Correction of β-thalassemia by CRISPR/Cas9 editing of the α-globin locus in human hematopoietic stem cells

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Key Points

- *HBA2* gene deletion in human HSPCs reduces α-globin precipitates in erythroblasts and ameliorates β^+-thalassemia phenotype.

- Synergistic *HBA2* gene deletion and *HBB* gene replacement in HSPCs ameliorate β^0-thalassemia phenotype

β-thalassemias (β-thal) are a group of blood disorders caused by mutations in the β-globin gene (*HBB*) cluster. β-globin associates with α-globin to form adult hemoglobin (HbA, $\alpha_2\beta_2$), the main oxygen-carrier in erythrocytes. When β-globin chains are absent or limiting, free α-globins precipitate and damage cell membranes, causing hemolysis and ineffective erythropoiesis. Clinical data show that severity of β-thal correlates with the number of inherited α-globin genes (*HBA1* and *HBA2*), with α-globin gene deletions having a beneficial effect for patients. Here, we describe a novel strategy to treat β-thal based on genome editing of the α-globin locus in human hematopoietic stem/progenitor cells (HSPCs). Using CRISPR/Cas9, we combined 2 therapeutic approaches: (1) α-globin downregulation, by deleting the *HBA2* gene to recreate an α-thalassemia trait, and (2) β-globin expression, by targeted integration of a β-globin transgene downstream the *HBA2* promoter. First, we optimized the CRISPR/Cas9 strategy and corrected the pathological phenotype in a cellular model of β-thalassemia (human erythroid progenitor cell [HUDEP-2] β^0). Then, we edited healthy donor HSPCs and demonstrated that they maintained long-term repopulation capacity and multipotency in xenotransplanted mice. To assess the clinical potential of this approach, we next edited β-thal HSPCs and achieved correction of α/β globin imbalance in HSPC-derived erythroblasts. As a safer option for clinical translation, we performed editing in HSPCs using Cas9 nickase showing precise editing with no InDels. Overall, we described an innovative CRISPR/Cas9 approach to improve α/β globin imbalance in thalassemic HSPCs, paving the way for novel therapeutic strategies for β-thal.

Introduction

Adult hemoglobin consists of 2 pairs of globin subunits ($\alpha_2\beta_2$), whose production is strictly regulated to ensure their balanced expression in erythroid cells. Disorders in hemoglobin synthesis cause thalassemia, a severe anemia requiring lifelong supportive treatments.[1] β-thalassemia is the most common and severe form of thalassemia, with >70 000 new patients per year worldwide, caused by mutations in the β-globin gene (*HBB*) cluster, which result in reduced or absent synthesis of β-globin chain.[2] As a consequence, the free α-globin in excess forms toxic precipitates that cause intramedullary apoptosis of erythroid precursors (ineffective erythropoiesis) and death of mature

red blood cells (hemolysis). Healthy individuals carry 4 α-globin genes per cell (αα/αα), 2 genes in *cis* on each chromosome 16 (*HBA1* and *HBA2*), which often undergo genomic rearrangements and *HBA2* gene loss because of their sequence homology (96.67%, GRCh38). Clinical data have shown that the severity of β-thalassemia directly correlates with the number of α-globin genes, with *HBA* deletions having a beneficial effect for patients.[3] This positive effect is especially pronounced in patients with hemoglobin variant E (HbE) β-thalassemia, where the HbE allele still allows residual expression of β-globin chain (~50% of all β-thalassemia patients).[4-7]

Current clinical management of β-thalassemia patients relies on regular blood transfusion and iron chelation therapy, required to reduce organ damage and iron overload, respectively.[8] Allogeneic hematopoietic stem cell (HSC) transplantation is a definitive cure, but it is limited by the availability of compatible donors.[9-12]

Two alternative strategies have been proposed to counteract the α/β globin imbalance in β-thal. The first consists in the expression/induction of β-globins or β-like globins, such as γ-globin, to complex with the α-globins in excess and produce adult or fetal hemoglobin.[13-18] In particular, ex vivo HSC gene therapy with lentiviral vectors (LV) encoding for *HBB* transgene significantly ameliorates or resolves β$^+$-thalassemia in patients, where the residual endogenous *HBB* expression contributes to the clinical benefit; however, its effects are less pronounced for β^0-thalassemia (no residual *HBB* expression).[13,14] The second approach to treat β-thalassemia aims at reducing α-globin expression to avoid the formation of toxic precipitates in erythroid cells.[3,19,20]

Here, we propose to use the CRISPR/Cas9 system to combine these 2 strategies: (1) α-globin chain reduction by recreating the natural α-thalassemia trait (-α$^{3.7}$ deletion, -α/αα or -α/-α) and (2) targeted integration and expression of a *HBB* transgene under the control of the endogenous *HBA* promoter. Concomitant downregulation of α-globin and upregulation of β-globin allows successful amelioration of α/β globin imbalance in both β$^+$ and β^0 thalassemia cells.

Methods

Plasmids

We designed 2 different cassettes for the expression of *HBB* (gene ID: 3043): (1) the first cassette contains a β-globin complementary DNA (cDNA), with 3 antisickling point mutations (β^{AS3}),[21] followed by a woodchuck posttranscriptional regulatory element and a SV40polyA; and (2) the second cassette contains the whole β^{AS3} gene with introns and its original 3′ untranslated region (UTR) and polyA (pA) site, with 3 antisickling point mutations.

Each cassette, followed by a green fluorescent protein (GFP) reporter gene under the control of the constitutive human phosphoglycerate kinase 1 promoter with an SV40 pA, was flanked by arms of homology (250 bp each) and cloned in a standard AAV vector backbone (AAV2) in sense orientation with respect to its inverted terminal repeat.

To generate the double reporter AAV, we substituted the β^{AS3} gene with the human low-affinity nerve growth factor receptor cDNA. Each cassette was synthetized by Genscript (Piscataway, NJ).

The LV encoding the β^{AS3} gene under the control of the erythroid specific β-globin enhancer/promoter was already described.[22]

All reagents and detailed sequence information are available upon request.

Vector productions

All recombinant single-stranded AAV2/6 used in this study were produced using a triple transfection protocol and purified by 2 sequential cesium chloride density gradients or chromatography, as described earlier.[23] The vector titer of each preparation was determined by real-time quantitative polymerase chain reaction (qPCR)–based titration method using primers and probe corresponding to the inverted terminal repeat region of the AAV genome.[24]

LV were produced by transient transfection of 293T using third-generation packaging plasmid pMDLg/p.RRE and pK.REV and pseudotyped with the vesicular stomatitis virus glycoprotein G envelope. LV were titrated in HCT116 cells and HIV-1 Gag p24 content was measured by enzyme-linked immunosorbent assay (PerkinElmer, Waltham, MA) according to the manufacturer's instructions.

Cell culture and reagents

K562 cells (ATCC CCL243) were maintained in RPMI 1640 medium containing 2 mM glutamine and supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1 mM sodium pyruvate, and penicillin and streptomycin (100 U/mL each; Gibco, Waltham, MA).[25]

HUDEP-2 β^0 cells were derived from a HUDEP-2[26] clone where both β-globin alleles were knocked out using CRISPR/Cas9 and a guide RNA (gRNA) targeting *HBB* exon 1 (HBB gRNA), as previously described.[17] Both HUDEP-2 and HUDEP-2 β^0 cells erythroid differentiation was performed in presence of doxycycline in 2 phases, with and without stem cell factor to promote erythroid maturation.[27]

HUDEP-2 β^0 single cell clones with different *HBA2* copy number were obtained by limiting dilution.

Streptococcus pyogenes Cas9 protein (with 2 SV40 nuclear localization signals) was provided by J.P. Concordet and was expressed and purified as previously described.[28] *Streptococcus pyogenes* Cas9 D10A protein (Alt-R S.p. Cas9 D10A Nickase V3; with 3 SV40 nuclear localization signals) was purchased from Integrated DNA Technologies (Coralville, IA).

Chemically modified single guide RNA were purchased from Synthego (with protospacer adjacent motif):

HBB gRNA: TCTGCCGTTACTGCCCTGT(GGG)

5′ UTR (HBA15) gRNA: GGGUUCUCUCUGAGUCUGUG(GGG)

HBA knockout (KO) gRNA: GUCGGCAGGAGACAGCACCA(TGG)

HBA20 gRNA: CATAAACCCTGGCGCGCTCG(CGG)

Primer and probes for PCRs were purchased from Sigma-Aldrich (St Louis, MO) and Integrated DNA Technologies (Coralville, IA).

CD34$^+$ cell culture, transfection, and transduction

Human umbilical cord blood (UCB) samples were provided by Centre Hospitalier Sud-Francilien (CHSF; Evry, France) and processed according to France bioethics laws (Declaration

DC-2012-1655 to the French Ministry of Higher Education and Research). CD34$^+$ cells were selected from patients affected by β-thalassemia as described[29] upon signed informed consent approved by the Ethical Committee of the San Raffaele Hospital (Milan, Italy).

CD34$^+$ cells were purified by immunomagnetic selection with AUTOMACS PRO (Miltenyi Biotec, Paris, France) after immunostaining with CD34 MicroBead Kit (Miltenyi Biotec). Mobilized peripheral blood (MPB) and UCB CD34$^+$ were also purchased from Cliniscience (Nanterre, France).

MPB- or UCB-derived HSPCs were thawed and cultured in prestimulation media for 48 hours (StemSpan, Stemregenin-1 0.75 μM, UM171 0.35 μM; StemCell Technologies, Vancouver, BC, Canada; rhSCF 300 ng/mL, rhFlt3-L 300 ng/mL, rhTPO 100 ng/mL, and rhIL-3 20 ng/mL; CellGenix, Freiburg, Germany).

Single guide RNA (sgRNA) was diluted following the manufacturer's instruction and ribonucleoprotein complexes were formed with 30 pmol of spCas9 (ratio 1:1.5). A total of 2.5×10^5 hematopoietic stem/progenitor cells (HSPCs) per condition were transfected with ribonucleoprotein (RNP) using a P3 Primary Cell 4D-Nucleofector Kit (CA137 program).[25] In knock-in experiments, 10 minutes after transfection, HSPCs were transduced with AAV6 for 6 hours (multiplicity of infection, 10 000-30 000), washed, and left in prestimulation media for additional 24 to 48 hours.

Lentiviral transduction was performed as previously described.[25]

After manipulation, HSPCs were cultured in erythroid differentiation medium (StemSpan, StemCell Technologies; rhSCF 20 ng/mL, rhEpo 1 U/mL, IL3 5 ng/mL, dexamethasone 2 μM and beta-estradiol 1 μM; Sigma-Aldrich) or in semisolid MethoCult medium (colony-forming cells [CFC] assay, H4435; StemCell Technologies) for 14 days. Colonies were counted and identified according to morphological criteria (burst-forming units-erythroid [BFU-E], colony-forming unit-granulocyte/granulocyte macrophage, and colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte). In some experiments, BFU-E were picked and cultured in erythroid progenitor expansion medium, as previously described.[30]

Flow cytometry

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

We used MoFlocell sorter (Beckman Coulter) to select live GFP$^+$ cells.

See supplemental Table 1 for the antibodies list and supplemental Figure 3A for gating strategy of human engrafted cells.

DNA analysis

Genomic DNA was extracted with QIAamp DNA Micro Kit, AllPrep DNA/RNA 96 Kit (Qiagen, Hilden, Germany) or QuickExtract DNA Extraction Solution (Lucigen, Middleton, WI).

Quantification of editing efficiency (InDels). Fifty nanograms 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). After Sanger sequencing (Genewiz, Takeley, United Kingdom), the percentage of insertions and deletions (InDels) was calculated using TIDE[31] and ICE (Synthego) softwares. See supplemental Table 2 for primer sequences.

ddPCR. Digital droplet PCR (ddPCR) was performed according to manufacturer's instruction using ddPCR Supermix for Probes No dUTP (BioRad, Hercules, CA) and 1 to 50 ng of genomic DNA digested with *Hind*III (New England Biolabs, Ipswich, MA). Droplets were generated using AutoDG Droplet Generator and analyzed with a QX200 Droplet Reader; data analysis was performed with QuantaSoft (BioRad).

To quantify *HBA2* copy number, primers and probes were designed on the 3′UTR of *HBA2* gene because it differs significantly from *HBA1*.

To quantify on-target transgene integration events, primers and probes were designed spanning the donor DNA-genome 3′ junction. Human albumin (*ALB*) or *ZNF843* genes were used as reference for copy number evaluation (assay ID: dHsaCP2506312, BioRad). See supplemental Table 2 for primer and probe sequences.

RNA extraction and reverse transcription-qPCR

Total RNA was purified using RNeasy Micro Kit or, in case of individual BFU-E colonies, AllPrep DNA/RNA 96 Kit (Qiagen). RNA was 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). Primers and probe were optimized using the standard curve method to reach 100% ± 5% efficiency. The expression of each target gene was normalized using human *GAPDH* as a reference gene (NM_002046.6) and represented as $2^{\Delta Ct}$ for each sample or as fold changes ($2^{\Delta\Delta Ct}$) relative to the control. See supplemental Table 2 for primer sequences.

Off-target analyses

GUIDE-seq. GUIDE-Seq experiments were designed as previously described.[32] Human embryonic kidney 293T/17 cells (2.5×10^5) were transfected using Lipofectamine 3000 (Life Scientific, Thermo Fisher Scientific) with 750 ng of SpCas9-coding and 250 ng of sgRNA-coding plasmid or an empty pUC19 vector (background control), 10 pmol of the bait double-stranded oligonucleotide containing phosphorothioate bonds at both ends and 50 ng of a pEGFP-IRES-Puro plasmid, expressing both enhanced GFP and the puromycin resistance genes. One day after transfection, cells were replated and selected with puromycin (1 μg/mL) for 48 hours to enrich for transfected cells.

Genomic DNA extraction was performed using DNeasy Blood and Tissue Kit (Qiagen) following manufacturer's instructions. Genomic DNA was sheared using a Covaris S200 sonicator to an average length of 500 bp and subsequently end-repaired. Library preparation was performed using adapters and primers previously described[32] and

quantified with Qubit double-stranded DNA High Sensitivity Assay Kit (Life Scientific, Thermo Fisher Scientific). The sequencing reaction was performed with MiSeq sequencing system (Illumina) using an Illumina Miseq Reagent Kit V2 - 300 cycles (2 × 150 bp paired-end) and raw sequencing data (FASTQ files) were analyzed using the GUIDE-Seq computational pipeline.[33] Briefly, after demultiplexing, putative PCR duplicates were consolidated into single reads and mapped to the human reference genome GrCh37. Reads with a mapping quality lower than 50 were filtered out. Upon identification of the genomic regions integrating double-stranded oligonucleotides in aligned data, off-target sites were identified if, at most, 7 mismatches against the target were present and if absent in the background controls. GUIDE-Seq datasets are available in the BioProject online repository (ID PRJNA676022; http://www.ncbi.nlm.nih.gov/bioproject/676022).

Amplicon-sequencing. A total of 200 ng of genomic DNA of RNP-edited HSPCs were used to amplify top 3 off-targets identified with Guide-Seq using Phusion High-Fidelity polymerase (New England Biolabs). After checking the correct amplification by Sanger sequencing (Genewiz), amplicons were purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Hoerdt, France), and 500 ng of DNA were used for library preparation. Illumina-compatible barcoded DNA amplicon libraries were prepared using TruSeq DNA PCR-Free Kit (Illumina). PCR amplification was then performed using 1 ng of double-stranded ligation product and Kapa Taq polymerase reagents (KAPA HiFi HotStart ReadyMix PCR Kit; Sigma-Aldrich). After purification (Ampure XP beads; Beckman Coulter), libraries were pooled and sequenced with Illumina NovaSeq 6000 (paired-end sequencing 100 × 100 bases). Data were analyzed using CRISPResso2.[34]

See supplemental Table 2 for primer sequences.

HPLC analysis of globin chains and tetramers

HPLC analysis was performed using a NexeraX2 SIL-30AC chromatograph (Shimadzu, Kyoto, Japan) and analyzed with LC Solution software. HSPC-derived erythroblasts were lysed in water and globin chains were separated using a 250 × 4.6-mm, 3.6-μm Aeris Widepore column (Phenomenex, Torrance, CA). Samples were eluted with a gradient mixture of solution A (water/acetonitrile/trifluoroacetic acid, 95:5:0.1) and solution B (water/acetonitrile/trifluoroacetic acid, 5:95:0.1), monitoring absorbance at 220 nm. Hemoglobin tetramers were separated by HPLC using a cation-exchange column (PolyCAT A; PolyLC, Columbia, MD). Samples were eluted with a gradient mixture of solution A (20 mM bis Tris, 2 mM KCN; pH 6.5) and solution B (20 mM bis Tris, 2 mM KCN, 250 mM NaCl; pH 6.8). The absorbance was measured at 415 nm.

In vivo experiments

NOD.Cg-Prkdc[scid]Il2rg[tm1Wjl]/SzJ (NSG) mice were purchased from The Jackson Laboratory, Bar Harbor, ME (strain 005557) and maintained in specific-pathogen-free conditions. This study was approved by Ethical Committee CEEA-51, and conducted according to French and European legislation on animal experimentation (APAFiS#16499-2018071809263257_v4).

Forty-eight hours after editing, 5 to 7 × 10^5 CD34+ cells were injected intravenously into 8-week-old female NSG mice after sublethal irradiation (150 cGy). Human cell engraftment and knockin (KI) levels were monitored at different time points in peripheral blood by flow cytometry using anti-human CD45 and HLA-ABC antibodies (supplemental Table 1). Sixteen weeks after transplantation, blood, bone marrow, and spleen were harvested and analyzed. Peripheral blood was stained and red blood cells lysed during sample fixation (VersaLyse Lysing Solution and IOTest3 Fixative Solution; Beckman Coulter).

Cell purification and enrichment

Human CD34+ or CD45+ cells were purified from mouse peripheral blood or bone marrow by immunomagnetic selection with CD34 or CD45 MicroBead Kit UltraPure in combination with AUTOMACS PRO (PosseID2 Separation Program; Miltenyi Biotec).

Statistical analyses

Statistical analyses were performed using GraphPad Prism, version 6.00, for Windows (GraphPad Software, La Jolla, CA). One- or 2-way analysis of variance (ANOVA) with Tukey's multiple comparison posttest for 3 or more groups was performed as indicated. Values are expressed as mean ± standard deviation (SD) as otherwise indicated, with "n" indicating the number of independent biological replicates used in each group. Differences were considered significant at *P < .05 **P < .01, and ***P < .001.

Results

Deletion of HBA2 gene reduces α-globin precipitates in a β⁰ adult erythroid cell line

Clinical data suggest that a reduction of α-globin to 75% to 25% of its physiological levels is safe and beneficial to patients with β-thalassemia.[3,35] The most common natural mutations affecting α-globin synthesis are gene deletions that remove a single HBA gene from 1 or both chromosomes generating -α/αα or -α/-α genotypes (-α^3.7 and -α^4.2 deletions[36]) (Figure 1A). To mimic these beneficial deletions with *Streptococcus pyogenes* (*Sp*)Cas9 nuclease, we designed a single gRNA that cuts both HBA1 and HBA2 alleles removing 1 HBA2 gene per chromosome. To minimize the possibility of generating a α-globin KO, the sgRNA was designed to target the 5′UTR (HBA15) of HBA1 and HBA2 (Figure 1A), where the presence of InDels resulting from double-strand breaks (DSBs) does not affect α-globin production.[37] As a β⁰-thalassemia cell model, we used immortalized HUDEP-2, which can differentiate and express adult hemoglobin (supplemental Figure 1A-B), and we knocked out β-globin genes (HUDEP-2 β⁰) (supplemental Figure 1C).

We transfected both wild-type HUDEP-2 and HUDEP-2 β⁰ with RNP targeting HBA and we achieved efficient editing (83.1% ± 12.1 and 77.3% ± 18.2, respectively, n = 3; Figure 1B) and genomic deletion of HBA2 gene (0.9 ± 0.2 and 0.9 ± 0.1 HBA2 copy per cell, n = 3; Figure 1C), which resulted in a decrease of α-globin messenger RNA (mRNA) expression upon erythroid differentiation (Figure 1D). To establish a correlation between α-globin expression and number of HBA genes, we generated multiple cell clones with mono- or biallelic HBA2 deletions (-α/αα and -α/-α, respectively, n = 3 per genotype) and we showed a significant amelioration of the α/β-like globin imbalance upon deletion of HBA2, with the -α/-α clones being indistinguishable from wild-type HUDEP-2 cells (Figure 1D; supplemental Figure 1D). Importantly, our HBA2 deletion strategy reduced but did not abolish
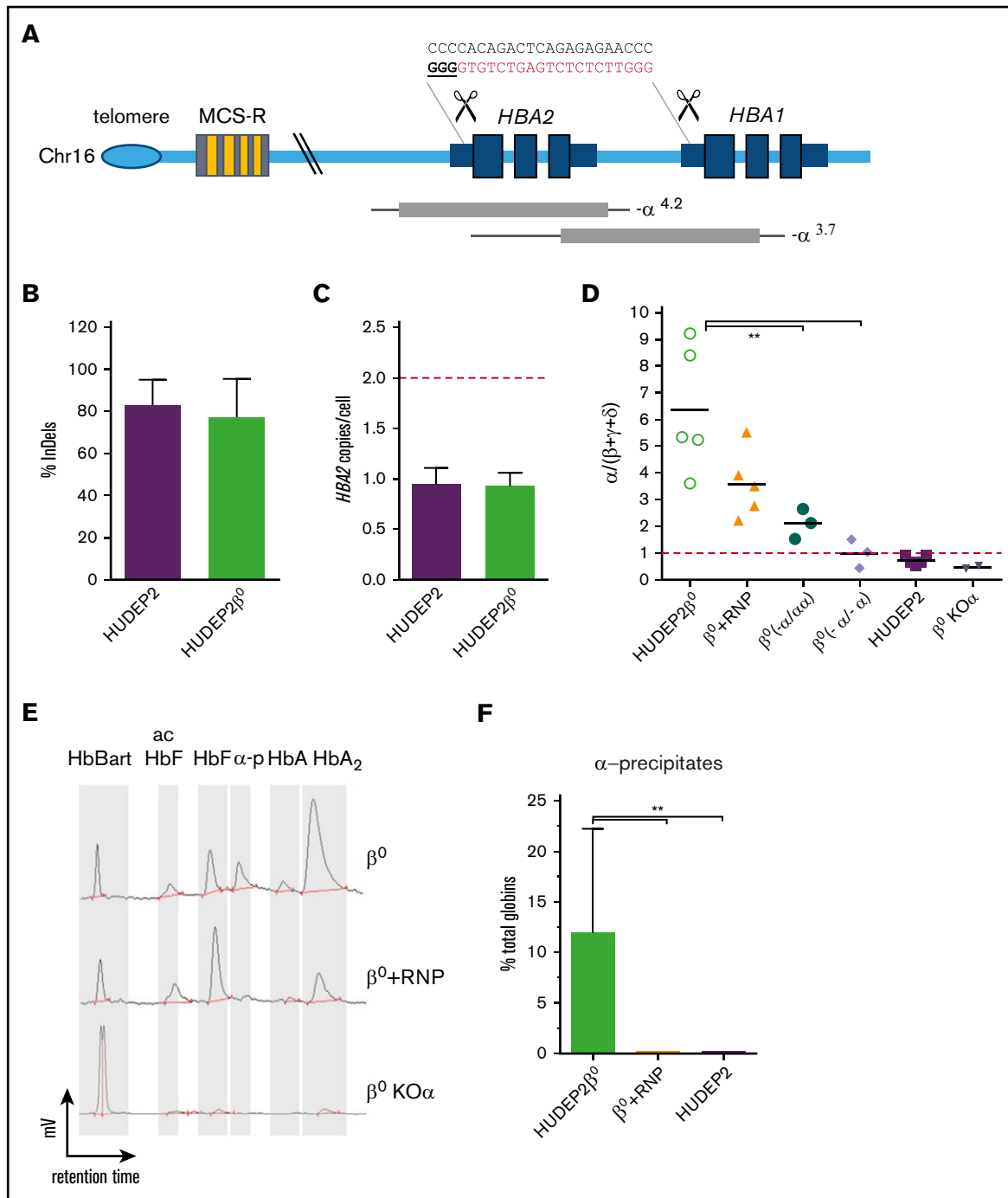
Figure 1. Deletion of *HBA2* reduces α-globin precipitates in HUDEP-2 β⁰ cells. (A) Schematic representation of the α-globin locus. *HBA1* and *HBA2* genes are located in the subtelomeric region of chromosome (Chr) 16. Expression is regulated by 4 erythroid-specific enhancers (multispecies conserved elements [MCS-R]) located 10 to 50 kb upstream. Common occurring deletions are shown as gray bars and thin lines indicate regions of uncertainty of the breakpoints (adapted from Harteveld and Higgs[98]). gRNA cutting sites are indicated as scissors. gRNA nucleotide sequence is indicated on top (in bold/underlined the protospacer adjacent motif sequence). (B) Editing efficiency in HUDEP-2 cells is expressed as percentage of modified HBA alleles. Bars represent mean ± SD (n = 3). (C) HBA2 copies in edited cells in panel B. Red dashed line indicates the number of expected HBA2 alleles in normal cells. (D) Reverse transcription-qPCR quantification of α/β-like globin mRNA ratios in HUDEP-2 β⁰ (n = 3-5) and in single-cell clones with monoallelic (α-/αα; n = 3) or biallelic (α-/α-; n = 3) *HBA2* deletions. β⁰ + RNP = HUDEP-2 β⁰ bulk population edited with gRNA HBA15/Cas9. β⁰ KOα = HUDEP-2 β⁰ bulk population edited with gRNA HBB/Cas9 to induce α-globin KO. Red dashed line indicates the physiological α/β-like ratio of 1. Bars represent mean ± SD (**$P < .01$, ANOVA, Tukey test). (E) Representative HPLC chromatograms of globin tetramer analysis in edited HUDEP-2 β⁰. γ₄, Hb Bart; acHbF, acetylated fetal hemoglobin; HbF, fetal hemoglobin (α₂γ₂); α-p, α-precipitates; HbA, adult hemoglobin (α₂β₂). (F) Quantification of α-precipitates. Every tetramer is reported as percent of total hemoglobins; bars show mean ± SD (n = 3; **$P < .01$; ANOVA, Tukey test).

α-globin production, as observed in α-globin KO control cells obtained with a gRNA targeting the coding sequence within the first exon of *HBA1* and *HBA2* genes (Figure 1D).

We also measured the relative abundance of the different hemoglobin forms by HPLC analysis. In the absence of β-globin, a portion of the α-globin pool complexes with β-like globins, such as γ- and δ-globins (to form HbF and HbA2, respectively), whereas the excess precipitates in insoluble aggregates (α-precipitates). Remarkably, RNP deletion of *HBA2* genes significantly reduced α-precipitates without affecting hemoglobin synthesis (Figure 1E-F). This is in sharp contrast with α-globin KO cells, where the predominant hemoglobin observed was the toxic HbBart (γ-globin tetramers), typical of severe forms of α-thalassemia (Figure 1E). This result clearly indicates that only a controlled reduction of α-globin results in a beneficial effect.

Targeted integration of β^AS3 transgene restores HbA expression in a β^0 adult erythroid cell line

To treat β^0-thalassemia patients, it is essential to express β-like globin chains; however, reaching expression levels to balance the endogenous α-globin has proven very challenging.[14] Therefore, we aim to lower the therapeutic threshold of β-globin expression by reducing α-globin abundance. For this purpose, we combined *HBA2* deletion with the KI of a β-globin transgene under the control of the endogenous *HBA* promoter. The same gRNA that deletes the *HBA2* gene will facilitate the integration of the β-globin transgene at this locus, whereas the endogenous *HBA* promoter will provide strong erythroid β expression, as previously suggested.[38-40] To discriminate exogenous vs endogenous β-globin expression, we used an *HBB* transgene containing 3 antisickling point mutations (β^AS3).[21]

To optimize β^AS3 expression, we designed 2 DNA donor cassettes: (1) a β^AS3 cDNA followed by a posttranscriptionally regulatory element[41] and SV40 polyadenylation signal (β^AS3 cDNA); and (2) a β^AS3 transgene that includes full-length *HBB* introns and the endogenous 3′UTR and polyadenylation signal (β^AS3 full). Both cassettes were cloned in an AAV6 vector with a GFP reporter gene under the control of a constitutive promoter and flanked by homology arms to favor homologous DNA recombination (HDR)[42] (Figure 2A).

To perform KI HUDEP-2 β^0 cells were transfected with RNP, transduced with AAV6, and then GFP sorted to enrich for β^AS3 integration (Figure 2B). A specific ddPCR confirmed on-target integration at molecular level (~0.8 β^AS3 on-target copies per cell), in good agreement with GFP expression (~95% GFP positive cells after sorting) (Figure 2C).

Analysis of β^AS3 mRNA expression in sorted cells showed an upregulation of about 100-fold for both cassettes upon erythroid differentiation, as expected from the endogenous α-globin promoter (Figure 2D). Although RNA levels were similar, we could detect β^AS3 globin protein only with the β^AS3 full, but not with the β^AS3 cDNA cassette (Figure 2E-F). This observation was further confirmed by hemoglobin tetramer analysis, in which only β^AS3 full cassette successfully restored ~40% of HbA ($\alpha_2\beta_2$) (Figure 2G-H). Overall, these results demonstrate that KI of β^AS3 full cassette under the endogenous *HBA* promoter can restore HbA expression in β^0 thalassemic cells.

HSPCs can be efficiently edited and retain long-term and multilineage engraftment potential

To evaluate this strategy in clinically relevant cells, we transfected human umbilical cord blood HSPCs with RNP and then transduced with AAV, as described for HUDEP-2 cells (Figure 3A). Without any selection, we achieved robust genome cutting (Figure 3B), with an InDel pattern consisting mostly of 1 T nucleotide insertion (supplemental Figure 2A-C), together with efficient *HBA2* deletion and donor DNA KI (Figure 3C-D).

Upon differentiation, HSPC-derived erythroblasts expressed β^AS3-mRNA, which accounted for ~15% (14.2 ± 9.4, n = 6) of total β globin RNA (Figure 3E-F).

We then plated HSPCs in methylcellulose containing cytokines supporting erythroid and myeloid differentiation (CFC assay), and we confirm that modified progenitors retained their multilineage potential, although some toxicity was observed (supplemental Figure 2D-E).

Single BFU-E genotyping showed that the majority of colonies had *HBA2* deletion (Figure 3G) and β^AS3 KI (Figure 3H) and 41% of them harbored both modifications (Figure 3I; supplemental Figure 2F).

Both *HBA2* deleted and β^AS3 KI HSPCs were then transplanted in immunodeficient NOD/SCID/γ (NSG) mice[43] to evaluate their in vivo homing, engraftment, and multilineage potential (Figure 4A). Both edited HSPCs showed successful engraftment in bone marrow, spleen and blood, and multilineage differentiation (Figure 4B; supplemental Figure 3B). Because NSG mice do not support human erythroid differentiation,[44] we confirmed differentiation by CFC assay of isolated human CD34^+ cells from bone marrow of engrafted mice (supplemental Figure 3C).

In addition, the percentage of InDels, as well as the extent of *HBA2* deletion in RNP-treated HSPCs, remained similar ex vivo and in vivo, confirming a similar efficiency in both stem and progenitor cells (Figure 4C-D).

β^AS3 KI HSPCs (GFP^+) were present at different time points (Figure 4E) in different lineages (supplemental Figure 3D); however, their engraftment was lower compared with unedited or *HBA2*-deleted HSPCs (Figure 4B,E), in accordance with previous reports describing AAV toxicity in HSPCs[45,46] and less efficient KI in noncycling HSCs.[47] Overall, these data show that we can achieve efficient *HBA2* deletion and β^AS3 KI in HSPCs while preserving their in vivo long-term engraftment.

Editing patients' HSPCs ameliorates β^+- and β^0-thalassemia phenotype

To test our strategy in therapeutic conditions, we assessed correction of globin imbalance in patients' HSPCs. In particular, we tested the *HBA2* deletion approach in β^+ cells and the combination of the α-deletion and β^AS3 KI strategy in β^+- and β^0-thalassemic HSPCs (supplemental Figure 4A-B).

HSPCs were transfected with RNP and transduced with AAV as described previously (Figure 3A). As positive control, HSPCs were transduced with a LV encoding for a β^AS3 transgene under the control of the β-globin gene promoter and its mini-locus control region, currently in clinical trial for thalassemia (LV β^AS3).[13,48]

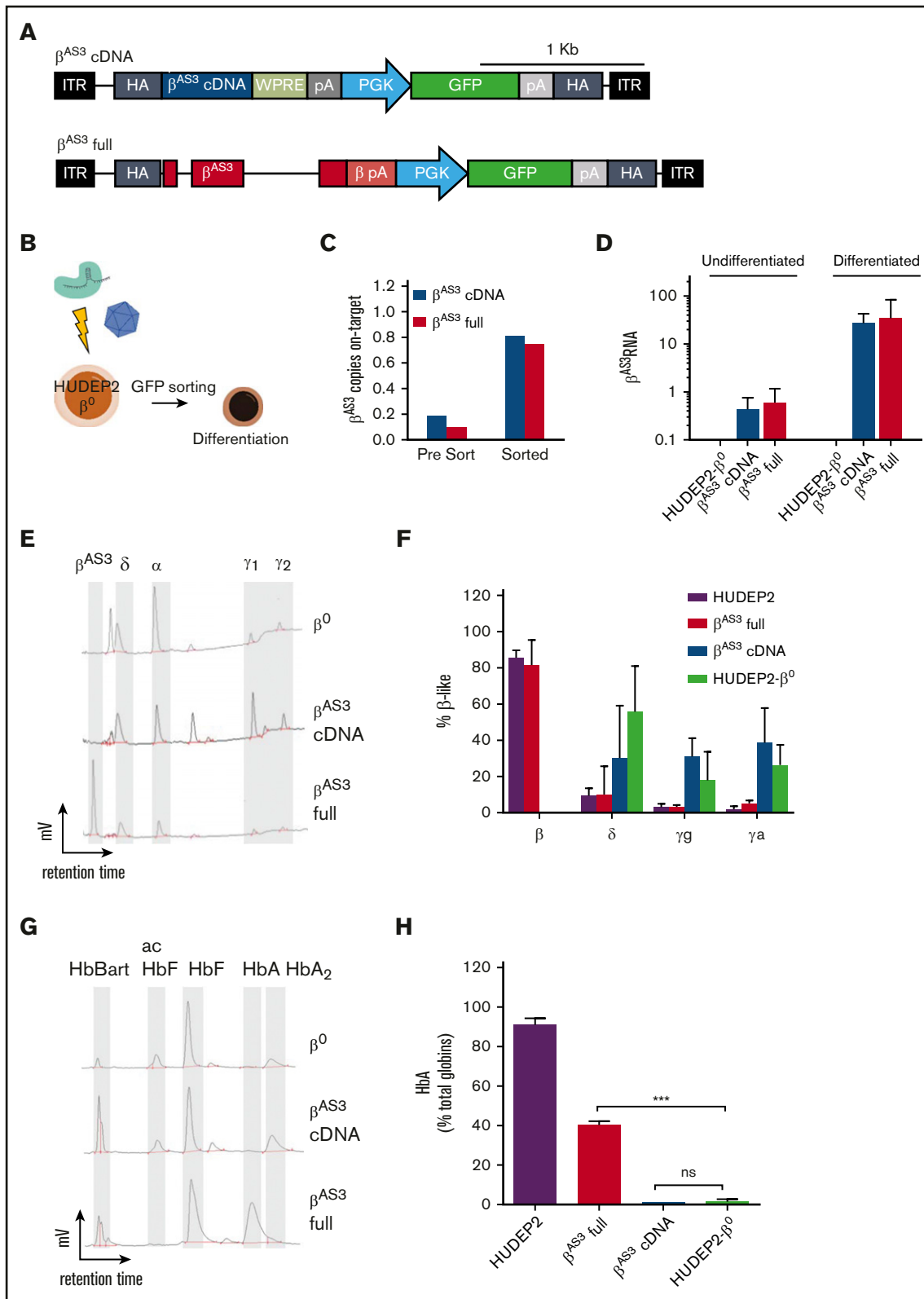**Figure 2. Targeted integration of a β^{AS3} transgene in the α-globin locus corrects thalassemic phenotype in HUDEP-2 β^0 cells.** (A) AAV6 donors used for KI experiments. Both vectors contain a promoterless β^{AS3} transgene, followed by a phosphoglycerate kinase (PGK) promoter with a GFP reporter and simian virus pA. This cassette is flanked by 250-bp homology arms (homology) to gRNA genomic target. ITR, inverted terminal repeats. Top: β^{AS3} cDNA followed by the woodchuck posttranscriptionally regulatory element (WPRE) and SV40 pA (β^{AS3} cDNA); bottom: β^{AS3} transgene that includes endogenous introns, 3′UTR and pA (β^{AS3} full). A 1-kb scale bar is indicated at top. (B) Schematic representation of HUDEP-2 β^0 targeting experiments. (C) KI efficiency of β^{AS3} cDNA (blue) or β^{AS3} full (red) in HUDEP-2 β^0 cells measured by

RNP transfection was very efficient both in erythroid liquid culture and CFC in generating InDels ($90.5\% \pm 7.1$ for RNP and $90.4\% \pm 9.0$ for RNP+AAV, mean $\pm$ SD; Figure 5A), *HBA2* deletion (0.95 ± 0.06 *HBA2* copies/cell for RNP and 0.77 ± 0.15 for RNP+AAV, mean $\pm$ SD; Figure 5B) and β^{AS3} KI (0.80 ± 0.21 copies/cell for RNP+AAV, mean $\pm$ SD; Figure 5C).

Globin expression was monitored during erythroid differentiation. mRNA globin imbalance (measured as α/β-like globins ratio) was ameliorated in all conditions (Figure 5D), with β^{AS3} KI cells performing better than *HBA2*-deleted erythroblasts because of β^{AS3} expression (Figure 5E; supplemental Figure 4C). Of note, modified HSPCs retained proper erythroid differentiation (supplemental Figure 4D) and multilineage potential (CFC assay; supplemental Figure 4F), although we observed some toxicity associated with the editing procedure (supplemental Figure 4E). Globin mRNA analysis of single BFU-E colonies derived from β^0- and β^+-edited HSPCs showed that α/β imbalance was improved in all conditions, in accordance with the reduced number of *HBA2* genes (supplemental Figure 4G) and with the stronger effect resulting from the concomitant α downregulation and β^{AS3} expression (Figure 5F).

Overall, these data show that we can modify β-thalassemia HSPCs and reduce their α/β globin balance, without affecting HSPCs potential.

Cas9 nickase represents a safe tool for HSPC editing

To reduce HSPC toxicity associated with nuclease-induced DSBs, we evaluated the use of Cas9 D10A nickase (Cas9 D10A). Because nicked genomic DNA is corrected by the endogenous base-excision repair pathway,[49] Cas9 D10A is expected to have minimal genotoxicty.[50,51]

To compare the efficiency of *HBA2* deletion and transgene targeted integration with single vs *trans* paired nicking of genomic DNA,[52] we selected another gRNA targeting α-globin 5′UTR (HBA20; supplemental Figure 5A-C) to combine with gRNA HBA15. K562 erythroleukemia cells were transfected with 1 or both gRNA complexed with Cas9 or Cas9D10A (RNP D10A) and transduced with a dual-reporter AAV vector to detect HDR (via a promoterless low-affinity nerve growth factor receptor) and non-HDR (via a phosphoglycerate kinase 1-GFP) integrations (supplemental Figure 5D). We first confirmed that gRNA HBA20 was comparable to HBA15 in generating InDels and *HBA2* deletions when complexed with wild-type Cas9 (RNP) (supplemental Figure 5E-F). When using RNP D10A, we observed that, without inducing any InDels (supplemental Figure 5E), both single and dual gRNA induced efficient HBA2 deletion and HDR integration, although to a lower extent compared with RNP (supplemental Figure 5F-H). Finally, we confirmed that RNP and RNP D10A gave the same ratio of HDR and non-HDR-mediated DNA integration (supplemental Figure 5I). Single gRNA generating 2 in *cis* nicks could induce genomic deletion by strand displacement, possibly facilitated by the homology of *HBA1* and *2* genes, whereas nick

induced AAV integration can proceed via genome-AAV alignment, followed by Holiday junction resolution and completion of homologous recombination.[53] Because we did not observe any difference between HBA15 and dual gRNA RNP D10A, we decided to continue on HSPCs using only HBA15.

HSPCs were transfected with RNP or RNP D10A and then transduced with the AAV6 β^{AS3} donor DNA. In both conditions, we observed *HBA2* deletion, β^{AS3} KI and, upon erythroid differentiation, β^{AS3} mRNA upregulation (Figure 6A-D), although on-target InDels were observed only with Cas9 as a result of DSB repair (Figure 6E). The lower number of DSBs could also explain the reduced toxicity observed in CFC edited with Cas9 D10A (Figure 6F-G).

By genotyping single BFU-E colonies, we observed that most of RNP D10A-edited BFU-E had 1 *HBA2* deletion, followed by 1 *HBA2* deletion and 1 β^{AS3} KI (Figure 6H; supplemental Figure 6A-B). In addition, we performed Sanger sequencing of PCR products spanning deletion junctions and observed that, whereas Cas9 left a composite pattern of InDels, Cas9 D10A deletions were seamless, suggesting absence of exonuclease processing (Figure 6I).

Last, in bulk populations of RNP and RNP D10A edited HSPC, deep sequencing of 3 top-scoring off-targets identified by GUIDE-Seq[32] in 293T cells (supplemental Figure 6C) showed low to undetectable off-target activity for both Cas9 and Cas9 D10A (supplemental Figure 6D). This analysis, together with our previous off-target analyses,[37] confirms the target specificity of the selected gRNA HBA15 sequence.

Overall, these data indicate that Cas9 D10A performs *HBA2* deletion and β^{AS3} KI, although less efficiently than Cas9, with no InDels and lower cellular toxicity, representing a promising alternative strategy for HSPC editing.

Discussion

In this study, we demonstrated the possibility of correcting α/β globin imbalance in β-thalassemia cells using the CRISPR/Cas9 system. In particular, we showed an amelioration in both β^+- and β^0-thalassemia patients' HSPC-derived erythroblasts by combining 2 approaches: α-globin reduction by deleting *HBA2* gene to mimic α-thalassemia (-α/$\alpha\alpha$ or -α/-α) and concomitant targeted integration and expression of a *HBB* transgene under the control of the endogenous *HBA* promoter. Because edited HSPCs retained engraftment and multilineage differentiation potential in vivo, the proposed strategy has clear potential for future clinical testing.

Inspired by clinical data showing that α-thalassemia ameliorates β-thalassemia,[3] several groups have proposed artificial down-regulation of α-globin expression as potential treatment of β^+-thalassemia, where residual β-globin expression guarantees sufficient hemoglobin formation (such as HbE, about one-half of all β-thalassemias).[3]

Figure 2. (continued) on-target ddPCR before and after sorting. (D) β^{AS3} transcript upregulation in targeted HUDEP-2 β^0 upon erythroid differentiation (qPCR, n = 2, mean $\pm$ SD). (E-F) HPLC analysis of globin monomers in differentiated HUDEP-2 β^0. Representative chromatograms (E) and relative quantification (F) of β-like subunits are shown; (mean $\pm$ SD; n = 3). (G-H) HPLC analysis of globin tetramers in differentiated HUDEP-2 β^0. Representative chromatograms (G) and HbA ($\alpha_2\beta_2$) quantification (H) are shown (mean $\pm$ SD, n = 3; **$P < .01$; ANOVA, Tukey test). ns, not significant.
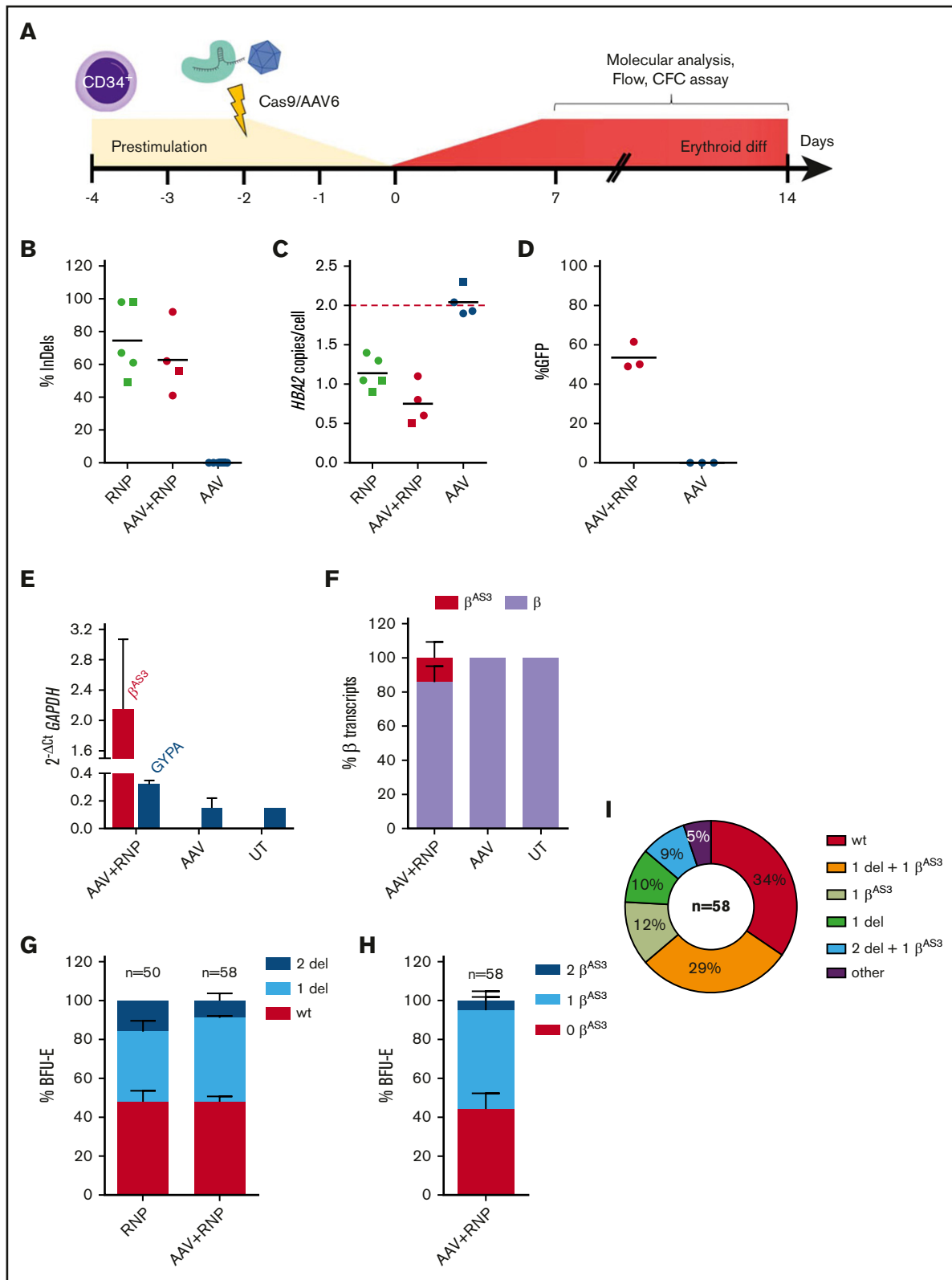
Figure 3. ***HBA2* deletion and β^AS3 integration efficiency in HSPCs.** (A) Schematic representation of HSPC targeting experiments. (B) Editing efficiencies in HSPCs at day 12 of erythroid differentiation. Lines represent mean. *HBA2* copies (C) and KI efficiency (D) in edited HSPCs in erythroid liquid culture (●) or in BFU-E (■). Black lines represent mean; red line indicates the number of expected *HBA2* alleles in untreated HSPCs. (E) β^AS3 and glycophorin A (GYPA) transcripts in HSPC-derived erythroblasts (qPCR, n = 2, mean ± SD). (F) Relative abundance of endogenous β and KI-β^AS3 mRNA at day 12 of erythroid liquid culture (n = 6, mean ± SD). *HBA2* deletion (G) and β^AS3 integration (H) pattern in single BFU-E. Bars represent mean ± SD (colonies derived from 2 independent experiments); no integration (0), monoallelic (1), and biallelic (2) KI or deletion (del). (I) Genotypes distribution of single KI-β^AS3 BFU-E. Percentages are indicated (n = 58).
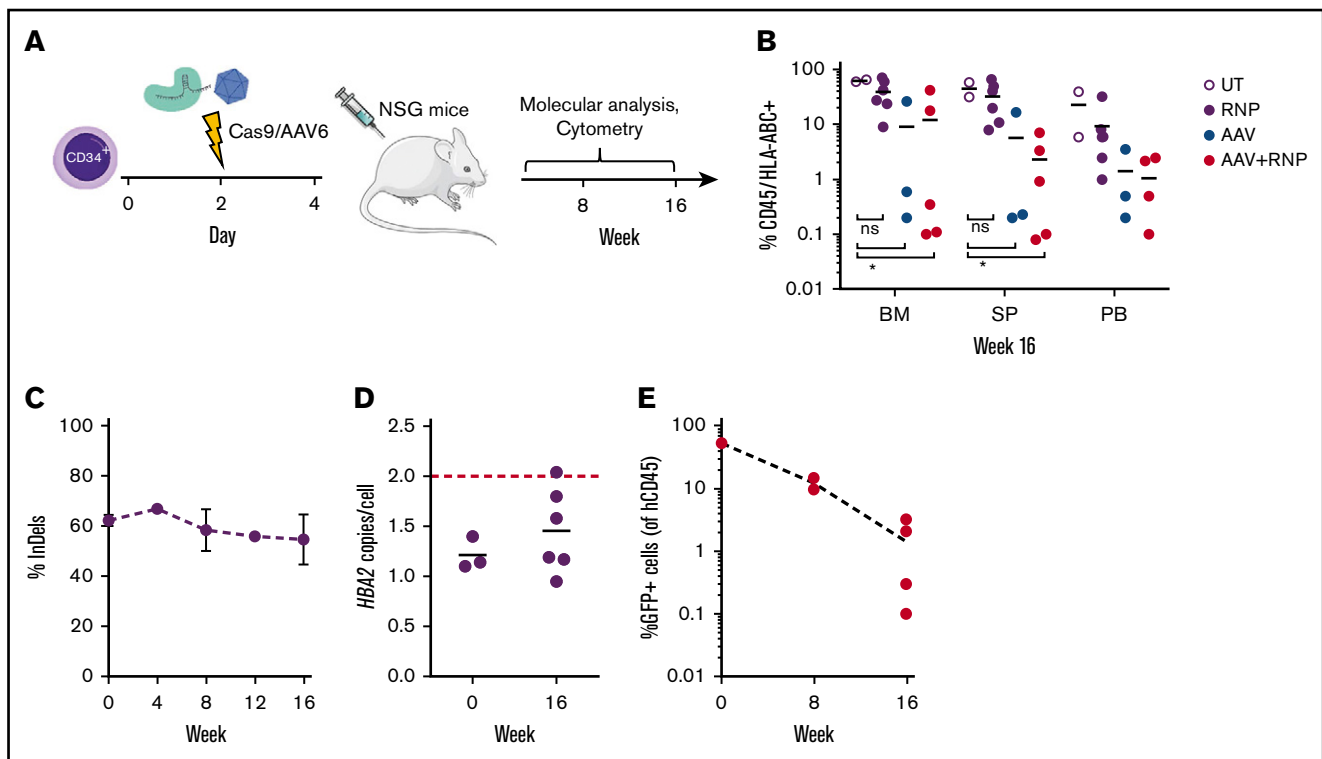
Figure 4. *HBA2*-deleted and β^AS3 KI HSPCs engraft NSG mice and maintain their multilineage potential. (A) Schematic representation of engraftment experiments. (B) Percentage of human CD45^{+}/HLA-ABC^{+} cells in hematopoietic organs of mice. BM, bone marrow; PB, peripheral blood; SP, spleen. Black lines indicate mean. (C) InDel efficiency in PB of RNP-engrafted mice at different timepoints (mean ± SD; n = 2-4). (D) *HBA2* copies in RNP-treated HSPCs at day 0 (injection) and in BM of engrafted mice at week 16. Black lines indicate mean; red line indicates the number of expected HBA2 alleles in untreated HSPCs. (E) GFP^{+} cells in PB of transplanted mice over time. GFP is expressed as percentage of CD45^{+} cells; line indicates mean.

Using a single gRNA targeting the 5′ untranslated region of both α-globin genes, we achieved efficient α-globin reduction by deleting 1 α-globin gene (*HBA2*), which corrected α/β imbalance and reduced α-precipitates in HUDEP-2 β[0]. Because *HBA1* and *HBA2* are similarly expressed,[54] removal of 1 or 2 *HBA2* genes should reduce α-globin expression to ~75% to 50% of its normal level, thus falling in the therapeutic window to benefit β-thal.[3] This α-globin reduction strategy could be used to treat moderate β[+]-thalassemia or to complement pharmacological treatment aimed at increasing expression of β-like globin in severe β[+] or β[0] patients.[55-57] To treat β[0]-thalassemia, where there is no residual β-globin expression, we combined α-globin reduction with β-globin replacement. We optimized, delivered, and integrated an AAV6 β^AS3 expression cassette under the control of the endogenous *HBA* promoter by HDR using the same gRNA deleting *HBA2* gene. Remarkably, the expression of 1 β^AS3 transgene was sufficient to reestablish almost one-half of normal hemoglobin level in HUDEP-2 β[0].

Hijacking the endogenous *HBA* promoter for the expression of β^AS3 transgene has 2 main advantages: (1) it does not require the genomic insertion of an exogenous promoter, which could result in transactivation of neighboring genes; and (2) compared with *HBB* gene correction strategies, it is a viable approach also for point mutations and deletions that inactivate regulatory elements in the *HBB* promoter.[58]

We confirmed that this "double" editing is feasible and efficient in primary human HSPCs from healthy donors and β[+]- or β[0]-thalassemia patients and does not affect erythroblast differentiation.

Both *HBA2* deletion and β^AS3 KI improved α/β globin imbalance to levels that were comparable or better than current *HBB* gene addition strategy based on LV. Additional experiments will be performed to systematically compare these 2 gene therapy strategies; however, our encouraging results support the development of a CRISPR/Cas9-based gene therapy approach. In addition, by transplanting HSPCs in humanized NSG, we demonstrated that both *HBA2* deletion and β^AS3 KI occur in long-term HSCs and that edited HSCs can give rise to multiple progenitors and differentiated hematopoietic lineages.

The idea of downregulating the expression of α-globin alone or in combination with increased expression of β-globin has already been proposed by several groups in mice and human cells with mixed outcomes.[19,59,60] The only test on thalassemic patient's HSPCs was performed using a foamy viral vector encoding an *HBB* transgene and a short hairpin against α-globin mRNA[61]; however, this approach is hindered by limitations of this RNA interference technology[62-64] and the mutagenic potential of semi-random integrating viral vector.[65,66]

Alternatively, α-globin mRNA downregulation was obtained in HSPCs with epigenetic drugs, such as broad-spectrum histone demethylase or deacetylase inhibitor; nevertheless, the effect on α-globin protein was not established and drug specificity, toxicity, and mechanism of action still need to be assessed.[67-69]
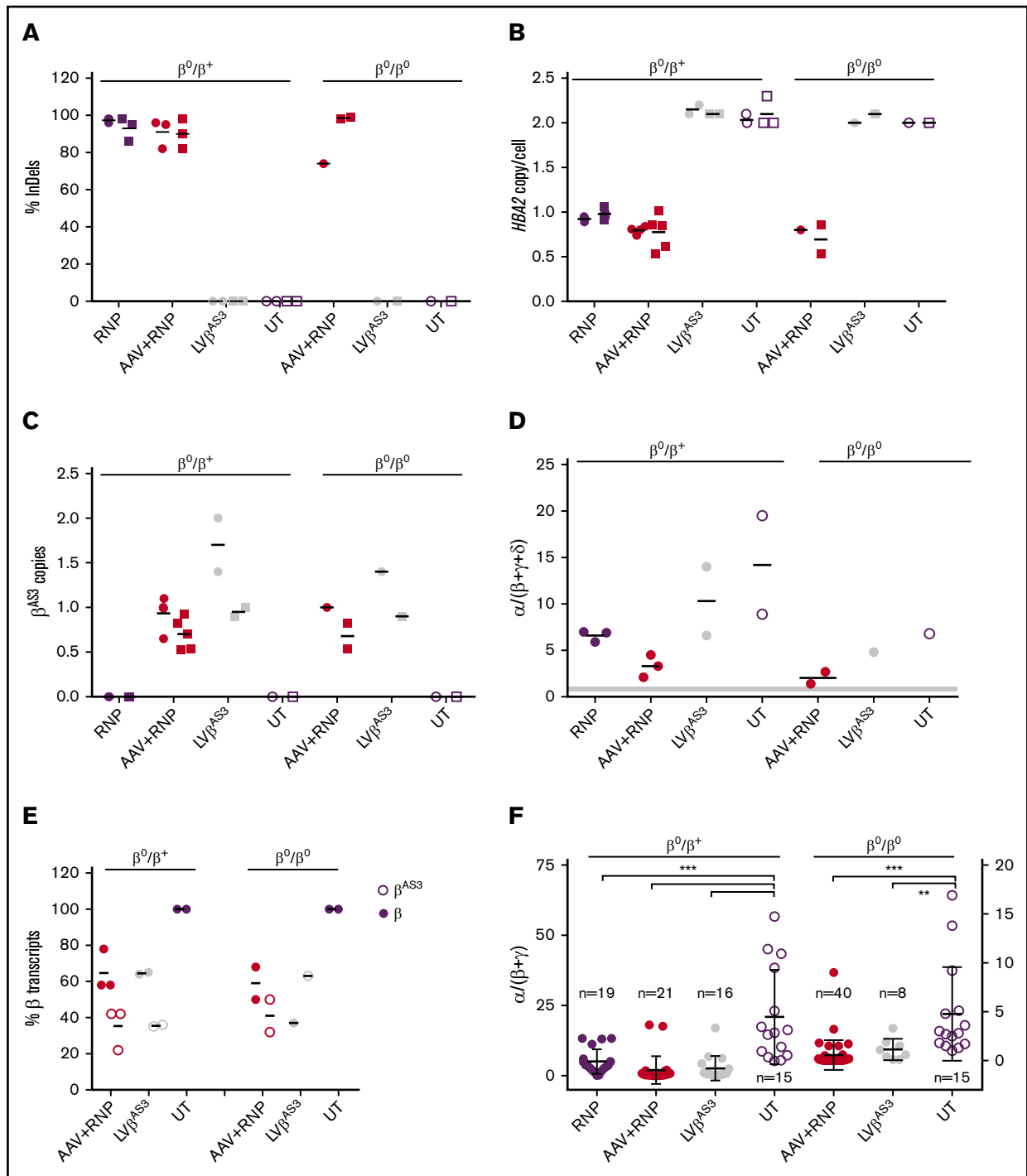
Figure 5. Genome editing of the α-globin locus ameliorates globin balance in thalassemic HSPCs. InDels (A), *HBA2* copies (B), and β[AS3] integrated copies (C) quantification in edited thalassemic HSPCs in erythroid liquid culture (●) or in BFU-E (■). Black lines represent mean. HSPCs from 1 patient of each genotype were used. (D) α/β-like globin mRNA ratios in edited thalassemic erythroblasts at day 12. Black lines indicate mean; gray line shows the range of α/β-like globin ratio in healthy donor MPB and UCB HSPCs (n = 5). (E) Relative abundance of endogenous β and integrated β[AS3] mRNA at day 12 of erythroid liquid culture. Black lines represent mean. (F) α/β-like globin mRNA ratios in edited thalassemic BFU-E. β[0]/β[+] colonies (n = 71) are plotted on the left axis, β[0]/β[0] (n = 63) on the right axis. Each dot represents a single colony. Black lines indicate mean ± SD (***P < .001; **P < .01; ANOVA, Tukey test). HSPCs derived from 1 patient of each genotype were used for this figure.

Using genome editing tools, Mettananda et al elegantly demonstrated the possibility of reducing α-globin expression in HbE patients' cells by deleting a powerful α-globin enhancer.[20] Although promising, this modification is beneficial only for β[+] patients and requires plasmid transfection combined with GFP-sorting enrichment, a strategy that is not suited for clinical translation.[25] Finally, the need for
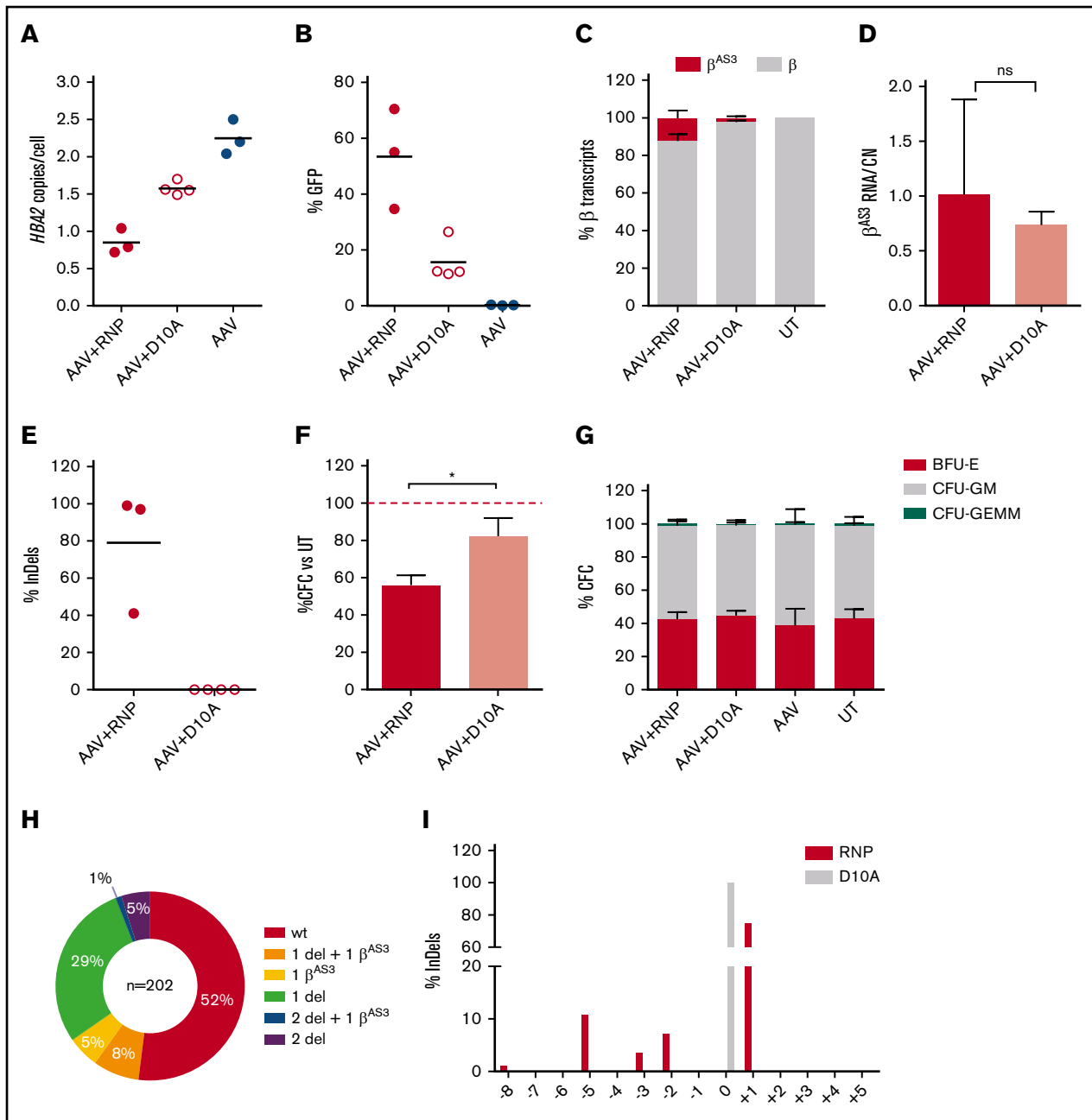
Figure 6. Cas9 nickase editing results in seamless *HBA2* deletion and reduced HSPCs toxicity. (A-B) *HBA2* copies (A) and KI efficiency (B) in Cas9 (RNP) or Cas9 nickase (RNP D10A) edited HSPCs in erythroid liquid culture. Black lines represent mean. (C) Relative abundance of endogenous β and KI-β^{AS3} mRNA at day 12 of erythroid liquid culture (mean ± SD, n = 3-4). (D) expression of β^{AS3} transcripts normalized by copy number (CN) in Cas9- or Cas9 nickase-treated erythroblasts (mean ± SD, n = 4-5). (E) InDel quantification at gRNA target site in edited HSPCs. Black lines represent mean. (F) CFC number expressed as percentage of untreated control (UT). Bars represent mean ± SD (n = 3-4); red dashed line indicates 100%. (G) CFU frequency in edited HSPCs. BFU-E, burst-forming unit-erythroid; CFU-GM, CFU-granulocyte, macrophage. Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte; Bars represent mean ± SD (n = 2-4). (H) Genotypes of BFU-E (n = 202) derived from RNP D10A + AAV HSPCs. Percentages are indicated. (I) Frequency of different InDel patterns in HBA2 deleted BFU-E treated with Cas9 (RNP, red; n = 28) or Cas9 nickase (RNP D10A, gray; n = 96). Editing was measured across HBA2 deletion junctions.

2 gRNAs to perform enhancer deletion increases the risk of on- and off-target side effects and can be technically challenging.

Our approach has the advantage of combining α reduction with β expression to restore α/β-globin balance and increase

hemoglobin level to treat both β⁰- and β⁺-thalassemia patients' HSPCs. Technically, we deliver Cas9/gRNA as RNP, which allows efficient HSPC modification without any selection and is already used in clinical trials (eg, NCT03164135, NCT03655678). In addition, our strategy requires only 1

gRNA, thus minimizing the possibility of on- and off-target side effects.

To further reduce this risk and general cellular toxicity associated with Cas9-induced DSB, we investigated the use of Cas9 D10A nickase.[51,70-72] Inspired by Metzger et al, who demonstrated that site-specific nicking enzymes can induce HDR with an AAV-delivered template in 293 cells,[53] we showed for the first time that a single Cas9 D10A/gRNA RNP can be used in HSPCs to achieve seamless genomic deletion and AAV targeted integration, without generating any InDels. Because the efficiency of targeted integration obtained with Cas9 D10A is lower than with Cas9, we will further optimize this approach by improving RNP transfection with nanoparticles,[73] reducing AAV donor size,[74] and using Cas9 nickase variants[75] and drugs affecting cellular DNA repair.[76,77] Further in vivo studies will be performed with this strategy to demonstrate effective editing of repopulating HSPCs.

By performing GUIDE-Seq analysis,[32] we verified the target specificity of the selected gRNA HBA15, confirming our previous off-target analyses.[37] However, additional safety aspects need to be carefully evaluated before clinical translation of this HSPC gene therapy platform. It will be particularly challenging to qualitatively and quantitatively assess all the possible on-target editing outcomes, especially low-frequency ones. We quantified *HBA2* deletions, but we could not detect any *HBA2* inversion by PCR analysis of edited HSPCs, although inversions are reported to be a frequent side product when generating CRISPR genomic deletions.[17,78] This observation could be a true negative result, as previously reported for *HBG* editing in HSPCs,[79,80] or because of technical difficulties associated with amplification of repetitive inverted sequences with high GC content present in the α-globin locus.[81] In addition, on-target cleavage can generate undesired chromosomal alterations,[70,82-84] whereas integration of AAV donor DNA can occur via HDR as well as via partial HDR or non-homologous end joining with or without the *HBA2* deletion.[85-87] A combination of whole genome sequencing,[88] long-range PCR,[89] long-read sequencing,[90] next-generation mapping,[91] and/or directional sequencing[92] will help to address these concerns by unraveling the full spectrum of editing outcomes.

In summary, we describe a novel CRISPR strategy to correct α/β globin imbalance in thalassemic HSPCs by α-globin downregulation with or without *HBB* transgene expression. Future experiments will elucidate the therapeutic potential of this strategy for treating β[+]- and β[0]-thalassemia and, eventually, sickle cell disease, where lower α-globin levels reduce HbS polymerization and sickle hemoglobin concentration and β[AS3] compete with β sickling for binding α-globin.[21,93-97]

Acknowledgments

Authorship

Contribution: G.P. conceived the study, designed and performed experiments, analyzed data, and wrote the manuscript; A.F. performed experiments and analyzed data on HSPCs; M.L. performed experiments and analyzed data on HUDEP-2 β[0] cells; F.A. performed experiments and analyzed data on Cas9 (D10A); E.C. performed experiments and analyzed data on HSPCs; A.C. performed HPLC analyses; G. M. and A.C. performed and analyzed GUIDE-Seq experiments; A.T. performed molecular analyses; J.-P.C. provided purified SpCas9 protein; F.M. provided scientific advice and financial support; G.F. provided β-thalassemia HSPCs; A.M. provided HUDEP-2 β[0] cells, performed and analyzed HPLC, and edited the manuscript; and M.A. conceived the study, designed experiments, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: G.P. and M.A. are the inventors of a patent describing this HSC-based gene therapy strategy for treating β-thalassemia (correction of β-thalassemia phenotype by genetically engineering hematopoietic stem cell; EP19305484.8). The remaining authors declare no competing financial interests.

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