Ex vivo editing of human hematopoietic stem cells for erythroid expression of therapeutic proteins

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Targeted genome editing has a great therapeutic potential to treat disorders that require protein replacement therapy. To develop a platform independent of specific patient mutations, therapeutic transgenes can be inserted in a safe and highly transcribed locus to maximize protein expression. Here, we describe an ex vivo editing approach to achieve efficient gene targeting in human hematopoietic stem/progenitor cells (HSPCs) and robust expression of clinically relevant proteins by the erythroid lineage. Using CRISPR-Cas9, we integrate different transgenes under the transcriptional control of the endogenous α-globin promoter, recapitulating its high and erythroid-specific expression. Erythroblasts derived from targeted HSPCs secrete different therapeutic proteins, which retain enzymatic activity and cross-correct patients’ cells. Moreover, modified HSPCs maintain long-term repopulation and multilineage differentiation potential in transplanted mice. Overall, we establish a safe and versatile CRISPR-Cas9-based HSPC platform for different therapeutic applications, including hemophilia and inherited metabolic disorders.
Many diseases require protein replacement therapy (PRT) to supplement a protein that is deficient because of a genetic defect. PRT is approved or under investigation to treat more than 40 inherited disorders, mostly involving blood factors and lysosomal enzymes. Although life saving for some patients, this therapy has several limitations, that lead to treatment failures and limited long-term efficacy.

Genome editing technologies have a great therapeutic potential for genetic disorders, as they can fix the underlying disease-causing mutation. However, this approach requires the development of countless gene-tailored editing strategies that can hinder clinical translation.

To overcome this issue, a single “safe harbor” or a highly transcribed genomic locus can be exploited to integrate and overexpress different therapeutic transgenes. Previous studies successfully used adeno-associated virus (AAV) for nucleasemediated targeting of transgenes under the control of the endogenous albumin promoter in liver. The striking transcriptional activity of this locus achieved therapeutic protein levels in different preclinical models and thus prompted the first in vivo genome editing trial in humans (NCT03041324). Although promising, this approach is hampered by: (1) presence of pre-existing antibodies against AAV capsid that precludes treatment to a significant portion of patients; (2) long-term expression of synthetic nucleases in vivo, which could result in genotoxicity and trigger immune responses against transduced hepatocytes; (3) liver conditions that can alter AAV transduction and hepatitis virus expression.

As therapeutic alternative, hematopoietic stem cells (HSCs) can be harnessed to overexpress transgenes in downstream hematopoietic lineages. Differently from liver, autologous HSCs can be easily accessed for ex vivo gene manipulation and re-administration, thus circumventing immunological issues; however, a suitable locus for transgene integration (knock-in, KI) still needs to be identified.

α-globin genes are expressed by the erythroid lineage at extremely high levels (~1.5 g/day), they are present in 4 copies per cells and the loss of up to 3 α-globin alleles is mostly asymptomatic, making this locus a promising candidate for KI in HSCs. In addition, erythroid cells are the most abundant hematopoietic progeny (~2 x 10^11 new erythrocytes per day) and can secrete relevant amounts of therapeutic proteins, as previously demonstrated by gene transfer using lentiviral vectors (LV).

Here, using CRISPR-Cas9 we integrate therapeutic genes under the transcriptional control of the endogenous α-globin promoter in human HSCs. We aim to combine strong transcription and abundance of transgene-expressing erythroblasts to maximize protein production, reducing the number of integration events required to reach therapeutic levels.

This HSC platform for robust erythroid-specific expression of therapeutic proteins opens possibilities for treating hemophilia and lysosomal storage disorders (LSD), as well as other genetic diseases.

Results
Selection of gRNA targeting the α-globin locus. To generate a DNA double-strand break (DSB) for transgene integration in the α-globin locus, we focused on Streptococcus pyogenes (Sp)Cas9 nuclease, the only Cas in clinical trials to edit HSCs (NCT03164135; NCT03655678). We designed 14 guide (g)RNAs targeting the non-coding sequences of α-globin genes, in particular the 5′ untranslated region and introns (5′UTR, IVS1 and IVS2 respectively), avoiding known regulatory elements (Fig. 1a and Supplementary Table 1A). gRNA were tested for on-target DNA cleavage (Indels) in K562 erythroleukemia cells constitutively expressing Cas9 (Fig. 1b). For the best candidates for each region we analyzed the indels pattern (Supplementary Fig. 1a) and we assayed their effect on α-globin production. As control, we designed a gRNA (KO) targeting the first exon of HBA1 and HBA2 genes, which abrogates α-globin production. In K562, 5′UTR and IVS2 gRNA did not alter α-globin protein level (Supplementary Fig. 1b) and were therefore selected for further investigation.

To evaluate DNA cleavage efficiency in clinically-relevant human HSCs, cells were transfected with Cas9/gRNA ribonucleoprotein complex (RNP). To control the effects of the editing procedure, we included a gRNA targeting an unrelated genomic locus (AAVS1). We observed efficient editing for 5′UTR and IVS2 gRNA in both erythroid liquid culture and methylcellulose-plated colony-forming cells (CFC) (Fig. 1c), which did not affect HSPC viability and multilineage potential (CFC assay; Fig. 1d and Supplementary Fig. 1c) or altered erythroid differentiation (flow cytometry analysis, Fig. 1e). Remarkably, 5′UTR and IVS2 gRNA did not modify α-globin expression, measured as ratio between α- and β-like globin chains (Fig. 1f and Supplementary Fig. 1d). In accordance with these data, adult hemoglobin (2α+2β globin chains, HbA) remained the predominant hemoglobin form in both 5′UTR and IVS2 erythroblasts, while it strongly decreased in KO controls where alternative homotetramers lacking α-globin chains appeared, as in α-thalassemic patients (Fig. 1g and Supplementary Fig. 1e). Lastly, since the two α-globin genes (HBA1 and HBA2) are the result of evolutionary duplication (96.67% sequence homology, GRCh38), we evaluated if simultaneous cleavage of both genes can induce loss of HBA2 in edited HSPCs. We observed a reduction of HBA2 copies per cell to 1.8 ± 0.3 for IVS2 gRNA, which selectively targets HBA2, and to 1.4 ± 0.3 for 5′UTR gRNA (Supplementary Fig. 1f); however, these rearrangements had minimal effect on globin production, as shown above. Detection and quantification of HBA2 inversions was not possible due to technical issues associated with the presence of repetitive sequences and the high GC content of the α-globin locus.

Overall, these results demonstrate that both 5′UTR and IVS2 gRNA efficiently cut α-globin genes without affecting HSPC viability, differentiation potential and hemoglobin expression, thus representing an interesting genomic locus to test KI.

Targeted integration. To evaluate if the α-globin promoter can drive the expression of an integrated heterologous transgene, we generated KI cassettes containing a promotorless GFP (Supplementary Fig. 2a). These cassettes were delivered in K562-Cas9 cells using integrase-defective lentiviral vector (IDLV) and integrated by transfecting a gRNA encoding plasmid. Interestingly, all gRNA/IDLV combinations resulted in GFP expression, which increased upon erythroid differentiation (Supplementary Fig. 2b). In addition, on-target integration by non-homologous end joining was confirmed in GFP positive clones by PCR (Supplementary Fig. 2c) and the presence of a chimeric messenger RNA showed correct splicing of intron traps (Supplementary Fig. 2d). Similar results were obtained upon KI in the β-globin gene, suggesting that KI in globin genes with different expression levels could be a viable strategy to modulate transgene expression (Supplementary Fig. 2e–j).

We further confirmed these α-globin KI data in immortalized human erythroid progenitor cells (HUDEP-2) which can differentiate to reticulocytes. To perform KI, HUDEP-2 cells were transfected with 5′UTR or IVS2 RNP and transduced with an AAV6 carrying the aforementioned expression cassettes flanked by homology arms to favor homologous DNA recombination
Fig. 1 Editing of selected sites in the α-globin locus minimally affects globin production. a Locations of gRNA on HBA2 gene. All gRNA except 74 (in purple) target both HBA1 and HBA2. Selected gRNA are highlighted. b K562-Cas9 screening of gRNA targeting different region of the α-globin locus (5' untranslated region/start codon (5'UTR/ATG), intron 1 (IVS1) or intron 2 (IVS2)). Each bar is a different gRNA, each dot a different experiment. Editing efficiency is expressed as percentage of modified HBA alleles (bars represent mean; data from 1, 2 or 3 biological replicates). The gRNA selected for each region are highlighted. c Editing efficiencies in HSPCs in erythroid liquid culture (circles) or in BFU-E (burst-forming unit-erythroid, squares). Lines show mean (2-4 donors; n = 5 KO, n = 6 AAVS1, n = 9 5’UTR and IVS2). d Colony-forming cell (CFC) frequency in edited HSPCs (mean ± SD; n = 2, n = 4 for IVS2). e Flow-cytometry analysis of erythroid markers upon differentiation of edited HSPCs, day12 (green 5’UTR, purple IVS2 and gray AAVS1). f HPLC analysis of hemoglobin monomers of erythroblasts derived from edited HSPCs (n = 6 5’UTR, n = 5 IVS2, n = 3 KO and AAVS1, n = 4 UT; 4 donors). Indels percentage mean is indicated. The ratio α/β-like globins in normal cells is close to 1 (red dashed line). Black lines show mean; BFU-E (squares), erythroid liquid culture (circles) ***p < 0.001 vs UT, 5’UTR, IVS2, AAVS1; one-way ANOVA, Tukey’s test). g HPLC analysis of hemoglobin tetramers of erythroblasts derived from edited HSPCs (same samples as in f). Every tetramer is reported as % of total hemoglobins (**p < 0.01, ***p < 0.001, two-way ANOVA, Dunnet’s test). **p < 0.017 5’UTR, p = 0.013 IVS2, p = 0.006 AAVS1 vs KO); HbH, βγ (p = 0.038 5’UTR, p = 0.035 IVS2, p = 0.032 AAVS1 vs KO); Hbs, fetal hemoglobin, αγββ (p < 0.001 5’UTR and AAVS1, p = 0.002 IVS2 vs KO); HbA, adult hemoglobin, α2β2 (p < 0.001 vs KO). Source data are in the Source Data file.
UTR or AAVS1 (negative control), and 29 top-scoring off-targets predicted in silico20,21 were PCR amplified for InDels quantification. Although on-target activity reached >85%, we could not detect any difference between HBA15 and control AAVS1 gRNA at any of the predicted HBA15 off-target sites (Supplementary Table 2) (with a technical threshold of >2% of TIDE software)22. To detect unpredicted off-targets, we also performed an unbiased genome-wide screening by IDLV capture analysis. This method exploits the tendency of IDLV genome to ligate into DSB, thereby stably tagging otherwise undetectable DSB23,24. To this purpose, K562 were transduced in triplicate with an IDLV encoding for a constitutively expressed GFP and transfected with 5’UTR or IVS2 RNP. The latter sample was used as positive control, since IVS2 gRNA has a known off-target in HBA1.
identified for 5’UTR gRNA after correction for random IDLV integration, further assuring the lack of any predominant off-target for this gRNA.

**Hemophilia B.** As first therapeutic target, we tested our platform for hemophilia B (OMIM #306990), a disease model for gene-based therapies caused by the absence of functional Factor IX (FIX, F9). Initially,HUDEP-2 cells were transfected with 5’UTR RNP and transduced with an AAV6 carrying two 250 bp homology arms flanking a promotorless human FIX-R338L (FIX Padua) and a constitutive GFP reporter to easily track KI cells (Fig. 3a). Concordance between DNA integration and GFP expression analyses before and after GFP sorting confirmed that most integrations were on-target (Fig. 3b), with a preference for HBA1 integration (Supplementary Fig. 4a). FIX expression was upregulated upon HUDEP-2 erythroid differentiation (Fig. 3c) and its secretion (median 1161 ng/10⁶ cells/FIX copy, 769.1-1885, interquartile range) correlated with the number of integrated FIX copies (Fig. 3d).

Editing of HSPCs showed that also in primary cells, without any selection, we could obtain high levels of Indels (Supplementary Fig. 4b) and KI of FIX as measured by GFP (Fig. 3e) and on-target ddPCR (Supplementary Fig. 4c), associated with a reduced number of HBA2 copies (Supplementary Fig. 4d).

Once more, we could demonstrate that F9 mRNA and protein secretion increased upon erythroid differentiation (Fig. 3f, g) and that secreted FIX was functional (Fig. 3h; Supplementary Fig. 4e). Interestingly, FIX expression achieved with targeted integration was higher compared to a state-of-the-art LV carrying an artificial β-globin promoter.⁷⁷ (Fig. 3i, j), highlighting one of the advantages of exploiting endogenous promoters in their chromatin context. Analysis of HSPC derived colonies, confirmed that high KI efficiency in CFC (both erythroid and granulocytemonocyte colonies, Supplementary Fig. 4f) did not affect HSPC clonogenic differentiation capacity (Supplementary Fig. 4g), although the total number of CFC was lower than control HSPCs due to known AAV toxicity (Supplementary Fig. 4h). In addition, by analyzing KI HSPC derived burst-forming unit-erythroid colonies (BFU-E) we showed that F9 integrations were mostly monoallelic (Fig. 3k) and HDR-mediated (19/19 colonies; Supplementary Fig. 4i), associated with a reduced number of HBA2 copies (Supplementary Fig. 4i). Importantly, also BFU-E derived erythroblasts were capable of secreting FIX (Supplementary Fig. 4m).

These results clearly indicate that this platform can express and secrete a functional protein with therapeutic relevance.

**Lysosomal storage disorders.** In light of these promising findings, we expanded our strategy to other genetic diseases eligible for PRT, such as LSD. These inherited metabolic conditions are characterized by an abnormal build-up of toxic metabolites in lysosomes as a result of enzyme deficiencies.⁶⁰ Here we tested three different human transgenes encoding for: α-L-iduronidase (IDUA; Hurler syndrome, OMIM #607014), α-galactosidase (GLA; Fabry disease, OMIM #301500) and lysosomal acid lipase (LAL; Wolman disease, OMIM #278000). To facilitate their detection, each enzyme was tagged with 3 copies of hemagglutinin epitope (HA) and cloned into AAV6 vectors (Fig. 4a). As for F9, these transgenes were integrated into the α-globin locus of HUDEP-2 and KI cells were enriched by GFP sorting. Both mRNA and protein analyses confirmed enzymes expression, which substantially increased upon erythroid differentiation (16–171 fold and 2.5–4.5 fold respectively, Fig. 4b, c). For additional experiments in HSPCs we focused on LAL transgene, since Wolman disease (WD) is a life-threatening genetic condition with a severe liver phenotype and no gene therapy options available.

Editing of HSPCs showed that, without any selection, we could obtain high levels of Indels (Supplementary Fig. 5a) and KI of LAL as measured by GFP (Fig. 4d) and on-target ddPCR (Supplementary Fig. 5b), associated with a reduced number of HBA2 copies (Supplementary Fig. 5c). In addition, LAL enzyme was strongly expressed and secreted upon erythroid differentiation (Fig. 4e, f) and retained its hydrolytic activity, in accordance with antigen levels (Fig. 4g).

By analyzing KI HSPC derived burst-forming unit-erythroid colonies (BFU-E) we showed that LAL integrations were mostly monoallelic (Fig. 4h), associated with a reduced number of HBA2 copies (Supplementary Fig. 5d). After aggregation of the genotypes of F9 and LAL BFU-E, we established that most of edited BFU-E (87%) had transgene integration and/or HBA2 deletion and 53% harbored both modifications (Supplementary Fig. 5e, f).

In order to be therapeutically relevant, secreted LAL enzyme should cross-correct LAL deficient cells and reduce pathological cholesterol accumulation in lysosomes. Thus, we exposed WD patient’s fibroblasts to conditioned medium from untreated (UT) or KI HSPC derived erythroblasts (LAL). After 3 days we observed LAL uptake in WD fibroblast lysates (Fig. 4i), which correlated with a significant decrease of total cholesterol (Fig. 4j) and lipid deposits (Fig. 4k), clearly showing that the secreted enzyme can ameliorate the metabolic dysfunction. Altogether, we demonstrated that our platform is versatile and can express several functional therapeutic proteins that require post-translational modifications.

**In vivo long-term analysis of edited HSPCs.** To evaluate if LAL-KI HSPCs maintain their homing, engraftment and multi-lineage potential, we transplanted immunodeficient NOD/SCID/γ⁻¹ (NSG) mice and monitored human cells for 16 weeks (Fig. 5a). All mice showed successful engraftment in bone marrow, spleen and blood (Fig. 5b). GFP positive cells were present at different time points (Fig. 5c, d; Supplementary Fig. 4m).
Fig. 3 F9 KI into the α-globin locus results in expression and secretion of functional enzyme. a AAV6 donor used for KI experiments of FIX Padua. b FIX KI efficiency in HUDEP-2 cells was measured by flow cytometry (light green) or ddPCR specific for on-target integration (dark green) before and after sorting (n = 1). c Quantification of FIX mRNA in KI HUDEP-2 upon differentiation (mean ± SD, n = 2 undifferentiated, n = 3 differentiated). d Quantification of FIX secretion in medium of HUDEP-2 clones (n = 28) with monoallelic or biallelic KI (ELISA), as detected by on target ddPCR analysis (AAV-genome junction amplification). Lines represent median. e KI efficiency in HSPCs at day 9 of erythroid differentiation. Lines represent mean (n = 4). f, g FIX expression during HSPC differentiation at RNA (f, qPCR; n = 2 day 9; n = 4 day 12) and protein level (g, ELISA on supernatants, n = 3 day 7; n = 4 day 9 and 12; 3 donors). Bars represent mean ± SD. h FIX antigen (ELISA) and activity (aPTT) in supernatants of KI HSPCs (mean; n = 2). i, j Comparison of FIX RNA at day 9 and 12 of erythroid differentiation (i) and protein (j) in KI HSPCs (AAV + RNP) vs HSPCs transduced with an erythroid-specific lentiviral vector (LvEry FIX). Bars represent mean (**p = 0.003 t-test Holm-Sidak correction for RNA at day 12; p = 0.08 for protein, n = 2). k Integration pattern in single BFU-E (2 donors): no integration (0), monoallelic (1) and biallelic KI (2). Source data are in the Source Data file.
Since NSG mice do not support human erythroid differentiation, we isolated human CD34$^+$ cells from bone marrow of engrafted mice and differentiated them ex vivo. In a CFC assay, KI HSPCs were still able to generate both erythroid and myeloid colonies, to express GFP (Supplementary Fig. 6b) and, most importantly, to produce LAL in erythroblasts (Fig. 5g and Supplementary Fig. 6c). Similar in vivo and ex vivo results were also obtained for FIX (Supplementary Fig. 6d–g).
**Fig. 4** Expression and therapeutic potential of different lysosomal enzymes. a AAV6 donor used for KI experiments. All enzymes were tagged with hemagglutinin tag (3xHA). b Transcript upregulation of different enzymes in targeted HUDEP-2 upon differentiation (qPCR, n = 2, mean). Fold increase is indicated. c Representative western blot detecting different enzymes (HA-tag and anti-β tubulin) of targeted HUDEP-2 upon differentiation (n = 2). d KI efficiency of LAL-AAV6 in HSPCs at day 9 (lines indicate mean; AAV n = 4, AAV + RNP n = 5). e Representative western blot of LAL in HSPC lysates, supernatants and BFU-E in untreated (UT), transduced (AAV) and KI-HSPCs (AAV + RNP). Anti-HA tag and anti-β tubulin antibodies were used. f Quantification of secreted LAL during erythroid differentiation. Anti-LAL antibody was used. Data are shown as fold increase over untreated cells (UT, donor = 2). g LAL activity in HSPC supernatants during erythroid differentiation, data are shown as fold increase over untreated cells (UT, n = 2). h Integration pattern in single BFU-E: no integration (0), monoallelic (1) and biallelic (2). Mean ± SD, donor = 2). i Uptake of erythroid-expressed LAL by WD fibroblasts, measured by western blot or activity assay (mean; n = 2). j Cholesterol levels in WD fibroblasts after incubation with conditioned medium from untreated (UT) or LAL KI-erythroblasts. WT fibroblasts are shown as control (n = 4; p = 0.003 WD-UT vs WD-LAL; p < 0.001 WD-LAL vs WT; one-way ANOVA, Tukey’s test). k Nile Red staining in WD fibroblasts after incubation with conditioned medium from untreated (UT) or LAL KI-erythroblasts. WT fibroblasts are shown as control. Black lines indicate mean ± SD; number of fibroblasts analyzed is indicated. (***p < 0.001 one-way ANOVA, Tukey’s test). Source data are in the Source Data file.

**Fig. 5** KI HSPCs engraft NSG mice and express LAL upon erythroid differentiation. a Schematic representation of engraftment experiments. b Percentage of human CD45+/HLA-ABC+ cells in hematopoietic organs of mice. BM = bone marrow. (UT and AAV n = 3; AAV + RNP n = 8). c GFP positive cells in peripheral blood of transplanted mice over time. Line indicates mean (n = 4 week 4; n = 8 week 8 and 16). d Edited cells in bone marrow of transplanted mice. GFP is expressed as percentage of CD45+ cells, mean is shown (*p = 0.012 AAV vs AAV + RNP, two-tailed Mann–Whitney test). e GFP positive cells in HSPCs (CD34), myeloid (CD33), B (CD19) and T (CD3) cells in bone marrow of transplanted mice. Each line represents one animal (n = 8). f GFP positive cells in HSPCs (CD34) and in a more primitive HSPC subset (CD34+/CD38−) in bone marrow of transplanted mice (n = 4; ns: p = 0.8, two-tailed Mann Whitney test). g Western blot on CD34-derived BFU-E from mice engrafted with untreated (UT), transduced (AAV) or KI HSPCs (AAV + RNP). Anti-HA tag and anti-β tubulin antibodies were used (n = 2, BFU-E pooled from 2 mice). Source data are in the Source Data file.
Overall, these data show that KI HSPCs can engraft NSG mice and reconstitute all hematopoietic lineages.

Discussion

We developed an ex vivo platform for efficient gene targeting in human HSCs and robust expression of therapeutic transgenes by the erythroid lineage. By inserting transgenes under the control of the endogenous α-globin gene promoter, we demonstrated that erythroblasts derived from KI HSPCs ex vivo can express and secrete different therapeutic proteins, which retain their enzymatic activity and cross-correct the metabolic defect of patient’s cells. In addition, KI HSPCs were able to engraft in vivo and maintained multilineage differentiation potential, we thus expect that our strategy can be used as platform to treat genetic and non-genetic disorders.

We demonstrated that the α-globin locus can be used as a safe harbor for transgene KI in HSCs. In particular, we showed that our selected Cas9/gRNA targeting α-globin 5’ UTR is: (i) efficient in inducing DSB in HSPCs (up to 80%); (ii) safe, as no effect on HSPC multipotency and hemoglobin expression was observed; (iii) specific for α-globin genes, as no predominant off-targets were detected. To further improve the safety profile of this approach, we can envisage the use of Cas9 variants, e.g., high-fidelity or nickase; nonetheless, ad hoc DNA analysis for major chromosomal alterations will be required before moving to clinical testing.

Using the described 5’UTR gRNA and an AAV6 vector carrying a promoterless transgene we achieved efficient HDR-based integration in the α-globin locus (above 50%). Although transgene integration will result in knockout of the targeted α-globin allele, this should not be a concern since α-globin genes are redundant and a reduction of 50% of α-globin chain is clinically asymptomatic. In addition, while it is theoretically possible to achieve 4 transgene integrations (1 for each HBA gene), KI efficiency is mostly limited to 1 transgene per cell (Figs. 3d, k, 4h), minimizing the risk of causing α-thalassemia.

Transgene expression was limited to the erythroid lineage and increased following erythroid maturation, as expected from the endogenous α-globin promoter. Importantly, we showed that erythroblasts are able to synthesize and secrete different functional enzymes; secreted LAL was uptaken by patient’s fibroblast and correctly sorted to lysosomes to reduce pathological cholesterologenesis, suggesting that secreted enzymes are properly processed to enter the mannose-6-phosphate pathway. Overall, these results show the versatility of our platform and support its application to other LSD.

By transplanting HSPCs in humanized NSG, we demonstrated that KI HSPCs can repopulate the bone marrow and give rise to progenitors and differentiated hematopoietic lineages. Unfortunately, since NSG and other immunodeficient mouse models do not support significant human erythropoiesis and prevent the in vivo assessment of this erythroid-based platform, we performed ex vivo erythroid differentiation of bone marrow isolated CD34+ cells confirming that HSCs can still differentiate and express the integrated transgene. Future experiment of KI in mouse HSPCs carrying the human α-globin locus will allow in vivo erythroid differentiation and direct assessment of the steady-state expression levels achievable with our strategy.

Protein replacement therapies have proven to be a life-saving therapy for patients affected by rare genetic diseases. However, PRT requires frequent costly injections with a peak-and-trough serum kinetics, which reduces patients’ compliance to the therapy and efficacy of treatment, and it is affected by development of anti-drugs antibodies, which negatively influence drug bioavailability and activity. Instead, gene therapy can provide constant serum level of therapeutic proteins with a single administration and can induce immune tolerance to the expressed transgene. In particular, the idea of integrating a therapeutic transgene in a safe and highly transcribed genomic locus has been already described for the albumin gene and is now under clinical evaluation (NCT03041324, NCT02695160). However, this in vivo approach is hampered by pre-existing liver conditions, pre-existing neutralizing antibodies and cell-mediated immune responses against AAV vectors used to deliver transgenes or nucleases, thus severely limiting the number of potentially treatable patients. To avoid these issues, autologous HSCs can be successfully engineered ex vivo by LV to express transgenes in ubiquitous or lineage-restricted manner, including erythroid lineage; however, the semi-random integration pattern of LV is intrinsically associated with the risk of inactivating an oncosuppressor and transactivating an oncogene. Our strategy promises to be a safer option since transgene integration is targeted to a safe locus, no exogenous promoter is required and transgene expression is truly restricted to erythroid cells, which can induce immune tolerance to exogenous proteins. In addition, transgene expression achieved by targeted integration outperformed a LV carrying an erythroid-specific promoter, which can only partially replicate the complex regulation of a genomic locus due to vector capacity limitations and different chromatin context. The benefit associated to our strategy is twofold: (i) need of fewer modified HSPCs; (ii) higher expression potential.

Our approach still requires bone marrow transplantation of HSPCs, but on-going improvements of HSC mobilization and conditioning regimens will facilitate this procedure. In addition, we will explore alternative DNA donor delivery system, e.g. IDLV or non-viral vectors, or transient p53 inactivation, means to avoid the negative effect of AAV6 on HSPC engraftment potential. Finally, we will have to assess in vivo if over-expression of transgenes in erythroid precursor cells can have an effect on the HSCs niche in the bone marrow or on erythrocyte differentiation, half-life and clearance. Previous experiments using LV to express different proteins from erythrocytes did not show any impact on erythropoiesis; however, transgene-specific effects should be carefully evaluated.

Finally, we will engineer transgene sequence with blood-brain barrier shuttle peptides to treat LSD with central nervous system involvement, a severe limitation of current PRT.

In conclusion, we identified the α-globin gene as a safe genomic locus for transgene KI in HSCs and erythroid-specific overexpression of therapeutic protein. Future in vivo tests will elucidate the therapeutic potential of this CRISPR-Cas9 based HSC-platform for PRT, especially for LSD and hemophilia.

Methods

Plasmids

Different gRNA protospacers were cloned in hU6-gRNA encoding plasmid (Addgene plasmid # 53188) after BbsI digestion. Promoter trap encodes for a promoterless GFP reporter (with bovine growth hormone polyA) followed by a puromycin resistance gene under the control of the human phosphoglycerate kinase 1 (PGK) promoter with a SV40 polyA. For intron traps, we added a synthetic intron with splice acceptor site (adapted from and a self-cleaving peptide from porcine teschovirus-1 (P2A) in frame (+0 or +2) at 5’ of GFP cDNA (Supplementary Fig. 2a and Supplementary Methods). These cassettes were inserted in a standard lentiviral vector (LV) backbone in antisense orientation with respect to its LTR.

According to the experiments, GFP and puromycin sequences were exchanged with enzyme cDNA and GFP respectively. f9 (Gene ID: 2158, R338L substitution), GLA (Gene ID: 2717), IDUA (Gene ID: 3432) and LAL (Gene ID: 3988) cDNA with a C-terminal 3xHA tag (1xHA: TATCCCTATGACGTGCCT GATTACGCC) and arms of homology (250 bp each) were synthesized by Genescript (Piscataway, NJ) and cloned in a standard AAV vector backbone (AAV2) in sense orientation with respect to its ITR. Upon successful HDR, transgene translation starts from the same ATG as the endogenous α-globin ATG for 5’ UTR integration or after translation of a fragment of α-globin chain for IVS2

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integration (α-globin-2A-GEF). Homology arms for 5’UTR α-globin integration are, upstream, chr1:16,712,642-172,892; downstream, chr1:16,712,893-173,142 (hg38). Homology arms for HBG1 α-globin integration are, upstream, chr1:16,713,135-173,385; downstream, chr1:16,738,366-173,636 (hg38).

Vector productions. LVs were produced by transient transfection of 293T using third-generation packaging plasmid pMDL/gp/RRE (or pMDL/gp/RRE.D64V for integrase defective vectors; IDLV), pK.REV, and pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) envelope. LV/IDLV were titrated in integrase defective vectors; IDLV), pK.REV, and pseudotyped with the vesicular Vector productions

173,385; downstream, chr16:173,386-173,636 (hg38).

chromatography (AVB Sepharose; GE Healthcare, Chicago, IL). The

penicillin and streptomycin (Gibco, Waltham, MA, USA). Mobilized peripheral blood (MPB) and UCB CD34

Paris, France) after immunostaining with CD34 MicroBead Kit (Miltenyi Biotec, Paris, France). Erythroid differentiation was induced with 50 μM Hemin (Sigma-Aldrich, St. Louis, MO, USA) and monitored for 4 days. As K562 differentiation is

drug-induced (α-globin integration

MOI 50 with integrase defective lentiviral vectors (IDLV) containing

using an adenovirus-free triple transfection method of HEK293 and puri

tracrRNA and crRNA were purchased from Integrated DNA Technologies, Coralville, IA, USA) or diluted (Synthego, CA, USA) following manu-

300 ng/ml, rhTPO 100 ng/ml and IL-3 20 ng/ml, CellGenix, Freiburg, Germany). Supplemental PD-15 µM and 2% human peripheral blood was also obtained by Cloniscience, (Nanterre, France). PBMC- or UCB-derived HSPCs were thawed and cultured in prestimulation media for 48 h ( StemSpan, Stemreugen-1

0.75uM, StemCell technologies, Vancouver, BC, Canada; rhSCF 300 ng/ml, Flt3-L 300 ng/ml, rHtPO 100 ng/ml and IL-3 20 ng/ml, CellGenix, Freiburg, Germany). Spatial and temporal intravital imaging of transduced donor DNA and scaffolds using fluorescent reporter genes and DNA Technol-

gies, Coralville, IA, USA) following manufacturer’s instructions. All recombining single-stranded AAV2/6 used in this study were produced using an adenosivirus-free triple transfection method of HEK293 and purified by two sequential cesium chloride (CsCl) density gradients or by single affinity chromatography (AVB Sepharose; GE Healthcare, Chicago, IL). The fluorescence

was measured in sterile phosphate buffered saline containing 0.01% of pluronic (F68; Sigma Aldrich, St Louis, MO), and stored at —80 °C.67

Cell culture and reagents. K562 cells (ATCC® CCL-243) were maintained in RPMI 1640 medium containing 2 mM glutamine and supplemented with 10% fetal bovine serum (FBS, Lonza, Basel, Switzerland), 10 mM HEPES, 1 mM sodium pyruvate and penicillin and streptomycin (100U/ml each; Gibco, Waltham, MA, USA). HEUDE-2 cells48 were maintained in StemSpan (StemCell Tech-

nologies, Vancouver, BC, Canada) supplemented with 2 mM glutamine, 100U/ml penicillin and streptomycin (Gibco, Waltham, MA, USA), Epo 3 U/ml, SCF 50 ng/ml (PeproTech, Rocky Hill, NJ, USA); 1 μg/ml doxycycline and 1 μg/mL dexamethasone (Sigma Aldrich, St Louis, MO, USA). Cells were differentiated in Isco’s Modified Dulbecco’s Medium (IMDM) with 2 mM glutamine, 100U/ml penicillin and streptomycin (Gibco, Waltham, MA, USA, Gibco, Waltham, MA, USA), Epo 3 U/ml, SCF 50 ng/ml (PeproTech, Rocky Hill, NJ, USA), 5% AB human serum (Biowest, Nuaille, France), 10 μg/ml insulin, 330 μg/ml Holo-

Transferrin, 2 U/ml heparin, 1 μg/ml doxycycline (Sigma Aldrich, St Louis, MO, USA). Doxycycline and SCF were removed after 5 days of differentiation68. HEUDE-2 single cell clones were obtained by limiting dilution. Human primary fibroblasts were obtained from the NIGMS Human Genetic Cell Repository at the Corell Institute for Medical Research: GM 11851 A (Wolman disease fibroblasts) and GM 6012 3 (healthy donor fibroblasts). Fibroblasts were maintained in DME Medium supplemented with 2 mM Glutamax (Gibco, Waltham, MA, USA), 15% of FBS (Lonza, Basel, Switzerland) and 100U/ml of penicillin/Strep-

tomycin (Gibco, Waltham, MA, USA). Streptococcus pyogenes (SpCas9) protein (with 2 nuclear localization signals, NLS; provided by J.P. Concordett) was expressed in E. coli strain BL21 Rosetta 2. The protein was purified as a combina-

of affinity, ion exchange and size exclusion chromatographic steps69. tracrRNA and crRNA were purchased from Integrated DNA Technologies, resuspended and annealed by manufacturer’s instructions. Chemically modified single guide RNA were purchased from Synthetic. Primer and probes for PCR were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Integrated DNA Tech-

nologies (Coralville, IA, USA).

Editing experiments in K562. gRNA screening: To generate a stable clone of Cas9-expressing K562 (K562-Cas9), K562 cells were transduced with a lentiviral vector (Addgene #52962) expressing SpCas9 and a bacterialin resistance cassette, selected with bacitracin (10μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and sub-

Erythroid differentiation was induced with 50 μM Hemin (Sigma-Aldrich, St Louis, MO, USA) and monitored for 4 days. As K562 differentiation is

MOI 10000-30000), washed and left in prestimulation media for additional 48 h. Lentiviral transduction of HSPCs was performed in retroenectin-coated plates (5ug/cm2; Takara, Japan) for 6 h in the presence of 4 μg/ml proteamine sulfate (Sanofi Aventis, Paris, France)35. After manipulation, HSPCs were cultured in erythroid differentiation medium (StemSpan, StemCell Technologies, Vancouver, BC, Canada; SCF 20 ng/ml, Epo 1 U/ml, IL3 5 ng/ml, Dexamethasone 2 μM and Betamethasol 1 μM; Sigma-Aldrich, St.Louis, MI, USA) or in semisolid Methocult medium (colony-forming cells (CFC) assay, H4435, StemCell Technologies, Vancouver, BC, Canada) for 14 days. Colonies were counted and identified according to morphological criteria (BFU-E, CFU-G/M, and CFU-GEMM). In some experiments, BFU-E were picked and cultured in erythroid progenitor expansion medium (StemSpan SFEM, StemCell Technologies, Vancouver, BC, Canada; Epo 20 ng/ml, SCF 100 ng/ml, insulin-like growth factor-1 (IGF-1) 50 ng/ml (Pepro-

Tech, Rocky Hill, NJ, USA); and 2 μM dexamethasone (Sigma Aldrich, St. Louis, MO, USA) for 3-5 days69.

Flow cytometry. Cells were fixed and/or permeabilized using Cytofix/Cytoperm™ (BD Bioscience, San Jose, CA, USA) according to manufacturer’s instructions. For live cell analysis, viability was assessed using Zombie Yellow dye (BioLegend, San Diego, CA, USA) as per manufacturers’ instructions to exclude dead cells from the analysis. Negative controls were obtained by staining cells only with the isotype control antibodies. For engraffment studies, an Fc Receptor Binding Inhibitor antibody was used to block unspecific binding of mouse Ab to human cells, as per manufacturers’ instructions. Cells were analyzed using CytoFLEX S (Beckman Coulter, Pasadena, CA, USA) or SP6800 Spectral Analyzer (Sony, Tokyo, Japan); data were elaborated with CytExpert (Beckman Coulter, Pasadena, CA, USA) or FlowJo software (Tree Star, OR, USA).

We used MoFlo sorter (Beckman Coulter, Pasadena, CA, USA) to select for live GFP positive cells. See Supplementary Table 4 for antibodies list and Supplementary methods for gating strategy of human engrafted cells.

DNA analysis. Genomic DNA was extracted with QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) or QuickExtract™ DNA Extraction Solution (Lucigen, Middleton, WI, USA). Quantification of editing efficiency (InDel): 50 ng of genomic DNA were used to amplify the region that spans the cutting site of each gRNA using KAPA2G Fast ReadyMix (Kapa Biosystem, Wilmington, MA, USA). After Sanger sequencing (Geneviz, Takeley, UK), the percentage of insertions and deletions (InDel) was calculated using TIDE software (Three Star, SF, USA)27.

To quantify HBA2 copy number, primers and probes were designed on the 3′ UTR of HBA2 gene, as it differs significantly from HBA1. To quantify on-target transgene integration events, primers and probe were designed spanning the donor DNA-genome 3′ junction. Human α-globin (ALB) or ZNF843 were used as reference for copy number evaluation (assay ID: dHsncP2506312, Biorad, Hercules, CA, US). Percentage of on-target integration obtained by ddPCR nicely correlated with GFP values obtained by FACS in K1 cells (Supplementary Methods). See Supplementary Table 3 for primer and probe sequences.

RNA extraction and RT-qPCR. Total RNA was purified using RNeasy Micro kit (Qiagen, Hilden, Germany) and reverse-transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qPCR was performed using Maxima Syber Green/Rox (Life Scientific, Thermo-Fisher Scientific, Waltham, MA, US). Primers and probes were optimized using the standard curve method to reach 100% ± 5% efficiency. The relative expression of each target gene was normalized using human GAPDH as a reference gene (NM_002046.6) and represented as 2-AACT for each sample or as fold changes (2-ΔΔCt) relative to the control. See Supplementary Table 3 for primer sequences.

Protein quantification and Western blot. FIX detection: FIX antigen in supernatants was measured with an ELISA assay using a standard curve with known amount of human FIX. A microtiter plate is coated with an anti-human FIX mouse mAb (BAF314, UCB Celltech, Brussels, Belgium, US) blocked with PBS-2% bovine serum albumin (BSA) and incubated with diluted supernatants. Protein is detected with a goat anti-human horseradish peroxidase (HRP)-
conjugated antibody (CL20040APHP; Cedarlane, Burlington, Canada)21. Samples were analyzed at different dilutions (1/20, 1/40 and 1/100). FX activity was measured by activated partial thromboplastin time (APTT)26. Protein concentration in diluted supernatant was calculated using a standard curve containing known quantities of hFIX spiked in FIX-deficient plasma.

Western blot: To detect intracellular proteins cells were lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MI, USA) supplemented with protease inhibitor (Roche, Basel, Switzerland), freezed/thawed and centrifuged 10 × at 4 °C. Total protein was quantified using BCA assay (Thermo-Fisher Scientific, Waltham, MA, US). 5–15 μg of protein or 2.5 ul of cell supernatants were denatured at 90 °C for 10’, run under reducing conditions on a 4–12% Bis-tris gel and transferred to a nitrocellulose membrane using (Blot2 system (Invitrogen, Waltham, MA, US). After Ponceau staining (Invitrogen, Waltham, MA, US) membranes were blocked for 2 h with Odyssey blocking buffer (Odyssey Blocking buffer (PBS), Li-Cor Biosciences, Lincoln, NE, USA) and incubated for 1 h with primary antibodies followed by secondary antibodies antibodies in PBS:Blocking buffer (see Supplementary Table 5 for antibodies list). β-Tubulin was used as loading control. Blots were imaged at 1 μm with Odyssey imager and analyzed with ImageStudio Lite software (Li-Cor Biosciences, Lincoln, NE, USA). After image background subtraction (average method, top/bottom), band intensities were quantified and normalized with tubulin signal. Antibody concentrations, suppliers and catalog numbers are provided in Supplementary Table 5.

LAL activity assay: Protease activity was detected in supernatants as previously described2,23 with some modifications. Briefly, samples were incubated 10 min at 37 °C with 42 μM Lalistat-2 (Sigma-Aldrich, St. Louis, MI, USA), a specific competitive inhibitor of LAL, or water. Samples were then transferred to a Optiplate 96 F Plate (PerkinElmer) where fluorometric reactions were initiated with 75 μl of 10 μg/ml 4-methyl umbelliferone buffer (300 mM Tris, pH 7.5; 200 mM cardiolipin in 135 mM acetate buffer pH 4.0). After 10 min, fluorescence was recorded (35 cycles, 30’ intervals, 37 °C) using SPARK TECAN Reader (Tecan, Austria). Kinetic parameters (average rate) were calculated using Magellan Software. LAL activity over untreated samples was quantified using this formula:

\[
\text{LAL activity} = \frac{\text{absorbance} - \text{blank absorbance}}{\text{time}}
\]

Data availability

The authors declare that all data supporting the findings of this study are available within the paper and Supplementary Information. The IDLV capture data that support the findings of this study have been deposited in NCBI Gene Expression Omnibus (GEO) under accession number GSE133861. [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE133861]. Any other relevant data are available upon reasonable request. Source data are provided with this paper.

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Author contributions
G.P. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. M.L. performed experiments and analyzed data for LAL. E.K., A.F. and A.S. performed experiments. G.C. performed bioinformatics analysis of Cas9/gRNA specificity. P.L. performed aPTT assay for FIX experiments. J.P.C. provided purified SpCas9 protein. M.T. produced and titrated AAV and LV. A.M. performed and analyzed HPLC. M.A. conceived the study, designed experiments, analyzed data and wrote the manuscript.

Competing interests
G.P. and M.A. are the inventors of a patent describing this HSC-based gene therapy platform (Genetically engineered hematopoietic stem cell as a platform for systemic protein expression; EP18303026.9). The remaining authors declare no competing interests.

Additional information
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