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Efficient and *in situ* correction of hemoglobin Constant Spring mutation by prime editing in human hematopoietic cells

Congwen Shao,[1,2,6] Qing Liu,[1,2,6] Jinchao Xu,[3,6] Jianxiang Zhang,[3] Chengpeng Zhang,[3] Ye Xin,[3] Yuhua Ye,[1,2] Bin Lin,[4] Xinhua Zhang,[5] Li Cheng,[3] Xiangmin Xu,[1,2] and Peng Xu[3]

[1]Innovation Center for Diagnostics and Treatment of Thalassemia, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China; [2]Department of Medical Genetics, School of Basic Medical Sciences, Guangdong Engineering and Technology Research Center for Molecular Diagnostics of Human Genetic Diseases, Southern Medical University, Guangzhou, Guangdong 510515, China; [3]Cyrus Tang Medical Institute, National Clinical Research Center for Hematologic Diseases, Collaborative Innovation Center of Hematology, State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou, Jiangsu 215123, China; [4]Guangzhou Jiexu Gene Technology Co. Ltd., Guangzhou, Guangdong 510535, China; [5]Department of Hematology, 923rd Hospital of the People's Liberation Army, Nanning, Guangxi 530021, China

Hemoglobin Constant Spring (Hb CS) is the most common non-deletional and clinically significant α-thalassemic mutation, and it is caused by an anti-termination mutation at the α2-globin gene stop codon. We developed a prime editing strategy for the creation and correction of Hb CS. We showed that prime editing could efficiently introduce Hb CS mutations in both human erythroblast cell lines (an average frequency of 32%) and primary hematopoietic stem and progenitor cells (HSPCs) from healthy donors (an average frequency of 27%). By targeting the established Hb CS homozygous erythroblasts, we achieved an average frequency of 32% *in situ* correction without selection. Notably, prime editing corrected the Hb CS mutation to wild type at an average frequency of 21% in HSPCs from three patients with hemoglobin H Constant Spring (HCS). Erythrocytes that differentiated from prime-edited erythroblasts or HSPCs exhibited a significant reduction in the amount of α^{CS}-globin chains. Insertions and deletions on *HBA2* locus and Cas9-dependent DNA off-target editing were detected with relatively low frequency after prime editing. Our findings showed that prime editing can successfully correct Hb CS in erythroblasts and patient HSPCs, which provides proof of principle for its therapeutic potential in HCS.

INTRODUCTION

Hemoglobin Constant Spring (Hb CS) is one of most clinically significant α-chain elongation mutants, with a prevalent frequency in southeast Asia and China.[1,2] Hb CS is caused by an anti-termination mutation at the α2-globin gene stop codon CD142 (UAA > CAA, STOP > Gln), which generates an unstable protein with an extension of 31 additional amino acids.[3] The co-inheritance of Hb CS with α^0-thalassemia results in non-deletional hemoglobin H (HbH) disease, called hemoglobin H Constant Spring (HCS) ($-/\alpha^{CS}\alpha$).[1] Most HCS patients exhibit more severe symptoms and require more frequent transfusions than patients with deletional HbH disease.[4] The routine treatment of HCS includes prevention approaches, such as annual influenza vaccination, prompt treatment of infections, transfusions, and iron chelation therapy when indicated.[5] Although allogenic hematopoietic stem and progenitor cell (HSPC) transplantation is curative for HCS patients, its use is limited because of graft-versus-host disease and a lack of immunologically matched donors.[1] Direct correction of gene mutations in patients' autologous hematopoietic stem cells (HSCs) provides an alternative and ideal strategy given the revolutionary development of precise genome editing.[6,7]

The revolutionary development of genome editing technologies has given rise to the possibility of creating and correcting various types of gene mutations.[6] Although Cas9 nuclease- or nickase-initiated homology-directed repair (HDR)-based strategies have recently been tested for their ability to correct the Hb CS in human induced pluripotent stem cells and fibroblasts,[8,9] the efficiency of these strategies in HSPCs is not known, and long-term safety issues may be caused by the introduction of double-stranded DNA breaks.[10,11] Notably, recent studies have developed a variety of new-generation gene editing tools

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[6]These authors contributed equally

Correspondence: Li Cheng, Cyrus Tang Medical Institute, National Clinical Research Center for Hematologic Diseases, Collaborative Innovation Center of Hematology, State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou, Jiangsu 215123, China.
E-mail: lcheng0514@163.com
Correspondence: Xiangmin Xu, Innovation Center for Diagnostics and Treatment of Thalassemia, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, China.
E-mail: xixm@smu.edu.cn
Correspondence: Peng Xu, Cyrus Tang Medical Institute, National Clinical Research Center for Hematologic Diseases, Collaborative Innovation Center of Hematology, State Key Laboratory of Radiation Medicine and Protection, Soochow University, Suzhou, Jiangsu 215123, China.
E-mail: pengxu@suda.edu.cn

at the single-base pair level, including cytosine base editors (CBEs) that are capable of C-G to T-A transitions,[12] adenosine base editors that are capable of A-T to G-C transitions,[13] and prime editors (PEs) that mediate targeted insertions, deletions, and all 12 possible base-to-base transitions/transversions.[14] Although each tool has distinct advantages and disadvantages, only CBEs and PEs may be used for correcting the Hb CS mutation, which requires a C-G to T-A transition. The newly developed PEs have shown therapeutic potential for different types of diseases.[15–19] However, the feasibility of prime editing to create and correct Hb CS mutations, especially in primary HSPCs, is not known.

Since the discovery of Hb CS mutation, its pathobiology in erythrocytes has been studied and has primarily focused on three layers of regulation: mRNA stability, protein stability, and membrane composition. Initially, α^{CS} mRNA is rapidly degraded in developing erythrocytes due to ribosomal entry into the 3′ UTR, which leads to α^{+}-thalassemia.[20–23] The accumulation of α^{CS}-globin chains in erythroid precursor cells causes considerable ineffective erythropoiesis.[24] Membrane and skeletal protein component analysis revealed that the deposition of α^{CS}-globin chains directly damages the red cell membrane, which leads to membrane dysfunction and homolysis.[25] A more recent study revealed that the extended translated 3′ UTR-encoded peptide in *HBA2* reduced mature protein expression in human cells, which was demonstrated using an exogenous reporter system.[26] The toxicity of the α^{CS}-mRNA and α^{CS}-globin chains in human erythroblasts is not clear due to the lack of a proper model. Therefore, an erythroblast cell line harboring the endogenous Hb CS mutation is needed.

We first performed prime editing and generated an endogenous homozygous Hb CS mutation in human umbilical cord blood-derived erythroid progenitor-2 (HUDEP-2) cells to provide a cellular model for prime editing correction and further study the pathobiology of Hb CS mutations.[27] The α^{CS}-globin chain (a larger protein with an extra 31 amino acids) was detected in the model cells and resulted in globin imbalance after induced maturation. Notably, we performed transient delivery of mRNA by encoding the PE with a prime editing guide RNA (pegRNA) and a nicking single-guide RNA (sgRNA) into Hb CS homozygous HUDEP-2 cells, and we achieved successful *in situ* correction with an average efficiency of 34% without enrichment, but no measurable editing occurred at the same loci of *HBA1*. Notably, prime editing resulted in a significant decrease in the amount of α^{cs}-globin chains without affecting normal erythroid maturation. We further successfully corrected the Hb CS mutation in HCS patient-derived $CD34^{+}$ HSPCs with an efficiency of 21%, accompanied by a 50% reduction in the amount of α^{CS}-globin chains. Notably, this editing efficiency was achieved with minimal generation of insertions and deletions and a low level of Cas9-dependent DNA off-target editing. Additionally, we successfully created the HCS mutation in normal HSPCs by prime editing and observed its long-term repopulation with persistent editing efficiency *in vivo*, suggesting the potential for future translational studies. Taken together, our results demonstrate the successful prime editing of the most common and significant α-thalassemic Hb CS mutation in human erythroblasts and patient HSPCs.

RESULTS

Prime editing mediates the successful generation of Hb CS mutations in a proerythroblast cell line

To evaluate the feasibility of prime editing and the consequent molecular manifestations, we created an endogenous Hb CS mutation in HUDEP-2 cells that primarily expressed HbA. According to the principles of the PE3 system,[14] an NGG-protospacer adjacent motif (PAM) targetable pegRNA, including a 13-nt prime binding site (PBS) and a 14-nt reverse transcription template (RTT), was designed for Hb CS mutant creation, with one nicking sgRNA targeting the 3′ UTR of *HBA2* (Figures 1A and S1A). For generation of the Hb CS mutation, we transiently delivered the pegRNA, PE2, and nicking sgRNA-expressing plasmids into wild-type HUDEP-2 cells via electroporation, followed by the enrichment of GFP^{+}-transduced cells and isolation of clonal cells (Figure 1B). In three biological replicate experiments, the T>C editing efficiency in the enriched cells was approximately 32.20% ± 3.25% for *HBA2* and 11.89% ± 1.44% for the highly homologous *HBA1* locus (Figure 1C). Due to the considerable amount of editing efficiency in the *HBA2* and *HBA1* alleles at the bulk level, we next evaluated the gene editing type and efficiency at the clonal level. Among the 42 total clonal HUDEP-2 cell lines obtained, four clonal HUDEP-2 cell lines with *HBA2* bi-allelic mutations and normal *HBA1* (referred to as Hb CS-Homo) and three clones with *HBA2* and *HBA1* bi-allelic mutations were confirmed using Sanger sequencing (Figures S1B and S1C). Nine of the remaining clones with at least one edited *HBA* gene were detected. Therefore, prime editing mediated nearly 38% of the Hb CS mutations at the clonal level (16 of the 42 clones were edited). Our data suggest that prime editing achieved the successful generation of Hb CS mutations in HUDEP-2 cells via a plasmid expression system.

Introduction of a homozygous Hb CS mutation causes globin imbalance in proerythroblast cells

Reported cases with homozygous Hb CS are as serious as Hb H hydrops fetalis.[28] Therefore, we explored whether the four clones carrying the *HBA2* bi-allelic mutation and normal *HBA1* reflected some clinical phenotypes in thalassemia patients. We evaluated the cellular growth, differentiation, and apoptosis of the Hb CS-Homo clones. Notably, compared with the wild-type controls, the four Hb CS-Homo clones exhibited significant reductions in *HBA2* mRNA but no change in *HBA1* (Figure S2A), which was consistent with their genotype (Figure S1C). Elongated α^{CS}-globin chains were detected in all clones with variable levels of other globin (Figure 2A). After induction of erythroid maturation, the expansion rate of the three clones remained largely similar to that of the control cells, but one clone exhibited significantly reduced expansion (Figure 2B). This finding was consistent with the frequencies of apoptotic cells (Figure S2B). We further tracked the erythroid maturation process using flow cytometry analysis of the erythroid surface markers CD105 and CD235a (glycophorin A [GYPA]). Notably, all four clones showed an increased proportion of mature $CD235a^{+}CD105^{-}$ erythroid cells accompanied by a reduced proportion of immature $CD235a^{+}CD105^{+}$ erythroblasts from days 0 to 8 (Figures 2C and S2C). Morphological analysis of mature

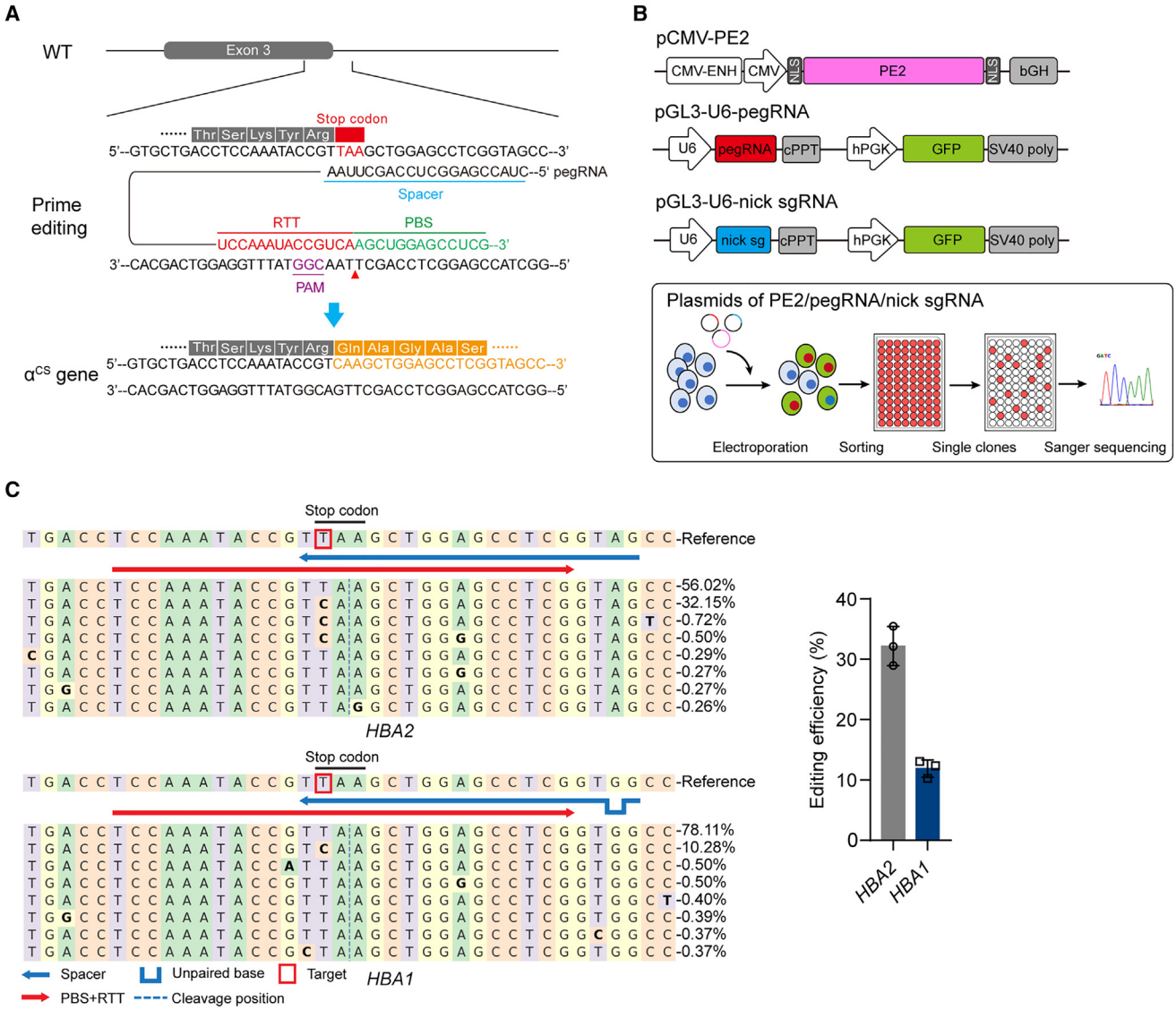

Figure 1. Prime editing mediates the successful generation of Hb CS mutations in a proerythroblast cell line

(A) Detailed strategy for creation of the T>C mutation at the stop codon of *HBA2* using prime editing. (B) Workflow of Hb CS mutation generation in HUDEP-2 cells. (C) Editing efficiency evaluation at the *HBA2* and *HBA1* loci in the bulk population by NGS (representative analysis at left). Values shown are means ± SDs of three independent experiments.

erythroid cells on day 8 revealed no difference between the four Hb CS-Homo clones and wild-type control cells, which suggested that the Hb CS homozygous mutation might not significantly affect erythroid maturation (Figures 2D and S2D). We also observed similar hemoglobinization in the cell pellets on day 8 (Figure 2E). To further determine the effect of Hb CS homozygous mutation on the expression of different globin genes, we quantified the relative expression levels of the *HBA1*, *HBA2*, *HBZ*, *HBB*, and *HBG* genes during erythroid maturation. Notably, the mRNA levels of all of these genes significantly increased, which further confirmed successful erythroid maturation (Figures 2F and S2E). More important, the ratio of α-globin genes relative to non-α-globin genes was significantly reduced in all four Hb CS-Homo clones after induction of erythroid maturation (Figure 2G). These results suggest that the introduction of a homozygous Hb CS mutation causes globin imbalance but may not significantly affect *in vitro* erythroid maturation in HUDEP-2 cells.

Prime editing mediates efficient correction of Hb CS mutation and restores α-globin expression in a proerythroblast cell line

Considering the robust editing efficiency of the *ex vivo* delivery of mRNA encoding base editors or PEs for sickle cell disease (SCD) in HSCs,[16,29] we hypothesized that PE2 mRNA with chemically

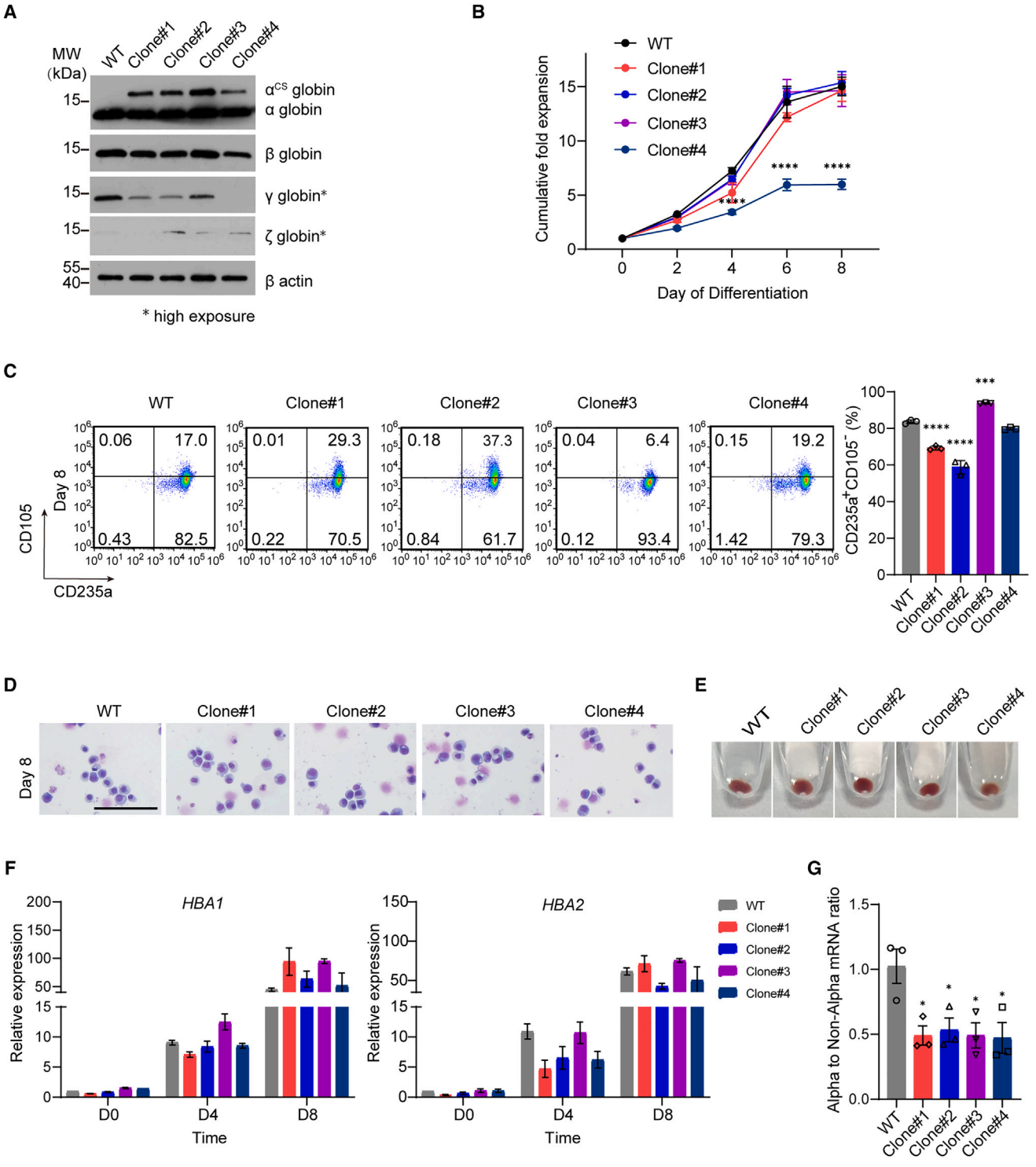

Figure 2. Introduction of a homozygous Hb CS mutation causes globin imbalance

(A) Western blot analysis of globin chains in differentiated clones. (B) The cell expansion profile in differentiation medium. (C) Flow cytometric analysis of erythroid maturation. Erythroid surface marker staining analysis revealed an increased proportion of mature CD235a$^+$CD105$^-$ erythroid cells. (D) Representative images of May-Grünwald

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synthesized pegRNA/nicking sgRNA would achieve efficient editing without enrichment in targeting Hb CS mutations. Similarly, an NGG-PAM-targeting pegRNA including 13-nt PBS and 14-nt RTT was designed for the correction strategy with one nicking sgRNA targeting the 3′ UTR of *HBA2* (Figure 3A). After optimization of the RNA dose and ratio, the average *in situ* correction efficiency in *HBA2* without enrichment achieved nearly 32% in four Hb CS-Homo clones (36.02% ± 5.19% in clone 1, 29.97% ± 1.02% in clone 2, 28.18% ± 2.28% in clone 3, 33.90% ± 4.04% in clone 4) (Figures 3B and S3A). Notably, no editing efficiency was detected at the *HBA1* gene locus (Figure S3B). We also confirmed the correction of the Hb CS mutation at the mRNA level in the Hb CS-Homo clone 4. Over 30% *in situ* correction of the α^{CS}-gene resulted in a 50% reduction in α^{CS}-mRNA synthesis and a nearly 50% increase in normal α2-globin transcript production (Figure 3C). Moreover, no substantial changes in *HBA2* gene copy number were detected after prime editing (Figure S3I), which suggests that the decrease in α^{CS}-mRNA transcript was not caused by *HBA2* deletion.

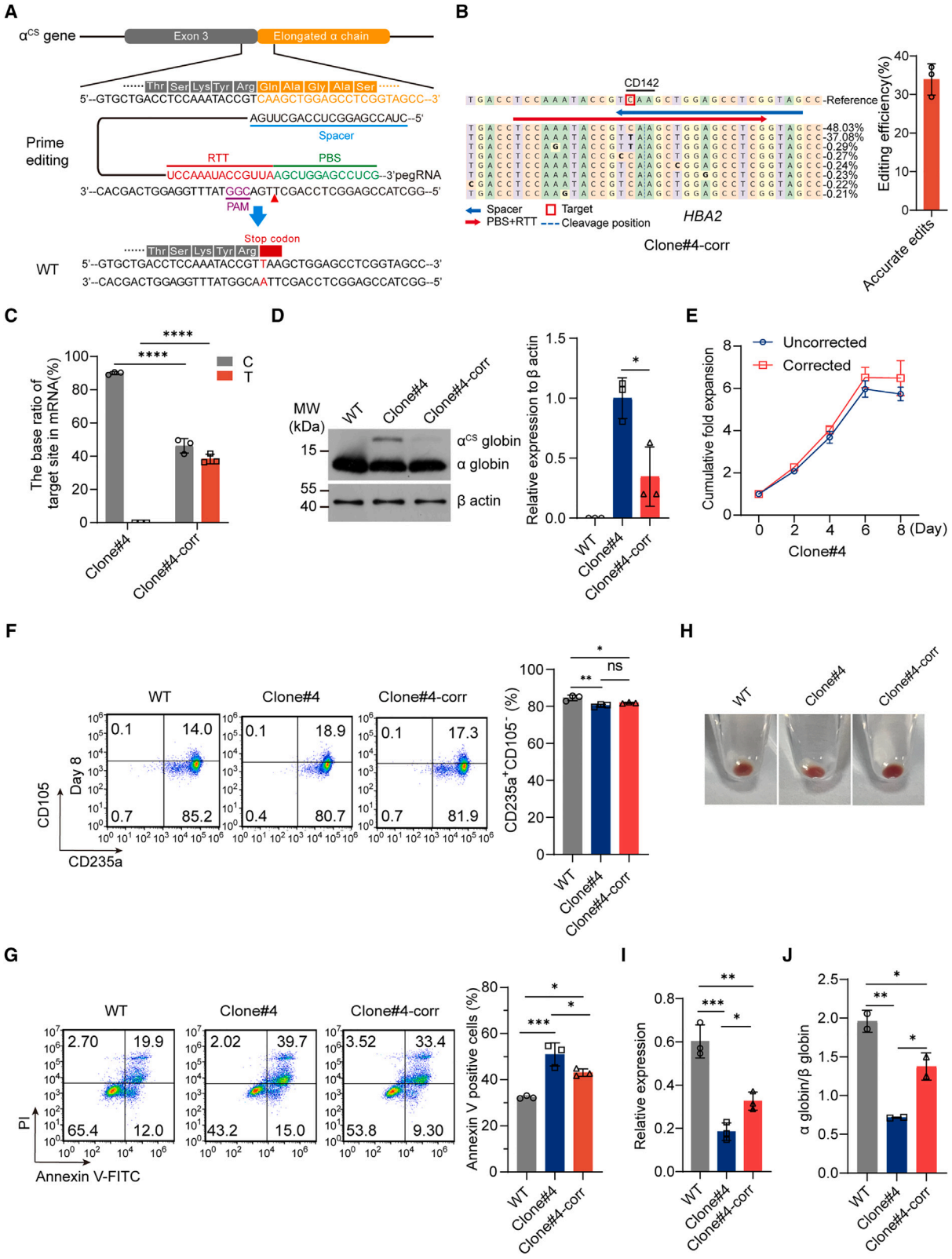
To further test whether this correction efficiency was sufficient to restore α-globin expression, we analyzed globin gene expression following erythroid maturation. The α^{CS}-globin chains of four individual clones were measured with largest extent of over 50% reduction in clone 4 (Figures 3D and S3C). Although the clonal heterogeneity exists, the cells exhibited a similar expansion and erythroid maturation profiles after prime editing (Figures 3E, 3F, S3D, and S3E). Importantly, higher apoptosis and significant growth defects was originally observed in the Hb CS-Homo clone 4, which partially mimic the clinical phenotype in some patients with homozygous Hb CS mutations.[24] Therefore, we chose clone 4 as a cellular model for further phenotypic evaluation after prime editing. Clone 4 after correction showed a lower proportion of apoptotic cells compared to uncorrected controls on day 8 of differentiation (Figure 3G). We also observed higher levels of hemoglobinization after prime editing but similar cell morphology (Figures 3H and S3F). Notably, the relative expression of *HBA2* was significantly increased but did not affect the expression of *HBA1* following prime editing (Figures 3I and S3G). Consistently, reverse-phase-high-performance liquid chromatography (RP-HPLC) analysis also confirmed the significant increase in the α-globin chains after prime editing (Figures 3J and S3H), which suggested the recovery of the globin balance. These results suggested that prime editing achieved highly efficient correction of Hb CS mutations and restored α-globin expression in the HUDEP-2 clonal model.

Prime editing efficiently corrected Hb CS mutations in HSPCs from HCS patients

Given the efficiency of prime editing in erythroblast cell lines, we further used a similar strategy to achieve *in situ* correction of Hb CS mutations in primary HSPCs. Considering the clinical severity, we mobilized and collected peripheral blood $CD34^+$ HSPCs from three HCS patients in a previous Chinese HbH disease cohort (Table S1).[30] Prior to *in situ* correction of Hb CS mutations, we compared the *in vitro* erythroid mature profiles of $CD34^+$ HSPCs from healthy donors and HCS patients. Of note, we did not observe a significant difference in cellular expansion, erythroid maturation, and apoptosis between three healthy and HCS patient donors (Figures S4A–S4E). To further confirm this observation, we performed the whole transcriptome analysis in erythroblasts from both healthy and patient donors. The differential gene expression was analyzed by a stringent cutoff (fold change >1.5 and false discovery rate <0.05): 506 dysregulated genes, including 356 decreased genes and 150 increased genes, were identified in HCS-derived erythroid cells compared to the healthy control (Figure S4F; Table S2). To further explore the gene function, we performed the Gene Ontology (GO) analysis for the dysregulated genes using the enrichR algorithm.[31] The GO analysis demonstrated that the significantly decreased genes were predominantly involved in antioxidant activity, while the increased genes were enriched in protein refolding (Figure S4G; Table S3). Most important, our datasets confirmed the decrease in *HBA2* and *HBA1* transcripts but an increase in *HBZ* transcripts in cells derived from HCS patient HSPCs (Figures S4F and S4H). However, quantification of globin balances showed no significant differences between healthy donor and patients' donor $CD34^+$ cell-derived erythroblasts (Figure S4I). The increase in embryonic ζ-globin expression might explain the similar *in vitro* maturation pattern between healthy and patient donors given its reported compensatory role.[32–34]

Furthermore, the *in situ* correction of Hb CS mutations in primary HSPCs from patients was determined. Subsequently, after the electroporation of PE2 mRNA with chemically synthesized pegRNA/nicking sgRNA, we obtained 21.21% ± 6.00% accurate editing of the pathogenic Hb CS mutation to the wild type at *HBA2* in $CD34^+$ HSPCs from three HCS patient samples (Figure 4A). However, no editing efficiency was detected at *HBA1* (Figure S5A). To confirm the effect of prime editing on erythroid differentiation and globin expression, we further performed erythroid maturation analysis of the edited primary HSPCs and compared them to the corresponding unedited controls. Notably, the edited cells exhibited similar expansion rates and proportions of apoptotic cells compared with unedited controls upon erythroid differentiation (Figures S5B and S5C). We also observed similar erythroid maturation processes, which were demonstrated by the levels of erythroid surface markers, cell morphology, and hemoglobinization (Figures 4B, 4C, and S5D). These results suggested no obvious cellular toxicity after prime editing. We confirmed globin gene expression after prime editing. Strikingly, 20% *in situ* correction of the α^{CS}-gene in HSPCs resulted in a 15% increase in normal α2-globin transcript production (Figure 4D). Notably, the

Giemsa-stained differentiated (day 8) cultures. Original magnification ×20. Scale bar, 50 μm. (E) Hemoglobinization analysis of cell pellets after erythroid maturation. (F) Relative expression of *HBA1* and *HBA2* on days 0 (D0), 4 (D4), and 8 (D8) of erythroid maturation. (G) Alpha to non-alpha mRNA ratio. Alpha to non-alpha mRNA ratio indicates the (α1+α2)/(β+γ)-globin mRNA ratio. Values shown are means ± SDs of three independent experiments. $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$ (one-way analysis of variance, Dunnett's multiple comparisons test).



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terminally matured erythroid cells showed an approximately 50% reduction in α^{-CS}-globin chain expression after correction (Figure 4E). These results suggested that 20% *in situ* correction of Hb CS mutations in HSPCs from HCS patients mediated an actionable reduction in the amount of pathogenic α^{CS}-globin chains.

Prime editing efficiently creates the Hb CS mutations in normal human HSPCs for long-term transplantation

To determine whether prime-edited HSPCs can repopulate bone marrow (BM) *in vivo*, we generated Hb CS mutations in healthy HSPCs considering the requirement of large amounts of primary cells (Figure 5A). In primary HSPCs from two independent donors, the T>C editing efficiency without enrichment was approximately 27.18% ± 0.90% for *HBA2* and 10.22% ± 1.16% for the highly homologous *HBA1* locus (Figure 5B). We further induced the primary edited HSPCs for erythroid maturation. Consistent with their genotype, the α^{CS}-globin chains were detected in primary erythroblasts from prime-edited HSPCs (Figure 5C). Interestingly, the edited cells exhibited a lower expansion rate but no significant defects in erythroid differentiation markers compared to unedited controls (Figures S6A–S6D), which is consistent with the above described HUDEP-2 Hb CS-Homo clone 4 model.

After confirming the editing efficiency *in vitro*, we transplanted the prime-edited bulk cells into immunodeficient NCG-Kit-V831M mice, which is a well-established mice model for long-term repopulation study of HSPCs.[35,36] The BM of the mice was collected and the editing efficiency was analyzed 16 weeks post-transplantation. Flow cytometry showed successful repopulation of edited healthy donor $CD34^+$ cells in the BM of recipient mice (Figure 5D). More important, editing efficiency in repopulating $CD34^+$ HSPCs from the engrafted BM exhibited 20% for *HBA2*, although with slight reduction compared to that before engraftment (Figure 5E). Overall, our results demonstrated that prime-edited HSPCs can repopulate BM. However, long-term repopulation of the prime-edited patient cells should be evaluated in future studies.

Prime editing induces detectable off-target events in erythroblasts and HSPCs

Compared to other CRISPR-based gene editing tools, prime editing causes considerably less off-target editing due to the requirement of base pairing events between the target DNA strand and the protospacer or primer binding site of the pegRNA. A recent study showed robust prime editing to correct the SCD allele in HSPCs from SCD patients with a considerable frequency of insertion or deletion (indel).[16] We first tested the indels at the pegRNA site and the nicking guide site in our settings. Notably, we detected 4.90% ± 0.76% indels and fewer than 0.5% unexpected editing events in *HBA2*, with an edit-to-indel ratio of 6.94 ± 0.29 after prime editing in HUDEP-2 cells (Figure 6A). We detected 1.68 ± 1.27% indels and fewer than 1% unexpected editing events in *HBA2*, with an edit-to-indel ratio as 27.47 ± 31.67 after prime editing in primary HSPCs (Figure 6B). To further assess off-target editing events of the *in situ* correction strategy, we performed targeted deep sequencing of the top five potential off-target sites of the pegRNA and the nicking sgRNA predicted by Cas-OFFinder.[37] Since target DNA amplification and DNA sequencing errors were the origins of DNA sequence differences between samples and the reference, sequences with the first mismatch encoded by the RTT of pegRNA were identified as putative pegRNA RTT-related off-target edits. CRISPR-Cas9-dependent off-target editing was calculated by quantifying the frequency of indels for the nicking sgRNA and by combining pegRNA RTT-related edits and indels for the pegRNA. No indel or pegRNA RTT-related edits were detected in 8 of the 10 potential off-target sites in HUDEP-2 cells (Figure 6C). The off-target sites were also evaluated in prime-edited HSPCs from HCS patients. Importantly, fewer than 0.1% indel or pegRNA RTT-related edits were detected in all 10 of the potential off-target sites in primary HSPCs (Figure 6D). Collectively, the analysis of potential off-target sites suggested that the prime editing strategy used to correct Hb CS resulted in relatively low but detectable off-target editing in the genome.

DISCUSSION

Hb CS is one of most common and clinically significant α-thalassemic globin variants in southeast Asia and the southern part of China.[1,2] Therefore, correction of the Hb CS mutation to reduce unstable α^{CS}-globin chains would, in principle, benefit transfusion-dependent HCS patients. The present study described a genome editing strategy to successfully correct Hb CS mutations in primary HSPCs from HCS patients. Notably, the prime-editing strategy yielded up to an average of 21% in HCS patient HSPCs and resulted in a significant reduction in elongated pathogenic α^{CS}-globin chains with detectable off-target events and cellular toxicity. We successfully created homozygous ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) forms in an erythroblast cell line and further corrected the mutation with an average frequency of 32% using prime editing. This cell model could serve as a cellular platform for further gene editing development and optimization.

Figure 3. Prime editing mediates efficient *in situ* correction of Hb CS mutation and restores α-globin expression in a proerythroblast cell line

(A) Detailed strategy to correct Hb CS mutations at the stop codon of *HBA2* using prime editing. (B) Representative NGS data showing the overall correction efficiency of Hb CS mutation at *HBA2* in a proerythroblast cell line. (C) Hb CS mutation revision editing detection at the cDNA level in a proerythroblast cell line using NGS. (D) Western blot analysis of α-globin chains in differentiated samples after correction of Hb CS mutation. (E) The cell expansion profile in differentiation medium. (F) Flow cytometric analysis of erythroid maturation on day 8 of differentiation. (G) Cellular viability analysis. The percentage of apoptotic cells was calculated by combining early-stage apoptotic cells (annexin V-$FITC^+$, PI^-) and late-stage apoptotic cells (annexin V-$FITC^+$, PI^+). (H) Hemoglobinization analysis of cells after erythroid maturation. (I) Real-time qPCR analysis of α2-globin mRNA levels in differentiated samples. The results were normalized to β-globin. (J) Quantification of α- and β-globin chain levels, as assessed by RP-HPLC. α-Globin expression was normalized to β-globin. Clone 4-corr indicates clone 4 with correction of Hb CS mutation. Values shown are means ± SDs of at least two independent experiments. $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. (C) and (E) two-way analysis of variance, Sidak's multiple comparisons test; (D) and (F)–(J), one-way analysis of variance, Tukey's multiple comparisons test).

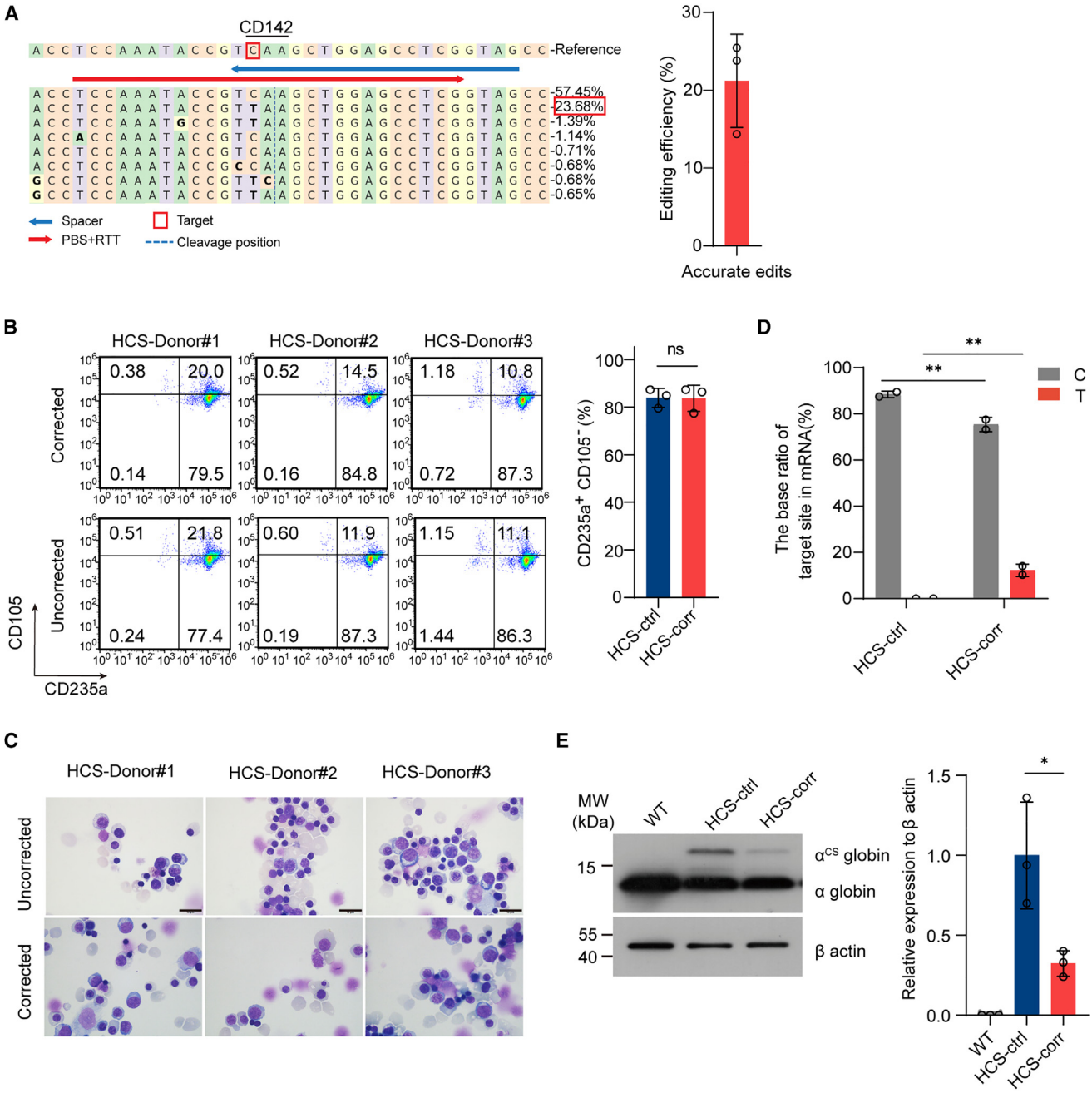

Figure 4. Prime editing-mediated efficient *in situ* correction of Hb CS mutations in HSPCs from HCS patients

(A) Representative NGS data showing the overall editing efficiency of *HBA2* in patient-derived HSPCs. (B) Quantification of erythroid maturation via flow cytometry on day 14 of differentiation. (C) Giemsa-stained differentiated (day 14) cultures. Original magnification ×100. Scale bars, 10 μm. (D) Hb CS mutation revision detection at the cDNA level in HSPCs using NGS. (E) Western blot analysis of α globin chains in differentiated samples. The error bars indicate the SDs of at least two patients. HCS-ctrl, HSPCs from HCS patients without correction, HCS-corr, HSPCs from HCS patients with Hb CS correction. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; ns, no significance (paired two-tailed Student's t test).

Notably, Hb CS contributes to α-thalassemia syndrome under different circumstances. When co-inherited with two α-globin deletional alleles (−/$\alpha^{CS}\alpha$), a severe form of HbH disease called HCS is produced.[4,38] When it occurs in its heterozygous ($\alpha^{CS}\alpha/\alpha\alpha$) or homozygous ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) forms, a more severe anemia phenotype results compared with the genotype in which the same α alleles are deleted.[25,28] Notably, even in patients with HCSs, there is no clear genotype-phenotype correlation because patients with identical

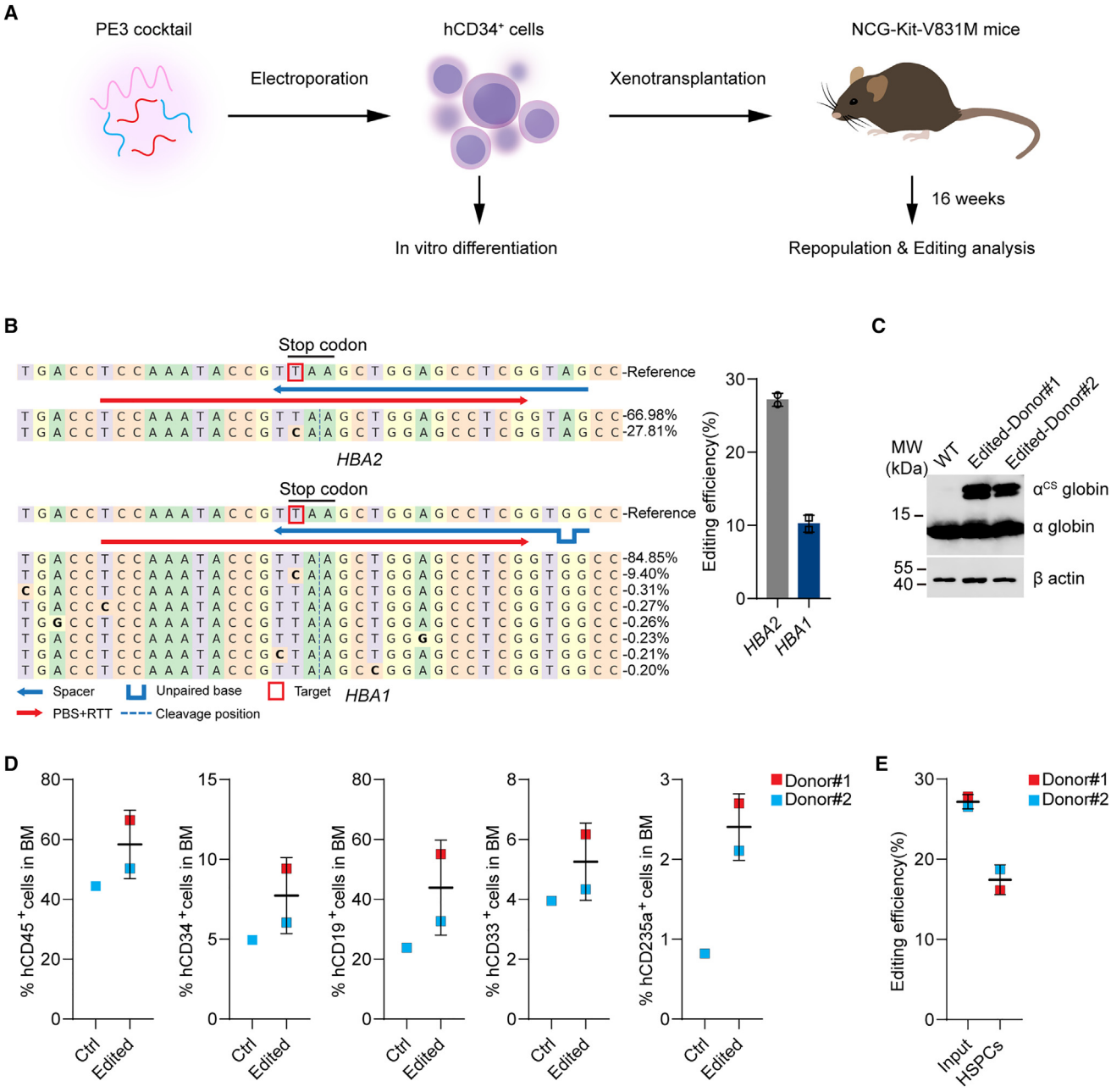

Figure 5. Prime editing efficiently creates the Hb CS mutations in human HSPCs for long-term transplantation

(A) To determine whether prime-edited HSPCs can repopulate BM *in vivo*, prime-edited HSPCs were electroporated into CD34$^+$ HSPCs and transplanted after 24 h into NCG-Kit-V831M mice by tail-vein injection. Bone marrow (BM) was harvested for further analysis at 16 weeks of transplantation. (B) Editing efficiency evaluation at the *HBA2* and *HBA1* loci in HSPCs before transplantation by NGS. (C) Abnormal α^{CS}-globin chains detection by western blot. (D) Flow cytometry analysis in mouse BM 16 weeks after transplantation for determination of human engraftment. (E) Editing efficiency analysis in CD34$^+$ human HSPCs derived from the BM of engrafted mice 16 weeks after transplantation. The error bars indicate the SDs of two healthy donors.

genotypes do not necessarily show the same severity.[5] Moreover, individuals with homozygous Hb CS have the phenotype of a mild α-thalassemia trait with very low levels of HbH.[39] Apart from mild to moderate microcytic hypochromic anemia, carriers are clinically asymptomatic and identified during a regular health check or during antenatal screening,[40] which indicates that two functional α-globin genes available in a homozygous Hb CS condition are capable of synthesizing considerable α-globin for hemoglobinization.

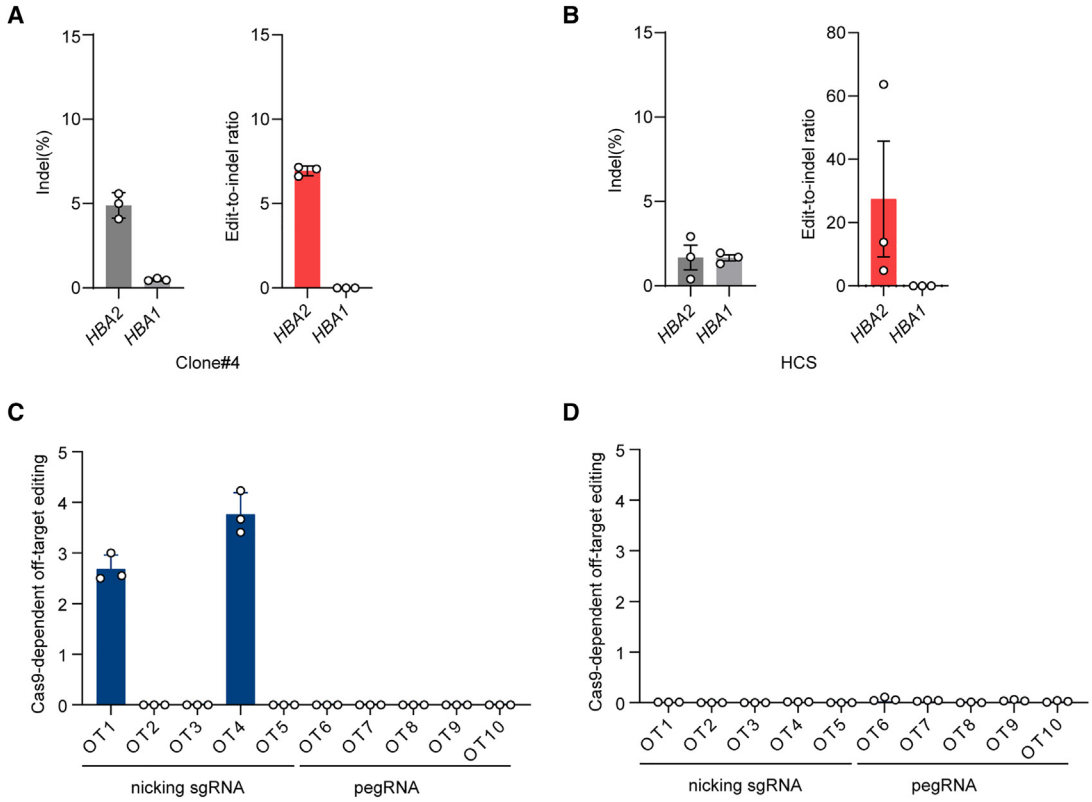

Figure 6. Prime editing induces detectable off-target events in erythroblasts and HSPCs

(A and B) Indels and edit-to-indel ratios in proerythroblast cell lines (A) and HCS patient-derived HSPCs (B) following correction of Hb CS mutation. (C and D) Analysis of the top 10 *in silico* predicted off-target (OT) sites in erythroblasts (C) and HSPCs (D) after prime editing. Off-target editing events in the erythroid cell line were detected using next-generation sequencing (NGS) at the top five off-target sites of the pegRNA and the nicking sgRNA predicted by Cas-OFFinder. CRISPR-Cas9-dependent off-target editing was calculated by quantifying the frequency of indels for the nicking sgRNA and by combing pegRNA RTT-related edits and indels for the pegRNA. The error bars indicate the SDs of three patients.

Our study collected primary HSPCs from three HCS patients with known severe clinical syndromes and observed obvious variations in cellular expansion and cell apoptosis after the induction of erythroid differentiation. No significant difference was observed in expansion rate between HSPCs from healthy donors and HCS patients. Moreover, expansion rate was not significantly changed after Hb CS correction in HUDEP-2 cell models and patient HSPCs. These results indicate that Hb CS mutation may not affect cell expansion *in vitro*. Among the four clones with the same homozygous ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) genotype in HUDEP-2 cells, only one clone exhibited delayed expansion and increased apoptosis after induced erythroid maturation. This result may be due to clonal heterogeneity. A previous study confirmed variations in γ-globin expression, erythroid maturation, and reticulocyte yield between HUDEP-2 clones and concluded that HUDEP-2 is a mixed clonal population.[41] Even in HUDEP-2 cells of the same clonal origin, γ-globin expression is highly variable.[42] Human embryonic ζ-globin is mainly synthesized in yolk sac-derived primitive erythroid cells, and decrease rapidly during definitive erythropoiesis.[43] Luo et al. showed that more than half of fetal erythrocytes were positive for ζ and approximately one-third of newborn red blood cells were ζ^{+}.[44] Persistent ζ-globin expression has been detected in carriers with $-^{SEA}$, $-^{MED}$, and $-^{SPAN}$ deletion.[45] The regulation of ζ-globin expression in HUDEP-2, a human umbilical cord blood-derived erythroid progenitor cell line, is not completely understood. Like γ-globin, induction of ζ-globin in certain clones may be due to clonal variation. Moreover, further investigations are needed to clarify whether DNA perturbation by prime editing at the *HBA2* would reactivate silenced ζ-globin expression. These observations also suggest that other genetic or epigenetic factors contribute to the degree of clinical severity in patients with Hb CS mutations. The exact phenotype of HSPCs with Hb CS mutations requires further comprehensive evaluation using other *in vivo* genetic or transplantation models.

Compared to intensive gene editing strategies in β-hemoglobinopathies, including SCD and β-thalassemia, the feasibility of gene editing in α-thalassemia is not known. The strategies for treating β-hemoglobinopathies primarily include the use of Cas9 nucleases or base editors to activate γ-globin gene transcription for the induction of fetal hemoglobin (HbF, α2γ2) and HDR-based *in situ* correction of SCD

by an AAV6 donor or a single-stranded oligodeoxynucleotide donor.[46–51] However, most of these strategies are in clinical trials, and the best strategy remains undetermined. In the context of α-thalassemia, the induction of embryonic ζ-globin may be an alternative strategy, and this approach should be comprehensively evaluated in the future.[32,34] Reverting the Hb CS allele back to the wild type is the most physiological approach. There are two gene editing tools, CBEs and PEs, which can correct Hb CS mutations. In this study, we tested the feasibility of prime editing in Hb CS mutation. Prime editing is an ideal technology for *in situ* correction of more than 80% of gene point mutations in diseases.[14] However, their wide application is limited primarily by large size for delivery and low efficiency in primary cells.[52] Several recent studies used a split construct of PE for adeno-associated virus-mediated delivery and successfully increased the efficiency to greater than 50%.[15,19] More recent studies have shown its great potential in correcting over 40% of SCD mutations *in vitro* and *in vivo*.[16,18] Our study revealed that the delivery of PE2 mRNA with synthesized pegRNA and nicking sgRNA is an alternative and efficient strategy for *ex vivo* HSC gene editing. Although the 20% correction efficiency achieved by prime editing is relatively low, this approach may be beneficial considering the therapeutic threshold from experience in SCD *in situ* correction.[47] The xenotransplant studies are widely acceptable models and the gold standard to evaluate the long-term repopulation capacity of primary HSPCs after gene editing.[16,29,50] In this study, we confirmed that the 20% of prime editing efficiency can be maintained over 16 weeks in the BM originally from normal HSPCs after prime editing. The editing efficiency in repopulating $CD34^+$ HSPCs is relatively lower compared to the efficiency for the recent reported SCD mutation by the similar strategy.[16] The discrepancy may come from the different target locus, the optimized prime editing strategy, or even the different electroporation system.[53] In addition, the long-term editing efficiency of patient-derived HSPCs *in vivo* had not been evaluated yet as a limitation of an adequate number for xenotransplant experiments. Therefore, further studies are required to define the cellular or *in vivo* phenotype in more patient samples after prime editing. Moreover, further comprehensive comparisons to other gene editing tools such as CBEs or HDR-based *in situ* correction are needed given recent advances.[54,55]

Another limitation of our study was the assessment of potential off-target effects of the PEs in erythroblasts. We used the widely used *in silico* prediction tool Cas-OFFinder to identify potential binding sites. Our targeted PCR analysis revealed 10 top-scoring sites and detected edits at 2 out of 10 off-target sites in Hb CS cell models. The off-target site 1 locates in intron 6 of *SHF*. SHF, an adapter protein, contains an SH2 domain and four putative tyrosine phosphorylation sites and is mainly expressed in skeletal muscle, brain, liver, prostate, testis, ovary, small intestine, and colon. SHF may play a role in the regulation of apoptosis in response to platelet-derived growth factor.[56] More recently, SHF was proved to act as a tumor suppressor in glioblastoma multiforme by disrupting STAT3 dimerization.[57] The off-target site 4 locates in intron 4 of *IQSEC2*, which is X-linked and mainly expressed in brain. This gene encodes a guanine nucleotide exchange factor for the ARF family of small guanosine triphosphate-binding proteins. Deficiency of *IQSEC2* is associated with intellectual disability, autism, and epilepsy.[58–60] To date, *SHF* and *IQSEC2* have not been reported to be involved in erythropoiesis. However, this technique was initially designed for Cas9 nuclease-directed double-strand breaks, and the potential off-target events introduced from the non-spacer sequence or the reverse transcriptase activity inherent to prime editors were not considered.[61,62] We were not able to assess all of the possible combinations and may have missed more off-target sites. Although there is no available method for prime editing, it would be feasible to evaluate the off-target sites of each spacer sequence by either CHANGE-seq (circularization for high-throughput analysis of nuclease genome-wide effects by sequencing) or CIRCLE-seq (circularization for *in vitro* reporting of cleavage effects by sequencing).[63–65] Both methods were designed for quick-testing the cutting efficiency of individual sgRNAs in the whole-genome sequence, but not in the cellular context. Recent studies had confirmed that the prime editing efficiency was highly dependent on the cellular chromatin context.[66,67] Moreover, Fiumara et al. reported the genotoxicity of Pes, which was mainly caused by the generation of DNA double-strand breaks and genotoxic by-products, including deletions and translocations.[68] Therefore, on-target and off-target effects, including the genome-wide indels, genome structural variations, and even epigenetic alterations, require further comprehensive assessment.

Overall, our study provided a successful case in which prime editing efficiently corrected Hb CS mutations in patient HSPCs, which provides proof of principle for its therapeutic potential in HCS.

MATERIALS AND METHODS

pegRNA and nicking sgRNA generation

For Hb CS generation in HUDEP-2 cells, pegRNA and nicking sgRNA were cloned and inserted into pGL3-U6-sgRNA-EGFP. Oligonucleotide duplexes containing the nicking sgRNA protospacer were ligated into BasI-digested pGL3-U6-sgRNA-EGFP. Oligonucleotide duplexes containing pegRNA protospacer, extension template, and scaffold sequences were ligated to the backbone of pGL3-U6-sgRNA-EGFP via Golden Gate assembly. For Hb CS mutation reversion editing in model cells and HSPCs, chemically modified synthetic pegRNA and nicking sgRNA were obtained from Integrated DNA Technologies. Each contained 2′-*O*-methyl modifications at the first three 5′ and 3′ nucleotides and 3′-phosphorothioate nonhydrolyzable linkages between the three first and last nucleotides.

Cell line culture

HUDEP-2 cells were cultured in StemSpan serum-free medium (SFEM; StemCell Technologies, 9650) supplemented with 1% penicillin/streptomycin, 50 ng/mL recombinant human stem cell factor (SCF; PeproTech, 300-07), 3 IU/mL erythropoietin (EPO; PeproTech, 100-64), 10 μM dexamethasone (Sigma-Aldrich, D4902-1), and 1 μg/mL doxycycline (DOX; Sigma-Aldrich). The cells were maintained at a density of less than 0.8 million per milliliter. Erythroid differentiation of HUDEP-2 cells was induced by growing the cells for

4 days in Iscove's modified Dulbecco's medium (Gibco, 12440053) supplemented with 3% AB serum (Gemini, 100–512), 2% fetal bovine serum (FBS; Gibco, 16000-044), 10 μg/mL insulin (Yeasen, 40112ES60), 3 IU/mL heparin (StemCell Technologies, 07980), 200 μg/mL holo-transferrin (Sigma-Aldrich, T0665), 3 IU/mL EPO, 10 μg/mL SCF, 1 ng/mL interleukin-3 (IL-3; Peprotech, 200-03), 1 μg/mL DOX, and 1% penicillin/streptomycin, as phase 1. The cells were transferred to phase 2 medium (phase 1 medium supplemented with 500 μg/mL holo-transferrin but without DOX) and cultured for another 4 days. Flow cytometric analysis of CD105 (BioLegend, 800507) and CD235a (GYPA) (BD Pharmingen, 559943) erythroid surface markers and May-Grünwald Giemsa staining were performed to monitor erythroid differentiation.

HSPC purification and culture

Three transfusion-dependent HCSs (with a transfusion frequency >4 times per year) were recruited from the 923rd Hospital of the People's Liberation Army. Peripheral blood was collected from granulocyte colony-stimulating factor-mobilized healthy donors and HCS patients following hospital ethics review board approval (923LL-2022KY-01-001), in accordance with the Declaration of Helsinki. Written informed consent was obtained from all the subjects and/or their family members.

$CD34^+$ HSPCs were enriched using the EasySep Human CD34 Positive Selection Kit (STEMCELL Technologies, 17856) according to the manufacturer's instructions. Twenty-four hours before electroporation, $CD34^+$ cells were thawed and maintained in stem cell medium containing SFEM medium supplemented with 100 ng/mL recombinant human SCF, 100 ng/mL recombinant human thrombopoietin (PeproTech, 300-18), and 100 ng/mL recombinant human FLT-3 ligand (PeproTech, 300-19). The electroporated cells were maintained in stem cell medium for 24 h then transferred to erythroid differentiation medium. $CD34^+$ cells were differentiated using a two-phase protocol. Cells were maintained in at 10^5 cells/mL for 6 days in phase 1 medium (SFEM medium supplemented with 10% FBS, 1 IU/mL recombinant human EPO, 10 ng/mL IL-3, and 50 ng/mL recombinant human SCF). Then, cells were switched to phase 2 medium (SFEM medium supplemented with 30% FBS and 3 IU/mL recombinant human EPO) and cultured for another 8 days. RNA and western blot samples were collected on day 14 of differentiation. Detailed information on all three HCS patients is listed in Table S1.

PE mRNA *in vitro* transcription

The mRNA transcription template was generated via digestion of the plasmid pCMV-PE2 (Addgene, 132775) overnight with PemI (NEB, R0560V). The linearized plasmid was purified using the phenol-chloroform method and eluted in DNase/RNase-free water. *In vitro* transcription of PE2 mRNA was performed using a mMESSAGE mMACHINE Kit (Invitrogen, AM1340), followed by polyadenylation (Invitrogen, AM1350) according to the manufacturer's instructions. The mRNA was precipitated using LiCl provided by the kit manufacturer.

PE electroporation

All electroporation procedures were performed with a Neon Transfection System. For Hb CS mutation generation, 2×10^6 HUDEP-2 cells were transfected with 1.5 μg PE plasmid, 500 ng pegRNA plasmid, and 166 ng nicking sgRNA plasmid using a Neon Kit (100 μL, Invitrogen, MPK10096) at 1,200 V for 40 ms with 1 pulse. Electroporated cells were sorted using a BD FACSAria II. Sorted HUDEP-2 bulk populations were cloned by limiting dilution. Genotyping of clones was performed via Sanger sequencing. Homozygous clones ($\alpha^{CS}\alpha/\alpha^{CS}\alpha$) were expanded for further analysis. For Hb CS mutation reversion editing in HUDEP-2 model cells, 2×10^6 cells were transfected with 1 μg PE mRNA, 1.5 μg pegRNA, and 500 ng nicking sgRNA using a Neon Kit (100 μL, Invitrogen) at 1,200 V for 40 ms with 1 pulse. For Hb CS mutation reversion editing in patient-derived HSPCs, 2×10^5 cells were transfected with 1 μg PE mRNA, 1.5 μg pegRNA, and 500 ng nicking sgRNA using a Neon Kit (10 μL, Invitrogen) with 1,600 V, 10 ms, and 3 pulses.

Western blot analysis

Western blotting was performed according to a standard protocol. Briefly, total protein was extracted from HUDEP-2 cells on day 8 of differentiation or from primary erythroblasts on day 14 of differentiation using cell lysis buffer (Beyotime, P0013) supplemented with 1× protease and phosphatase inhibitor cocktail (Beyotime, P1045) and 1 mM phenylmethanesulfonylfluoride (Beyotime, ST506). Equal amounts of proteins were loaded onto 12.5% SDS-PAGE gels (NCM Biotech, P2013) and separated in 1× SDS running buffer. The separated proteins were transferred onto polyvinylidene fluoride membranes and incubated with primary antibody overnight at 4°C. The primary antibodies used were anti-hemoglobin α (Santa Cruz, sc-514378), 1:1,000; anti-hemoglobin β (Santa Cruz, sc-21757), 1:1,000; anti-hemoglobin γ (Abcam, ab137096), 1:2,000; anti-hemoglobin ζ (GeneTex, GT1544), 1:2,000; and anti-β-actin (Santa Cruz, sc-47778), 1:2,000. Protein quantification was performed using densitometry with ImageJ Software, and protein expression was normalized to β-actin.

Quantitative PCR analysis of globin

Total RNA was extracted from HUDEP-2 cells on day 8 of differentiation or from primary erythroblasts on day 14 of differentiation with a total RNA isolation kit (Vazyme, RC112-01) following the manufacturer's instructions. Total RNA (500 ng) was reverse-transcribed to cDNA using PrimeScript RT Master Mix (TaKaRa, RR036A). qPCR products were prepared with AceQ Universal SYBR qPCR Master Mix (Vazyme, Q511-02) and run on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Quantification was performed using the ΔΔCT method. β-Actin was used as the endogenous control. The primers used for real-time qPCR are listed in Table S4.

Evaluation of editing efficiency

Editing efficiency was evaluated via PCR amplification of sequences of interest followed by Sanger sequencing or next-generation sequencing (NGS). Genomic DNA was extracted from control and

PE3-edited cells using lysis buffer (10 mM Tris-HCl, pH 8.5; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 0.45% NP-40; 0.45% Tween 20; and 100 μg/mL Proteinase K). Lysed cells were incubated at 55°C for 1 h, followed by heat inactivation at 95°C for 10 min. For single-clone genotyping, PCR amplification of target regions was subjected to Sanger sequencing. The ABI files were analyzed using Synthego ICE (https://ice.synthego.com/). For the on- and off-target site editing efficiency evaluation, following the initial PCR amplification of the target sequences with CloneAmp HiFi PCR Premix (TaKaRa, 639298), a second PCR was performed using CloneAmp HiFi PCR Premix to add Illumina-compatible adapters with multiplexed indices. After purification using a TIANgel Midi purification kit (TIANGEN, DP209), the libraries were pooled and paired-end (2 × 150 bp) sequenced using an Illumina MiSeq platform. The NGS data were analyzed using CRISPResso2.[69] The primers used for deep sequencing are listed in Table S4.

cDNA sequencing

To investigate the edits at the RNA level, the cDNA prepared above was PCR amplified and subjected to NGS.

RP-HPLC analysis of globin chains

RP-HPLC analysis was performed using an Agilent 1260 Infinity II chromatograph. Hemolysates were prepared by lysing 10^5 cells in 50 μL of HPLC-grade water for 10 min. Cell debris was removed via centrifugation at 13,200 rpm for 10 min. Globin samples were eluted with a gradient mixture of solution A (water/acetonitrile/trifluoroacetic acid, 700:300:0.7) and solution B (water/acetonitrile/trifluoroacetic acid, 450:550:0.5). Globin chains were separated via HPLC using a 150 × 4.6 mm, 3.6 μm Aeris WIDEPORE LC column (Phenomenex). The absorbance was measured at 220 nm.

Cas-OFFinder off-target editing analysis

Computational prediction of potential off-target sites was performed using Cas-OFFinder[37] with the following settings: no bulges and three or fewer mismatches sharing canonical PAMs with nicking sgRNA or pegRNA. The top 10 off-target sites are listed in Table S5.

Cell viability analysis

The viability of differentiated HUDEP-2 cells was detected using flow cytometry and an Annexin V-FITC (fluorescein isothiocyanate) Apoptosis Detection Kit (Beyotime, C1062) following the manufacturer's instructions. Cells with negative annexin V-FITC and phosphatidylinositol staining were used as controls. Annexin V^+ cells including Annexin V^+/PI^- cells and Annexin V^+/PI^+ were calculated to evaluate the effect of Hb CS mutation on cell viability.

Prime-edited HSPCs xenotransplantation in NCG-Kit-V831M mice

Animal experiment was approved by the Institutional Animal Care and Use Committee of GemPharmatech. pegRNA, nicking sgRNA, and PE2 mRNA were electroporated into $CD34^+$ HSPCs and transplanted after 24 h into NCG-Kit-V831M mice by tail-vein injection. Transplantation experiments were performed as described.[29] At 16 weeks after transplantation, recipient mice were sacrificed. Cells were harvested from BM; stained with antibodies against human surface markers human CD45 (BioLegend, 304008), human CD235a (BD Pharmingen, 559943), human CD34 (Elabscience, E-AB-F1143E), human CD19 (BioLegend, 392504), human CD33 (BioLegend, 366614); and analyzed by flow cytometry using a Gallios analyzer and FlowJo software (BD Biosciences). Human $CD34^+$ cells were sorted by immunomagnetic selection with CD34 MicroBeads (STEMCELL Technologies, 17856).

Statistical analysis

The data are presented as means ± SDs. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad). $p < 0.05$ was considered to indicate statistical significance.

DATA AND CODE AVAILABILITY

Data are available from the authors upon reasonable request.

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AUTHOR CONTRIBUTIONS

C.S., P.X., L.C., and X.X. planned the experimental design, analyzed the data, and wrote the manuscript. Q.L. and J.X. performed the PE optimization and erythroid maturation under the guidance of C.S. and P.X. Y.X. performed the bioinformatics analyses for NGS. J.Z., Y.Y., B.L., and X.Z. prepared and collected the patient-derived $CD34^+$ HSPCs and performed the clinical data analysis. J.X. and C.Z. performed the transplant study. P.X., L.C., and X.X. supervised the study. All authors discussed the results and contributed to preparation of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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