheters for intravascular infusion and expression of SB transposons in the liver of dogs as a large animal model for human therapy. Balloon-catheters were introduced under fluoroscopic guidance through either the jugular or the femoral vein and positioned in the inferior vena cava for occlusion of venous outflow of the and retrograde infusion through the left hepatic vein. Infusions were 10 seconds in duration and delivered volumes of DNA solution equal to about three times the estimated blood volume of the left side of the liver. Using canine secreted alkaline phosphatase (cSEAP) as a reporter we have developed effective protocols for infusion of transposons into canine liver as a scale-up for preclinical gene therapy studies in large animal models of human disease. We tested our protocol on both Factor IX (FIX)-deficient and beta-glucuronidase (GUSB)-deficient young dogs by hydrodynamically infusing two plasmids, one that harbors a T2-SB transposon containing either a canine FIX gene or a canine GUSB gene under transcriptional direction of a CAGGS or liver specific promoter (LSP) and a second carrying either the SB11 or SB100X transposase regulated by the CMV early promoter. Duration of FIX and GUSB expression in the treated dogs was transient, lasting only about 1 – 8 weeks. PCR analysis of plasmids in liver suggest that the efficiency of catheter-mediated delivery to canine livers is approximately 0.1-1 % that in the mouse, which accounts for the relatively poor levels of transgene activity in the larger animals. We discuss our hypothesis that the larger vascular net with many more bifurcations in the livers of large animals lowers both impulse and shear forces necessary for effective hydrodynamic delivery in the liver.

127. Detection of Vector Integration Sites by Targeted Sequencing

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For biosafety assessment and the understanding of viral integration mechanisms the determination of the exact position and distribution of viral integration sites in the host genome is crucial. Current methods for mapping of vector insertion sites are based on primer binding within the vector genome, its elongation into the host genome, ligation of a common adapter on the host part, and subsequent PCRs. Thus, these methods depend on the presence of the primer binding site in the viral genome and biases are introduced during restriction digest (if applied), ligation, and PCR. Here, we present results from a targeted sequencing approach (SureSelect, Agilent) in which we enriched for genomic regions including vector sequences. This approach has major advantages over primer based approaches like LAM PCR:

- As less PCR cycles are needed, a more quantitative estimation of cell clonality (clones with the sample integration site) should be possible.
- Genomic regions that are captured together with the vector allow for relative quantification of vector copies per genome.
- Integrations of incomplete vectors (common for adeno-associated vectors) can be detected without the need of specific primers.
- As the entire vector is sequenced, mutations within the vector (and transgene) can be detected.

Using control samples we defined the sensitivity, background, and dynamic quantitative range of this method. As the entire vector is sequenced, mutations within the vector (and transgene) can be detected. In summary, targeted sequencing provides an important complementary tool to primer based approaches (like LAM-PCR) for mapping of vector/viral integration sites.

128. Deletion of GAA Repeats Expansion from the Intron 1 of the Frataxin Gene Using CRISPR/Cas9 System

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In the past few years, the CRISPR/Cas9 system has rapidly proven its efficiency as a tool to modulate gene expression in mammalian cells. Modified Cas9 proteins can cut or nick DNA with a lot of specificity when used with appropriate RNA guides expressed from polymerase III promoters. We use this technique in Friedreich Ataxia (FRDA), an inherited autosomal disease known to be caused by a decrease of the mitochondrial frataxin protein. Genetic analysis revealed a GAA repeat expansion within the intron 1 of the frataxin

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