range, at 24 months post-infusion both patients have normal-range hemoglobin (13.2 g/dL and 14.7 g/dL, respectively), and no red blood cell transfusion requirements post-engraftment. Other parameters of hemolysis and anemia (LDH, bilirubin, erythropoietin) are improved. Peripheral blood mononuclear cell (PBMC) vector copy numbers (VCNs) were 1.75 and 1.43 at 24-months. Both patients reported improved quality of life (QOL), also demonstrated by increases in both FACT-An and SF-36 scores, with marked improvement in SF-36 energy/fatigue, physical functioning, and general health components. No serious adverse events (SAEs) have been attributed to RP-L301. Hematopoietic reconstitution occurred within 2 weeks of administration. ISA in PB and BM for both patients up to 12 months following therapy indicate highly polyclonal patterns; longitudinal results delineating clonal diversity will be presented. **Conclusion:** Clinical efficacy and safety data indicate that RP-L301 is a potential treatment for patients with severe PKD, including those who did not derive benefit from available therapies. Robust and sustained efficacy in both patients at 24 months post-treatment was demonstrated by normalized hemoglobin, improved hemolysis parameters, and transfusion independence.

219 Unraveling the Effect of Proliferative Stress *In Vivo* in Hematopoietic Stem Cell Gene Therapy Mouse Study

Francesco Gazzo[1,2], Daniela Cesana[1], Pierangela Gallina[1], Laura Rudilosso[1], Giulio Spinozzi[1], Giulia Pais[1], Eugenio Montini[1], Andrea Calabria[1]

[1]San Raffaele Telethon Institute for GT (Sr-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Electronics, Information and Bioengineering, Politecnico di Milano, Milan, Italy

The hematopoietic system of patients enrolled in hematopoietic stem cells (HSC) gene therapy (GT) treatments is fully reconstituted upon autologous transplantation of engineered stem cells. HSCs highly proliferate up to full restoration of homeostasis and compete for niche homing and engraftment. The impact of the proliferation stress in HSC on genetic instability remains an open question that cured patients advocate for characterizing long-term safety and efficacy. The accumulation of somatic mutations has been widely used as a sensor of proliferative stress. Vector integration site (IS) can be used as a molecular tool for clonal identity, inherited by all HSC progeny, to uncover lineage dynamics in vivo at single-cell level. Here we characterized at single-clone granularity the proliferative stress of HSCs and their progeny over time by measuring the accumulation of mutations from the DNA of each IS. To test the feasibility of the approach, we set-up an experimental framework that combines tumor-prone *Cdkn2a*$^{-/-}$ and wild type (WT) mouse models of HSC-GT and molecular analyses on different hematopoietic cell lineages after transplantation of HSCs transduced with genotoxic LV (LV.SF.LTR) or GT-like non-genotoxic LV (SIN.LV.PGK). The *Cdkn2a*$^{-/-}$ mouse model provided the experimental conditions to detect the accumulation of somatic mutations, since the absence of p16^{INK4A} and p19^{ARF} enhances the proliferative potential of cells that have acquired oncogenic mutations. As expected, mice transplanted with *Cdkn2a*$^{-/-}$ Lin^{-} cells marked with LV.SF.LTR (N=24) developed tumors significantly earlier compared to mock (N=20, $p<0.0001$), while mice treated with SIN.LV.PGK (N=23) did not. On the other side, mice that received WT Lin^{-} cells treated with LV.SF.LTR (N=25) or SIN.LV.PGK (N=24) vector have not developed tumors. Given this scenario, we expect that *Cdkn2a*$^{-/-}$ Lin^{-} cells transduced with LV.SF.LTR are associated with higher mutation rates compared to the SIN.LV.PGK group and wild type control mice. The composition of peripheral blood, lymphoid (B and T) and myeloid compartments was assessed by FACS on samples collected every 4 weeks and IS identification. More than 200,000 IS have been recovered. To identify the presence of somatic mutations, the genomic portions of sequencing reads flanking each different IS were analyzed with VarScan2. The accumulation rates of mutations have been evaluated by our new Mutation Index (MI) which normalizes the number of mutations by clones and coverage. Considering that a large portion of IS has been discarded since not covered by a minimum number of 5 unique reads (genomes), the remaining number of IS contained >90% of reads in each group. The MI increased over time in both LV.SF.LTR groups, with higher values for the *Cdkn2a*$^{-/-}$. On the other hand, treatment with SIN.LV.PGK resulted in lower MI in both groups compared to LV.SF.LTR groups, reflecting the higher clonal composition of the cells treated with the SIN.LV.PGK and the phenomenon of insertional mutagenesis in the LV.SF.LTR. Moreover, the higher MI values of the SIN.LV.PGK *Cdkn2a*$^{-/-}$ group compared with the WT group proved the induction of DNA fragility. Our results showed that the analysis of the accumulation of somatic mutations at single clone unraveled HSC proliferation stress in vivo, combining for the first time the analysis of acquired mutations with IS. We are now applying our model to different clinical trials, and studying HSCs subclonal trees by symmetric divisions, previously indistinguishable by IS only. Our study will open the doors to in vivo long-term non-invasive studies of HSC stability in patients.

220 Highly Efficient Correction of the Sickle Cell Disease Mutation in Patient HSC Using an RNA Gene Writing System, an RNA-Based, Nuclease-Free Approach to Genome Editing

Giulia Schiroli[1], Yaser Heshmati[1], Aamir Mir[1], Yu Cao[1], Shallu Kutlehria[1], Jicheng Duan[1], Lorenzo Tozzi[1], Rui Zhang[1], Felipe Gazos Lopez[1], William E. Salomon[1], James B. Rottman[1], Jane Wang[1], Anne Bothmer[2], Jean-Antoine Ribeil[3], John P. Manis[4], Cecilia Cotta-Ramusino[1], Michael C. Holmes[1]

[1]Tessera Therapeutics, Somerville, MA,[2]Tessera Therapeutics, Cambridge, MA,[3]Boston University, Boston, MA,[4]Boston Children's Hospital, Boston, MA

The ability to introduce new DNA sequences into the genome with high specificity and efficiency would provide a critical complement to existing gene editing approaches. Here, we developed Gene Writing systems, leveraging target-primed reverse transcription (TPRT) biochemistry evolved by non-LTR retrotransposon mobile genetic elements to edit genetic information at a specific genomic site without introducing DNA breaks in hematopoietic stem cells (HSC). Programmable TPRT requires the enzymatic steps of DNA nicking and reverse transcription. Using a large throughput screening, we developed synthetic Gene Writer enzymes that can catalyze a variety of editing reactions, such as the introduction of gene-length DNA sequence and substitutions, including the correction of *HBB* sickle mutation