964. Site Specific Knock-In Genome Editing in Human HSCs Using Baboon Envelope gp Pseudotypedviral Derived "Nanoblades" Loaded with Cas9/sgRNA Combined with Donor Encoding AAV-6

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Programmable nucleases have enabled rapid and accessible genome engineering in eukaryotic cells and living organisms. Here, we have designed "Nanoblades", a new technology that will deliver a genomic cleaving agent into cells. These are genetically modified Murine Leukemia Virus (MLV) or HIV derived virus like particle (VLP), in which the viral structural protein Gag has been fused to the Cas9. These VLPs are thus loaded with Cas9 protein together with the guide RNAs. Thus, nanoblades are devoid of any viral-derived genetic material. Highly efficient gene editing was obtained in cell lines, IPS cells and primary mouse and human cells (REF 1). However, their delivery into target cells can be technically challenging when working with primary immune cells. Here, we showed that nanoblades were remarkably efficient for entry into human T, B and hematopoietic stem cells thanks to their surface co-pseudotyping with baboon retroviral and VSVG envelope glycoproteins. We were able to induce efficient, transient and very rapidly genome-editing in human induced pluripotent stem cells reaching up to 70% in the empty spiracles homeobox 1(EMX1) and muscular dystrophy (MD) gene locus. A brief nanoblade incubation of primary human T and B cells resulted in 40% and 20% editing of the Wiskott-Aldrich syndrome(WAS) gene locus, while hematopoietic stem cells treated for 18 h with nanoblades allowed 30-40% gene editing in the WAS gene locus and up to 80% for the Myd88 genomic target. Moreover, for the HIV- and MLV-derived nanoblades no cell toxicity and low to undetectable off-target effects were demonstrated. Finally, we also treated hHSCs with nanoblades in combination with an AAV-6 donor encoding vector resulting in over 20% of stable expression cassette knock-in into the WAS gene locus. Currently, we are evaluating these gene modified HSCs for their long-term reconstitution of NOD/SCIDgammaC-/- mice. Summarizing, this new technology is simple to implement in any laboratory, shows high flexibility for different targets including primary immune cells of murine and human origin, is relatively inexpensive and therefore have important prospects for basic and clinical translation in the area of gene therapy. **Ref 1:** P.E. Mangeot, V. Risson, F. Fusil, A Marnef, E. Laurent, J. Blin, V. Mournetas, E. Massourid, T. J. M. Sohier, A. Corbin, F Aub, C. Pinset, L. Schaeffer, G. Legube, FL. Cosset, E.Verhoeyen, T. Ohlmann, E. P. Ricci. Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Nature communications, 2019 Jan 3;10(1):45.

965. CRISPR/Cas9-Engineered Transgenesis of Hematopoietic Stem Cells via NHEJ-Mediated Targeted Integration Retains Engraftment Potential

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The ability to edit the genome of long-term-engrafting hematopoietic stem cells (LT-HSCs) would provide a curative therapy for a wide range of hematological disorders. While mixed LT/ST-HSC (ST, short-term) pools edited via non-homologous end joining (NHEJ) yield engraftable transgenic cells, pools made transgenic via homology-dependent repair (HDR) do not. This observation is likely explained by the absence of DNA replication required for HDR in LT-HSCs. Quiescence renders LT-HSCs refractory to transgene insertion via HDR, with the transgene-positive population consisting overwhelmingly of actively dividing, short-term-engrafting HSCs. In contrast to HDR, NHEJ is a DNA repair mechanism active in non-dividing cells. We hypothesized that CRISPR/Cas9-mediated transgenesis of HSC pools via NHEJ-mediated targeted integration (NHEJ-TI) would allow modification of LT-HSCs and produce edited, long-term engrafting progeny. First, we investigated whether NHEJ-TI is a feasible method of genome editing in HSCs. Utilizing CRISPR/Cas9 to induce a DSB in the *AAVS1* (*PPP1R12C*) locus and a self-complementary rAAV6 (scAAV6) to deliver a GFP expression cassette dependent on the endogenous promoter, we optimized RNP nucleofection, scAAV6 infection, and HSC culture conditions for efficient NHEJ-TI in HSCs. Flow cytometry for eGFP confirmed transgene integration at frequencies ranging from 4-6%. Droplet digital PCR of the 5' junction of integrated transgene into the AAVS1 locus corroborated the flow cytometry results. The addition of the endogenous protospacer sequence flanking the donor transgene decreased integration of the AAV ITRs while increasing the integration rate of the donor sequence in the correct orientation. Next, we investigated whether the NHEJ-TI edited pool of mixed LT/ST-HSCs contained engraftable, edited cells by injection into irradiated NOD/SCID/IL2Rg$^{-/-}$ (NSG) mice. We compared the engraftment potential of transgenic HSCs cultured for either 2 days (2D) or 2 hours (2H) prior to editing. While the overall editing efficiency was higher in the 2D condition versus the 2H condition (GFP+ of hCD34+: 3.07%, 2H; 2.58%), the near absence of cell pre-stimulation in the 2H condition resulted in increased levels of overall engraftment, doubling the total engraftment level of edited hematopoietic cells at 16 weeks post-injection. Importantly, the percentage of edited cells among the engrafted human hematopoietic cell population remained essentially unchanged in the peripheral blood over the course of 16-weeks post-injection (GFP+ of hCD45+ population: 2D; 4.64±0.33%, 2H; 3.03±0.53%). Analysis of bone marrow-resident human CD34-positive cells at 16 weeks post-injection reveal the persistence of edited LT-HSCs (GFP+ of hCD34+ population: 2D; 4.43±0.49%, 2H; 2.65±0.80%). Our results show that NHEJ-TI is a feasible method of inserting transgenes into LT-HSCs whilst retaining their engraftment potential. To our knowledge, this is the first study to achieve successful NHEJ-mediated targeted integration in HSCs while retaining engraftment potential and transgenesis.