or in the vector backbone and outside the gene expression cassette (mirT-LV). In our rationale, the mirT sequences when incorporated in an aberrantly generated mRNA would be selectively degraded through the miRNA pathway. Thus, by taking advantage of our *in vivo* models, we assessed the genotoxicity of these LVs with mirT sequences. Systemic injection of mirsT-LTR.LV (N=34) and mirT-LV (N=39) in *Cdkn2a*$^{-/-}$ mice did not cause any significant acceleration in hematopoietic tumor onset compared to un-injected mice (N=37) or mice injected with a SINLV that does not harbor mirT sequences (N=24). Similar results have been obtained after injection of the same vectors in *Cdkn2a*$^{+/-}$ mice (N=29 mirsT-LTR.LV, N=25 mirT-LV, N=40 un-injected and N=15 injected control mice). To gain additional information on the safety profile of these vectors, we performed IS analysis (N>10,000) in tumor-derived DNA. By this analysis, we previously found that *Map3k8* activation by LV insertions was the major mechanism of genotoxicity when prototypical SINLVs were injected into *Cdkn2a*$^{-/-}$ mice. Now, we found that mice treated with mirsT-LTR.LV and mirT-LV did not show any *Map3k8* activating insertions, suggesting that the new vectors are efficient in preventing its activation and confirming their superior safety profile. Furthermore, as expected, *Pten* was the most frequently targeted gene in tumors derived from *Cdkn2a*$^{-/-}$ mice injected with the LVs harboring mirT sequences. *Pten* insertions mainly targeted exons, suggesting the potential inactivation of its transcription unit. Finally, we found that *Sfi1* was the major Common Insertion Site (CIS) in *Cdkn2a*$^{+/-}$ mice injected with LVs harboring mirT sequences. This CIS gene however appears to be the product of an intrinsic bias of LV integration, rather than the result of a selection process. Overall, our studies showed that these new advanced design LVs have a significantly improved safety profile and could represent the vector design of choice in future gene therapy applications.

530. Lentiviral Vector Particles Pseudotyped with Wild-Type Baboon Endogenous Retrovirus (BAEV) Glycoprotein Outperform VSV-G Particles in Transducing Human CD34+ Cells Isolated from Cytokine-Mobilized Peripheral Blood or Bone Marrow

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Lentiviral vector gene therapy for hematopoietic disorders caused by single gene mutation or deficiency has demonstrated success in clinical trials. However, efficient gene delivery is still challenging, requiring high multiplicity of infection (MOI) to achieve average vector copy numbers of at least 1 per cell. Conditions utilizing high MOI result in improved transduction rates and adequate copy number but these benefits can coincide with potential insertional mutagenesis. Attempting to achieve optimal gene transfer at reduced MOI, we directly compared self-inactivating (SIN) lentiviral vector particles encoding eGFP under control of a murine stem cell virus (MSCV) promoter/enhancer pseudotyped with envelope glycoproteins for BaEV or vesicular stomatitis virus (VSV). BaEV proteins were either full-length (BaWT) or lacked the terminal R-peptide (BaR-less), a version previously reported to improve infectious titer (TU, transducing units per mL) using transient production procedures. Using a standardized four-plasmid transient production protocol, cells transfected with R-less expression plasmids produced syncitia; an issue that resolved using BaWT. Titers were significantly higher for virus packaged with BaWT compared to R-less (BaR-less: $3.6 \times 10^5 \pm 6.6 \times 10^4$ N=12; BaWT $2.4 \times 10^6 \pm 4.5 \times 10^5$; n=10; $p<0.001$) determined by flow cytometry analysis of GFP expression in transduced HEK-293T cells. However, these values were 10-to 20-fold less than those observed for VSV-G ($3.5 \times 10^7 \pm 4.5 \times 10^6$). Human CD34+ hematopoietic stem/progenitor cells isolated from bone marrow (BM) or cytokine-mobilized peripheral blood (mPB) of healthy donors were prestimulated and transduced overnight on retronectin-coated plates with BaWT particles at an MOI of 0.5, 1, 2, or 4. The following day, cells were either plated in methylcellulose to assess colony-formation (CFU), maintained in liquid culture (Bulk), or transplanted into NOD-*scid* IL2Rg^{null} (NSG) mice pre-conditioned with busulfan (n=10 mice/$1x10^6$ cells each). Gene transfer efficiency was gauged by expression of GFP following flow cytometry analysis of cells maintained in culture or visual inspection of colonies growing in methylcellulose using an inverted fluorescence microscope after 6 or 12 days, respectively. Transduction efficiency increased with viral MOI reaching peak levels using an MOI of 2 (Bulk, 93% GFP+; CFU, 98% GFP+). Six NSG mice survived to 14 weeks post-transplant and demonstrated engraftment of CD45+ cells ranging from 19 to 54% (39 ± 13%; mean ± SD) and GFP marking ranging from 32 to 59% (41 ± 10%) determined by flow cytometry. Finally, we compared GFP-encoding particles pseudotyped with VSV-G or BaWT normalizing for levels of p24 quantified by ELISA (BaWT, 1155.0 pg/ml, 3.3×10^6 TU; VSV-G, 1164.9 pg/ml, 5.1×10^7 TU) to account for differences in calculated titer. Prestimulated mPB CD34+ cells were transduced overnight and placed into methylcellulose or transplanted into 15 NSG mice each. Methylcellulose colonies were analyzed for GFP after 12 days of growth (BaWT, 95.6%; VSV-G, 76.6%). Transplanted mice will be analyzed at 16-18 weeks post-transplant to measure engraftment and GFP expression. We believe the stability of producer cells expressing BaWT, the high efficiency of transduction at low MOI, and the ability to transduce adequate numbers of cells with unconcentrated virus supernatant warrant continued optimization of transient production methods and development of a stable producer cell line utilizing BaWT envelope as an alternative to VSV-g.

531. Computational Pipeline for the Identification of Integration Sites and Novel Method for the Quantification of Clone Sizes in Clonal Tracking Studies

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Gene-corrected cells in Gene Therapy (GT) treated patients can be tracked in vivo by means of vector integration site (IS) analysis, since each engineered clone becomes univocally and stably marked by an individual IS. As the proper IS identification and quantification is crucial to accurately perform clonal tracking studies, we designed a customizable and tailored pipeline to analyze LAM-PCR amplicons sequenced by Illumina MiSeq/HiSeq technology. The sequencing data are initially processed through a series of quality filters and cleaned from vector and Linker Cassette (LC) sequences with customizable settings. Demultiplexing is then performed according to the recognition of specific barcodes combination used upon library preparation and the sequences are aligned to the reference genome. Importantly, the human genome assembly Hg19 is composed of 93 contigs, among which the mitochondrial genome, unlocalized and unplaced contigs and some alternative haplotypes of chr6. While previous approaches aligned IS sequences only to the standard 24 human chromosomes, using the whole assembled genome allowed improving alignment accuracy and concomitantly increased the amount of detectable ISs. To date, we have processed 28 independent human sample sets retrieving 260,994 ISs from 189,270,566 sequencing reads. Although, sequencing read counts at each IS have

been widely used to estimate the relative IS abundance, this method carries inherent accuracy constraints due to the rounds of exponential amplification required by LAM-PCR that might generate unbalances on the original clonal representation. More recently, a method based on genomic sonication has been proposed exploiting shear site counts to tag the number of original fragments belonging to each IS before PCR amplification. However, the number of cells composing a given clone could far exceed the number of fragments of different lengths that can be generated upon fragmentation in proximity of that given IS. This would rapidly saturate the available diversity of shear sites and progressively generate more and more same-site shearing on independent genomes. In order to overcome the described biases and reliably quantify ISs, we designed and tested a new LC encoding random barcodes. The new LC is composed of a known sequence of 29nt used as binding site for the primers upon amplification steps, a 6nt-random barcode, a fixed-anchor sequence of 6nt, a second 6nt-random barcode and a final known sequence of 22nt containing sticky ends for the three main restriction enzymes in use (MluI, HpyCH4IV and AciI). This peculiar design allowed increasing the accuracy of clonal diversity estimation since the fixed-anchor sequence acts as a control for sequencing reliability in the barcode area. The theoretical number of different available barcodes per clone (4^{12}=16,777,216) far exceeds the requirements for not saturating the original diversity of the analyzed sample (on average composed by around 50.000 cells). We validated this novel approach by performing assays on serial dilutions of individual clones carrying known ISs. The precision rate obtained was averagely around 99.3%, while the worst error rate reaches at most the 1.86%, confirming the reliability of IS quantification. We successfully applied the barcoded-LC system to the analysis of clinical samples from a Wiskott Aldrich Syndrome GT patient, collecting to date 50,215 barcoded ISs from 94,052,785 sequencing reads.

532. Stable Transcriptional Repression, Gene Excision, and Parastism of HIV by Conditionally Replicating Lentiviral Vectors

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Gene-based therapies represent a promising therapeutic paradigm for the treatment of HIV, as they have the potential for sustained viral inhibition via reduced treatment interventions. One innovative approach developed here involves using conditionally replicating vectors (CR-Vectors), as these vectors utilize HIV-expressed proteins to replicate. These vector payloads can spread along with HIV into the budding viral particles, and co-infect target cells, essentially disseminating to HIV infected cells. We have generated and characterized CR-Vectors carrying therapeutic payloads consisting of various non-coding RNA regulatory expression cassettes, which modulate HIV both transcriptionally and post-transcriptionally, as well as CRISPR directed excision machinery. Notably, we have followed both virus and vector expression in T-cells and *in vivo* in the presence and absence of mycophenolic acid (MPA) selection. We find here that CR-Vectors functionally suppress HIV expression in a long-term stable and potent manner in both the presence and absence of MPA; and that transcriptional targeting is more potent at modulating stable suppression of HIV than post-transcriptional targeting or CRISPR directed excision of HIV. This suppression may be physiologically relevant, as it appears to drive HIV to a sustained non-pathogenic set point. Our findings suggest that CR-Vectors with modulatory non-coding RNAs may be a viable approach to achieving long-term stable suppression of HIV leading ultimately to a functional cure.

533. Genomic Excision of *PiggyBac* Transposon Cassettes by Lentiviral Protein Transduction of GagPol-Fused, Excision-Only *PiggyBac* Transposase

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The *PiggyBac* (PB) transposon system is a potent nonviral gene delivery tool with relevance in both gene therapy and cell reprogramming for production of induced pluripotent stem cells (iPSCs). Moreover, by taking advantage of the ability of the PB transposase to excise transposon-embedded gene cassettes from the genome without leaving footprints, the PB system is unique in facilitating seamless genome editing. The need for intracellular production of the transposase, however, raises concerns related to delivery and to cytotoxicity caused by sustained transposase expression and insertional mutagenesis. Furthermore, transposon re-integration may decrease the overall efficiency of the PB-mediated excision as well as increasing the risk of adverse secondary insertions. Based on our previous work, we present a new approach for lentivirus-based delivery of PB transposase. By fusing the hyperactive PB transposase, hyPBase, to the C-terminus of the GagPol polyprotein, we show robust incorporation and subsequent release of the transposase in matured lentiviral particles. Furthermore, in an effort to limit transposon re-integration, we engineered a hyPBase variant carrying three missense mutations. This novel hyPBase variant, hyPBase$^{Exc+/Int-}$, demonstrates integration levels very close to background levels, thus limiting the risk of reintegration. Notably, the ability of hyPBase$^{Exc+/Int-}$ to excise transposons from plasmid-borne as well as from genomically integrated PB transposon cassettes is increased up to 6.3-fold relative to the original hyPBase transposase. By fusing the hyPBase$^{Exc+/Int-}$ to the C-terminus of GagPol, transposase protein can be efficiently delivered to cells by lentiviral protein transduction and, in our model system, performs seamless genomic excision of PB transposon cassettes in a copy number and dose-dependent manner, resulting in excision in up to 23.6% of virus-treated cells. Using a transposon containing the puro-deltaTK transgene cassette, we furthermore show that cells with successful excision can be enriched 17-fold by negative selection with FIAU. We believe that protein transduction of hyPBase$^{Exc+/Int-}$ may increase the applicability and safety of transposase-directed genomic excision in iPSCs and in hard-to-transfect cell types including hematopoietic cells.