CIRCULARIZATION OF SINGLE-STRANDED DNA DONOR TEMPLATE UNLEASHES THE POWER OF NON-VIRAL GENE DELIVERY FOR LONG-TERM HSC EDITING

Gil Letort[1], Aymeric Duclert[1], Diane Le Clerre[1], Isabelle Chion-Sotinel[1], Roger Salvatori[2], Emilie Dessez[1], Margaux Sevin[1], Marco Rotondi[1], Cosimo Ducani[2], Philippe Duchateau[1], and Julien Valton[1]

[1]Cellectis S.A., 8 Rue de la Croix Jarry, 75013 Paris, France; [2]Moligo Technologies, Anderstorpsvägen 16, 171 54, Solna, Sweden

1-Background

- Engineered nucleases enable to generate sequence-specific double-strand breaks (DSBs) at targeted genomic loci. This facilitates the inactivation (knockout, KO) of genes or the insertion (knock-in, KI) of therapeutic genes in the presence of a DNA donor template and enables development of cell and gene therapy products for a wide array of indications. While viral vectors, particularly Adeno-Associated Viruses (AAVs), are prevalent carriers of donor templates, recent reports have raised safety and efficacy concerns over AAV related to potential genotoxicity. Thus, exploring and identifying new alternatives to vectorize DNA donor templates for engineered nucleases-mediated gene insertion purposes is highly regarded.

- Non-viral DNA donor templates such as linear single-stranded DNA (lssDNA) and circular single-stranded DNA (cssDNA) are emerging as promising options to edit Hematopoietic Stem and Progenitor Cells (HSPCs) for therapeutic applications. Capitalizing on TALEN technology, we have devised a gene editing process that incorporates non-viral DNA donor template delivery (LssDNA or CssDNA) to enhance gene insertion in HSPCs.

2-TALEN-mediated Knock-in process using lssDNA and cssDNA

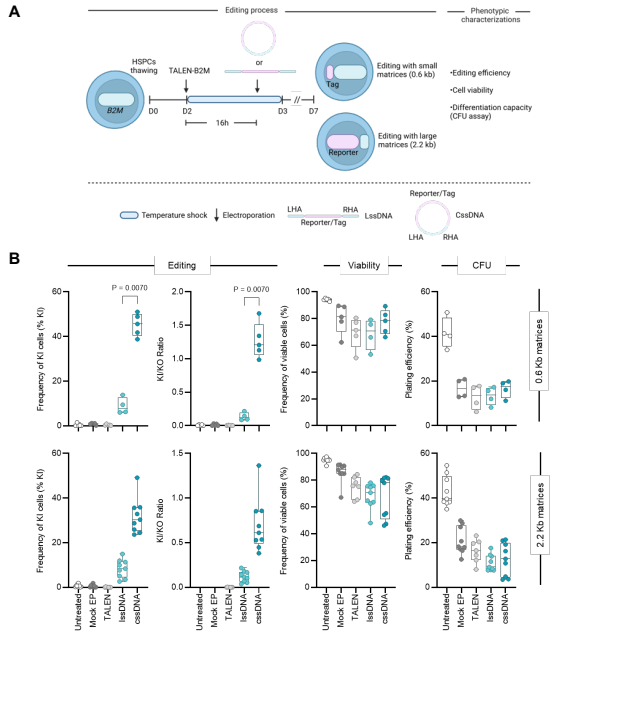

Figure 1. Circularization of ssDNA increases the overall efficiency of TALEN-mediated Knock-in in HSPCs.

A. Representative schema of HSPCs editing protocol using an mRNA encoded TALEN targeting the *B2M* locus and lssDNA or cssDNA as DNA donor templates to insert a tag (0.6 kb) or a reported gene (2.2 kb) via non-disruptive and disruptive insertions, respectively. mRNAs encoding a viability enhancer and a HDR enhancer (Via-Enh01 and HDR-Enh01, respectively) were also incorporated in the process. The timing is indicated in days (D0-D7). Edited HSPCs obtained 7 days post thawing were characterized by flow cytometry to assess the level of knock-in (KI) of DNA donor templates and knock-out (KO) of *B2M* as well as their viability. Their differentiation capacity into erythroid and myeloid progenitors was also assessed by colony forming unit (CFU) assay. **B.** Experimental results illustrating the frequency of cells harboring KI events, the ratio KI/KO, the viability and plating efficiency of HSPCs either untreated, electroporated (Mock EP), edited with TALEN only (TALEN), or edited with TALEN and lssDNA or cssDNA donor templates (lssDNA or cssDNA, respectively). **Top and bottom panels** illustrate results obtained with 0.6 kb and 2.2 kb DNA donor templates, respectively. On each box plot, the central mark indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data point. Each dot represents data obtained from one HSPCs donor.

3-Comparison of AAV- and cssDNA-mediated Knock-in processes

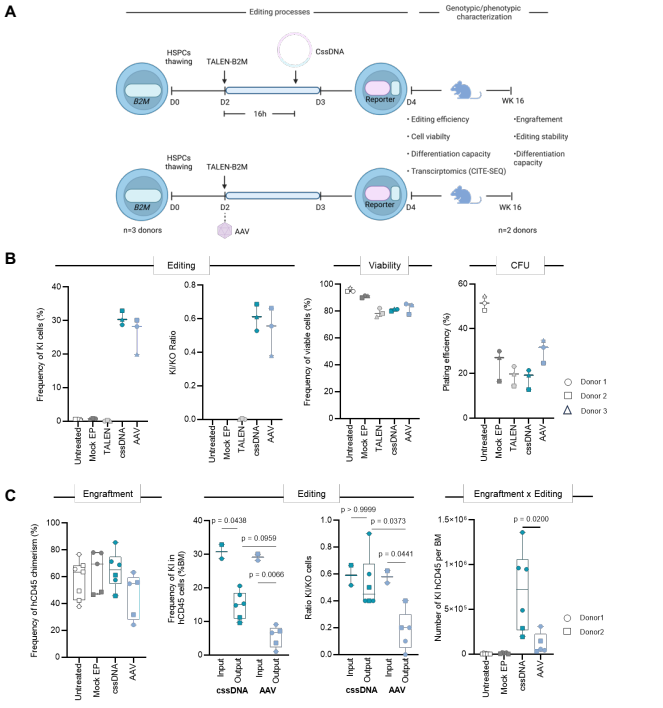

Figure 2. CssDNA/TALEN-mediated knock-in process leads to a higher engraftment of edited HSPCs than does the AAV/TALEN reference process. A. Representative schema of HSPCs editing protocol using an mRNA encoded TALEN targeting the *B2M* locus and cssDNA or AAV (MOI=350 vg/cell) as DNA donor templates to insert a reported gene (2.2 kb) via disruptive insertion. mRNAs encoding a viability enhancer and a HDR enhancer (Via-Enh01 and HDR-Enh01, respectively) were also incorporated in the process. The timing is indicated in days (D0-D7). Edited HSPCs retrieved 7 days post thawing were characterized by flow cytometry to assess the level of knock-in (KI) of DNA donor templates and knock-out (KO) of *B2M* as well as their viability. Their differentiation capacity into erythroid and myeloid progenitors as well as their transcriptomics profile were also assessed by colony forming unit (CFU) assay and CITE-seq, respectively. Edited HSPCs retrieved 4 days post thawing, were also injected in NCG mice to assess their ability to engraft, differentiate and keep their editing events, 16 weeks after injection onset. **B.** In vitro experimental results illustrating the frequency of cells harboring KI events, the ratio KI/KO, the viability and plating efficiency of HSPCs either untreated, electroporated (Mock EP), edited with TALEN only (TALEN), or edited with TALEN and cssDNA or AAV donor templates (cssDNA or AAV respectively). **C.** In vivo experimental results illustrating the level of human CD45+ cells (hCD45) engraftment and of KI and KO frequencies determined either before mice injection (input), or in hCD45+ cells engrafted in the bone marrow (BM) of NCG mice, 16 weeks after cells injection onset (output). Two-way ANOVA followed by Bonferroni multi-comparison test. P-values are indicated. The product of the frequency of hCD45+ cells engraftment and frequency of KI is also shown to illustrate the overall efficiency of each protocol of HSPC editing. Mann–Whitney two-tailed non-parametric unpaired test with a confidence interval of 95%. P-value is indicated. On each box plot, the central mark indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data point. Each dot represents data obtained from one HSPCs donor.

4-Comparative CITE-Seq transcriptomics analysis of HSPCs edited by the TALEN/AAV or TALEN/cssDNA Knock-in processes

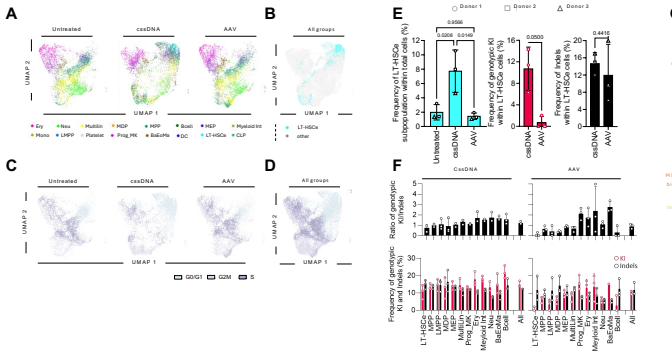

Figure 3. CssDNA/TALEN-mediated knock-in process leads to a higher frequency of gene edited HSC-enriched subpopulation than does the AAV/TALEN reference process.

A. UMAP plots showing aggregated 5'scRNA CITE-Seq data obtained from HSPCs either untreated, edited with TALEN and AAV or cssDNA donor templates (AAV or cssDNA respectively), 4 days post thawing (D4) and at the time of NCG mice injection onset (n = 3 independent biological donors). The different cell subpopulations identified are illustrated by a color code indicated at the bottom of the graph. The Long-term HSC-enriched cell subpopulation is indicated as LT-HSCe and definition of each subpopulation is documented in the methods section **B.** UMAP plots aggregated from 5'scRNA CITE-Seq data obtained from all experimental groups showing the position of LTHSCe subpopulation. **C** and **D.** UMAP plots showing the cell cycle phases (G0/G1, G2M and S) of each cell identified in each experimental groups and in all groups, respectively. **E. left panel,** illustrate the frequency of LT-HSCe within all subpopulations. Two-way ANOVA with Bonferroni post-tests. P-values are indicated. **E. middle and right panels**, illustrate the frequency of KI and KO within the LT-HSCe subpopulation, respectively. Paired t-test. P-values are indicated. Each dot represents data obtained from one HSPCs donor. **F.** plot showing the frequency of KI(+) and KO(+) in each subpopulation found in the CssDNA and AAV-edited HSPCs (n=3 donors aggregated, subpopulations identified with fewer than 100 cells are not displayed). **G.** Gene Set Enrichment Analysis (GSEA) obtained in LT-HSCe to compare the AAV and CssDNA experimental groups to the untreated reference group and to directly compare the cssDNA group to the AAV reference group. Normalized Enrichment Score (NES) as well as Log P value obtained for each donor (n=3 independent HSPC donors) are illustrated by a red/white/blue color code and size of the dots, respectively. The different pathways found to be significantly up (red) or down (blue) regulated in the different experimental group comparisons, were numbered and aggregated in subgroups shown in the right side of the figure for the sake of clarity.

Conclusions

-lssDNA or cssDNA can be used as DNA donor template to promote targeted gene insertion at the *B2M* locus in HSPCs using the TALEN technology.
-cssDNA yields a 3- to 5-fold higher gene insertion frequency than lssDNA, with up to 49% of HSPCs harboring a precise targeted gene insertion event.
-HSPCs edited with the cssDNA/TALEN process efficiently engraft in the bone marrow of NCG mice and retain 50% of the gene insertion events detected before injection onset. This process generates HSPCs harboring higher in vivo engraftment capacity and editing stability than those edited with the AAV/TALEN reference editing process, even when AAV is transduced at low MOI.
-The cssDNA/TALEN editing process promotes higher levels of correctly edited HSC-enriched subpopulation than the AAV/TALEN reference editing process.