Molecular Therapy
Nucleic Acids
Original Article

Precise correction of a spectrum of β-thalassemia mutations in coding and non-coding regions by base editors

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β-thalassemia/HbE results from mutations in the β-globin locus that impede the production of functional adult hemoglobin. Base editors (BEs) could facilitate the correction of the point mutations with minimal or no indel creation, but its efficiency and bystander editing for the correction of β-thalassemia mutations in coding and non-coding regions remains unexplored. Here, we screened BE variants in HUDEP-2 cells for their ability to correct a spectrum of β-thalassemia mutations that were integrated into the genome as fragments of *HBB*. The identified targets were introduced into their endogenous genomic location using BEs and Cas9/homology-directed repair (HDR) to create cellular models with β-thalassemia/HbE. These β-thalassemia/HbE models were then used to assess the efficiency of correction in the native locus and functional β-globin restoration. Most bystander edits produced near target sites did not interfere with adult hemoglobin expression and are not predicted to be pathogenic. Further, the effectiveness of BE was validated for the correction of the pathogenic HbE variant in severe β^0/β^E-thalassaemia patient cells. Overall, our study establishes a novel platform to screen and select optimal BE tools for therapeutic genome editing by demonstrating the precise, efficient, and scarless correction of pathogenic point mutations spanning multiple regions of *HBB* including the promoter, intron, and exons.

INTRODUCTION

β-thalassemia represents the most common inherited monogenic disorder caused by a spectrum of mutations in the β-globin gene, *HBB*. These mutation results in a quantitative decrease in the production of the functional β-globin chain, which creates an imbalance in the α/β globin-chain ratio leading to ineffective erythropoiesis.[1,2] Depending upon the severity of the phenotype, β-thalassemia is categorized as (β^0/β^0) major, (β^0/β^+) intermedia, and ($\beta/\beta^{+/0}$) minor.[3]

At the molecular level, β-thalassemia encompasses over 350 disease-causing variants, including single-nucleotide changes, deletions, and insertions. Most of the pathogenic variants reside in the *HBB* coding and non-coding regions, including the promoter, intron, and exons.[4,5] Notably, the *HBB* promoter region contains sequences crucial for the binding of transcriptional regulators that finely modulate β-globin expression.[1] Specific variants like *HBB* -88 (C>T) and *HBB* -28 (A>G) in the promoter region significantly reduce *HBB* mRNA production, leading to a β^+ phenotype.[5] In the coding region, mutations such at the initiation codon (ATG>ACG) affect the *HBB* protein synthesis,[5] and CD15 (TGG>TAG) result in premature stop codons, causing severe β^0-thalassemia.[1] Another common mutation at CD26 (GAG>AAG) leads to the HbE variant, resulting in a severe disease phenotype when inherited as compound heterozygous HbE/β-thalassemia.[6] Non-coding intronic mutations also play a crucial role in β-thalassemia pathogenesis, such as IVS1-5 (G>A) which disrupt conventional β-globin splicing by affecting the consensus sequence, present around the splice donor sites,[1] and IVS1-110 (G>A), which disrupts normal β-globin splicing by generating a de novo splice acceptor site.[7,8] Additionally, mutations like *HBB* IVS2-849 (A>G) inactivate normal splice acceptor sites, causing improper

Received 28 April 2023; accepted 26 April 2024;
https://doi.org/10.1016/j.omtn.2024.102205.

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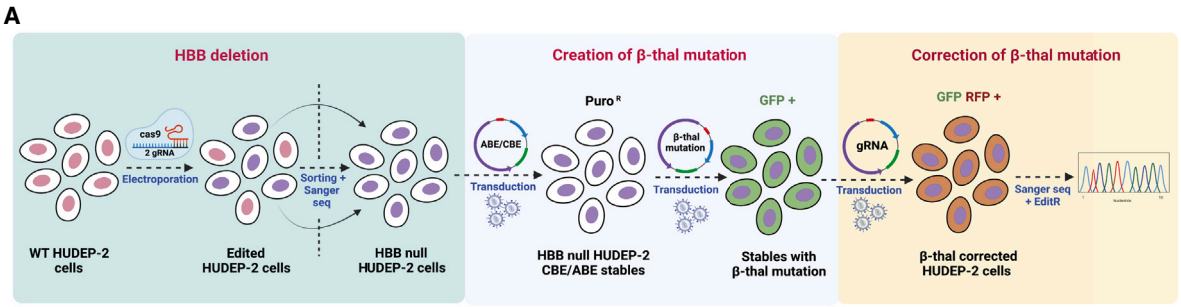

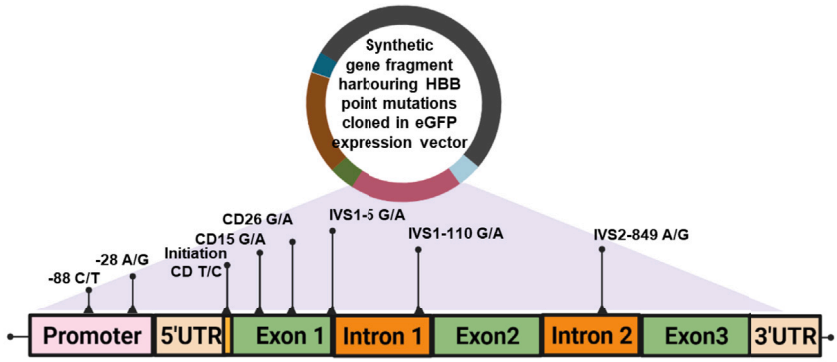

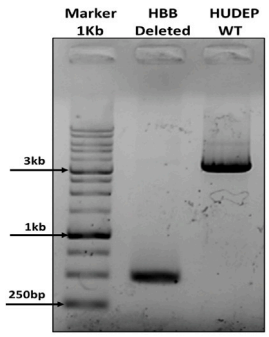

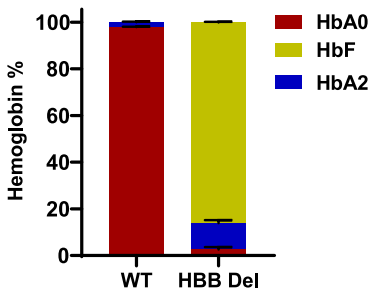

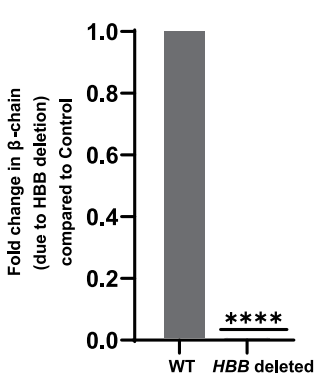

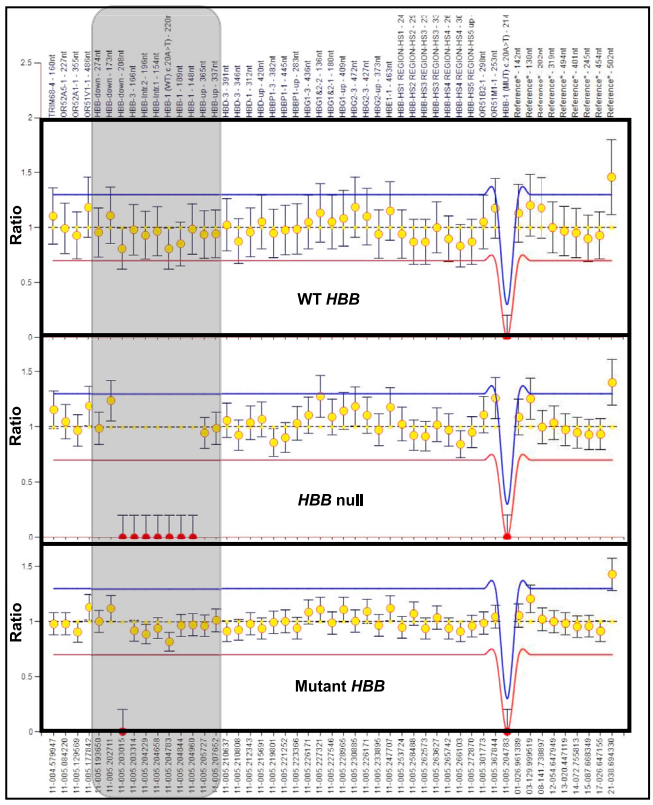

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splicing and intron retention during transcription.[9] These insights into the molecular mechanisms underlying β-thalassemia mutations are vital for understanding disease pathogenesis and developing targeted therapeutic interventions.

Current gene-editing strategies focus on *ex vivo* modification of patient's hematopoietic stem and progenitor cells (HSPCs) using CRISPR-Cas9 for either the precise homology-directed repair (HDR) or error-prone non-homologous end-joining (NHEJ)-mediated correction of the β-thalassemia mutations.[10–12] However, HDR-mediated gene repair is inefficient in primary cells, and a high indel/HDR ratio might be detrimental in coding areas. Even after achieving high HDR efficiency using small molecules, the long-term engraftment of primary cells was shown to be compromised.[13–15] On the other hand, the NHEJ-based approach has successfully corrected the intronic mutations such as IVS1-110 and IVS2-654[16,17] but is not suitable for the correction of mutations in highly conserved sequences where defined editing outcomes are required. Nevertheless, the unintended effects of double-strand breaks (DSBs), such as chromothripsis, large-deletions, translocations, and p53 activation hamper the prospects of Cas9-mediated approaches.[18,19]

Base editors (BEs) harness the advantage of CRISPR-Cas9 by combining a Cas9-nickase targeting domain with either adenosine deaminase (ABE) or cytosine deaminase (CBE) that enables efficient, scarless single-nucleotide conversion (A>G or C>T) at the desired target site without the generation of DSBs.[20–22] However, a limitation of BE is their propensity to generate bystander mutations. Some bystander mutations can introduce new disease-causing variation even as the original disease-causing variant is corrected, but this can often be circumvented by careful selection of single guide RNA (sgRNA) and BE variants.[23,24] The limited availability of patient cells with desired mutations for the preclinical studies due to disease complications emphasizes the need to develop more relevant β-thalassemia disease models. Patient-derived induced pluripotent stem cells (iPSCs) could serve as an ideal disease model, but these are not available for most disease-causing variants because iPSC generation is labor-intensive and the process of confirming patient consent and sharing such research models is challenging.[25,26] Base editing provides a direct single-step approach for mutagenesis and has been used efficiently to create various cellular and animal models of genetic diseases.[27,28]

In the current study, we have explored the potential of BEs to correct eight β-thalassemia mutations in the coding and non-coding regions. Toward this end, we generated human erythroid cells harboring a spectrum of β-thalassemia mutations for initial screening of BEs and sgRNA. Using this model, we showed that BE mediated highly efficient and precise correction of multiple point mutations present in *HBB* promoter, intron, and exon. The best identified targets from the primary screening were validated in new cellular models we generated to harbor individual β-thalassemia point mutations. The outcomes were confirmed to correct the disease-causing HbE/CD26 (G>A) mutation in β^0/β^E-thalassemia patient cells. Overall, we demonstrated the effectiveness of BE in precise modeling and correction of the structural as well as functional β-globin variants in established human erythroid cell lines and human primary cells.

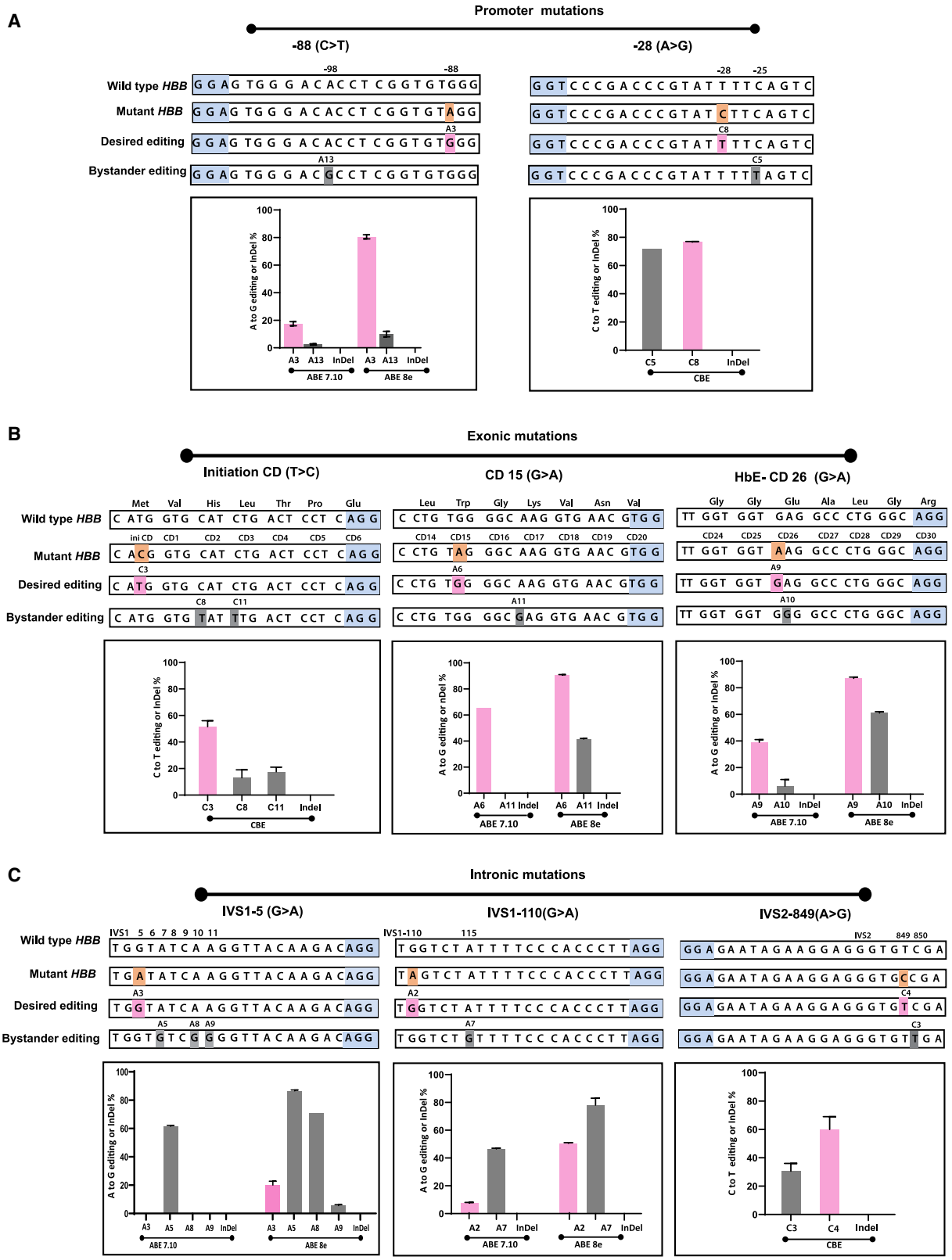
RESULTS

Engineering the human erythroid cells with a spectrum of β-thalassemia mutations

First, as proof of principle, we evaluated the efficiency of BEs to correct eight key-point mutations present in coding and non-coding regions of *HBB* and assessed the bystander editing. The exact outcome of BE is unpredictable and is often specific to the sgRNA, base editor variant, and genomic locus. Therefore, we screened different variants of BEs for their potential to precisely correct β-thalassemia mutations in HUDEP-2 cells. HUDEP-2 cells are an immortalized human erythroid progenitor cell line derived from CD34+ hematopoietic cells which can differentiate into the erythroid lineage and expresses adult hemoglobin (HbA0).[29] For the primary screening, we generated HUDEP-2 cells with a spectrum of β-thalassemia mutations (Figure 1A), as it is often challenging to obtain a β-thalassemia patient CD34+ HSPCs with particular point mutations. We incorporated the globally prevalent β-thalassemia point mutations (Table S1) that could be corrected using BEs into an *HBB* gene fragment (Figure 1B) and integrated it into the HUDEP-2 cells by lentiviral transduction. Before incorporation of the *HBB* gene fragment, we deleted the endogenous *HBB* gene, including the promoter, from the HUDEP-2 cells to avoid the potential bias in editing outcomes while validating the efficiency of sgRNAs (Figures 1C and S1A and S1B). Single-cell clones with homozygous *HBB* deletion (five clones) were pooled and expanded for further experiments (referred to as *HBB* null cells) (Figure S1C). A drastic decrease in the levels of HbA0 expression with a compensatory increase in the fetal hemoglobin (HbF) was observed in *HBB* null cells upon erythroid differentiation as previously reported[30] (Figures 1D and S1D–S1E). We performed qRT-PCR as well as multiplex ligation-dependent probe amplification (MLPA) assay in the pool of *HBB* null cells, a commonly used method for analyzing the copy number variations in β-thalassemia patients,

Figure 1. Generation of human erythroid cells with a spectrum of β-thalassemia point mutations

(A) Schematic representation of the workflow for creating engineered HUDEP-2 cells expressing base editor. The engineered HUDEP-2 cells are devoid of the native *HBB* locus while harboring *HBB* gene fragment with desired β-thalassemia point mutations and expressing base editors. (B) Layout of *HBB* gene fragment harboring β-thalassemia point mutations targeted in this study. (C) Confirmation of endogenous *HBB* gene deletion in HUDEP-2 cells by PCR amplification in control (WT HUDEP) and *HBB* null HUDEP-2 cells; the PCR bands at 500 bp and 3330 bp represent the *HBB*-deleted and control samples, respectively. (D) Evaluation of hemoglobin variants expression after erythroid differentiation of *HBB* null HUDEP-2 cells by HPLC. (E) Quantification of *HBB* gene deletion in genomic DNA using qRT-PCR in HUDEP-2 cells (unpaired t test). (F) Validation of *HBB*-mutant fragment integration in *HBB* null HUDEP-2 cells using MLPA analysis (gray shaded box indicates probes targeting *HBB*). Data are expressed as mean ± SEM triplicates. Asterisks indicate levels of statistical significance (****$p < 0.0001$).



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which further confirmed the *HBB* deletion with no signals detected by the probes targeting at the *HBB* locus (Figures 1E and 1F). *HBB* null cells expressing ABE (ABE 7.10 and ABE8e) and CBE were generated to facilitate base editing for further experiments. Base editing potential of these stable cell lines was confirmed using a previously reported sgRNA targeting the *BCL11A* binding site in the *HBG* promoter[31] (Figure S1F). These cells were then transduced with a lentiviral vector, encoding the *HBB*-mutant fragment and GFP reporter. MLPA analysis showed the integration of approximately one copy of mutated *HBB* gene fragment in the *HBB* null cells (Figure 1F). Thus, we generated HUDEP-2 cells with a spectrum of β-thalassemia mutations expressing different BE variants.

Primary screening of base editor variants for the correction of various β-thalassemia mutations in the *HBB* promoter, introns, and exons

To investigate the on-target and bystander editing efficiency of BE variants when correcting disease-causing variants, we used our engineered HUDEP-2 cells harboring various β-thalassemia mutations (Figure S2.1A). We used two variants of ABE (7.10 and 8e) for A>G conversion and CBE (BE3) for C>T conversion. The sgRNAs were selected based on the position of the target nucleotide within the editing window of the BE and were delivered by lentiviral transduction (Figure S2.1B).

The two promoter mutations, *HBB* -88(C>T) and *HBB* -28(A>G), resulting in β^+-phenotype, were corrected efficiently using ABE and CBE, respectively. (Figure S2.1A). ABE8e showed ∼80% on-target conversion at −88 site, while ABE7.10 showed very low editing (∼20%), and both the variants showed minimal bystander editing at the −98 site (Figure 2A). This result indicated that careful selection of BE variants can effectively correct the *HBB* promoter mutation with high precision and efficiency. CBE-mediated correction of *HBB* -28(A>G) mutation showed equivalent editing efficiency at both on-target and bystander sites (Figure 2A). Although the bystander editing at *HBB* -25(G>A) has not been reported to cause the β-thalassemia phenotype, another variant, *HBB* -25(G>C), is known to be pathogenic.[4] The ramifications of this G>A bystander edit will require further exploration.

Next, we aimed to reverse the severe β^0-thalassemia phenotype caused due to mutations in the coding region, beginning with the transcription start codon (ATG>ACG) of *HBB* using CBE (Figure S2.1A). We achieved 51% of C>T base substitution at the desired target site with a lower percentage of bystander deamination in the codon2 (CD2) (CAT>TAT) and CD3 (CTG>TTG) (Figure 2B). Interestingly, previous clinical reports indicate the existence of non-pathogenic mutations co-inherited with initiation codon of *HBB*. Bystander editing at CD2 (CAT>TAT) resulted in Hb-Fukuoka, a normal variant in heterozygous form, whereas at CD3 (CTG>TTG) created a silent mutation[32] (Figure S2.1C).

Further, we corrected another important severe β^0-thalassemia mutation at CD15 (TGG>TAG) using ABE variants (Figure S2.1A). Both variants of ABE showed high on-target conversion, 65% using ABE7.10 and 90% using ABE8e (Figure 2B). However, there was bystander editing (41%) at CD17 that produced a naturally occurring hemoglobin variant, Hb-Nagasaki with ABE8e[32] (Figure S2.1D). Although the on-target editing was lower with ABE7.10 at this locus, the bystander editing that introduced Hb-Nagasaki was absent.

In addition, we attempted to correct the second most frequent pathological structural Hb variant, HbE/CD26 (GAG>AAG) mutation using ABE variants (Figure S2.1A). Correction of the HbE mutation alone would be sufficient to reduce the severity of compound heterozygote HbE/β-thalassemia symptoms. Two potential sgRNAs (sgRNA1 and sgRNA2) were designed for HbE correction. However, the incorporation of IVS1-5 (G>A) mutation in the *HBB* gene fragment altered the protospacer adjacent motifs (PAM) (TGG>TGA) of sgRNA1, which hindered its validation in the engineered HUDEP-2 cells. Using sgRNA2, we achieved 39% and 87% on-target conversion of CD26 (G>A) mutation using ABE7.10 and ABE8e variants, respectively (Figure 2B). Although the bystander effect was observed for both the variants of ABE, the on-target editing was 8-fold higher while using ABE7.10 and ∼2-fold higher when editing with ABE8e. Nevertheless, the bystander edit resulted in the creation of Hb-Aubenas (CD26 (AAG >GGG) (Lys>Gly)), which is a naturally occurring hemoglobin variant with normal hematological features in heterozygous conditions[32] (Figure S2.1E).

The base substitution at the consensus flanking regions surrounding the exon-intron junction (*HBB* IVS1-5 (G>A) and *HBB* IVS1-110 (G>A)), and invariant dinucleotides (GT/AG) at the donor/acceptor splice sites at the *HBB* locus (*HBB* IVS2-849 (A>G)) affect the mRNA processing, leading to severe/mild β-thalassemia phenotype[1] (Figure S2.1A). Here, we employed both ABE7.10 and ABE8e variants to correct the IVS1-5 (G>A) mutation. Unlike the other validated target regions, we observed that the bystander edits (at IVS1-7 and 1–10 positions) were more prominent (∼4-fold) compared with on-target conversion at the IVS1-5 site utilizing ABE8e, while ABE7.10 failed to correct the desired base and only created bystander mutations in the IVS1-7 site (Figure 2C). Notably, the bystander edit creates a point mutation outside the consensus splice site within the intron, which might be permissive for normal splicing.

Figure 2. Correction of various β-thalassemia point mutations in the engineered HUDEP-2 cells by base editors

(A–C) The point mutations in the engineered *HBB* gene fragment were corrected using different base editor variants. The targeted point mutations were classified into three categories based on their positions in the *HBB* gene: Promoter (A), Exonic (B), and Intronic (C) regions. A graphical representation of the DNA sequences representing β-thalassemia mutation (orange), precise correction (pink), and undesired bystander editing (gray); highlights in blue represent the PAM sequence of the correction sgRNA. The intended base conversion efficiency for all eight different *HBB* point mutations is represented in the pink bar, while the gray bar represents the unintended bystander editing. Data are expressed as mean ± SEM from duplicates.

Next, we aimed to correct a cryptic splice site mutation, *HBB* IVS1-110 (G>A) using ABE variants (Figure S2.1A). Here, we targeted specific correction of IVSI-110 (G>A) using ABE variants. At the IVSI-110 (G>A) site, ABE7.10 delivered lower editing due to the location of the target base outside the editing window, but ABE8e yielded 50% conversion (A>G) (Figure 2C). Although utilizing ABE8e improved editing efficiency, it led to substantial bystander editing at IVSI-115 (A>G). Based on the earlier results of Cas9-induced non-specific disruption and as per the recently published article showing edits at IVSI-115 (A>G) site, we expect that the bystander edit will not have a functional consequence.[8,17]

Finally, we attempted to correct the common intron-exon splice junction mutation, *HBB* IVS2-849 (A>G) using CBE (Figure S2.1A). We achieved 60% on-target conversion (G>A) along with 30% bystander editing at IVS2-850 at the splice junction, where variant have been reported to cause β^0-thalassemia[4] (Figure 2C). Even though half of edited alleles also have bystander edits, we anticipate that the remaining 30% of G>A conversion at IVS2-849 with no bystander edits would be sufficient to restore functional β-globin production.

Although we observed several unintended bystander edits while utilizing BEs for gene correction, we did not observe any indels at any of the target sites in the coding and non-coding regions. Interestingly, most bystander edits resulted in a normal phenotype or were not implicated in functional *HBB* expression, except for bystander edits in consensus splice sites. Collectively, our data demonstrate a BE-mediated correction for multiple β-thalassemia point mutations located in promoter, exonic, and intronic regions of the *HBB* gene using engineered HUDEP-2 cells harboring various β-thalassemia mutations.

Multiplexed base editing facilitates simultaneous correction of two distinct β-thalassaemia mutations

Previous attempts of genome editing for correction of β-thalassaemia mutations have primarily focused on correcting single point mutation anticipating that even correcting one allele would result in a normal phenotype. However, since $\beta/\beta^{+/0}$ genotypes can still lead to β-thalassemia minor, it would be preferable to correct both mutant alleles simultaneously.[33,34] Therefore, we attempted to correct two β-thalassemia pathogenic variants simultaneously using BEs.

To this end, we selected two pairs of β-thalassemia mutations (HbE (G>A)/CD15 (G>A) and initiation CD (T>C)/IVS2-849 (A>G)) that have been already validated in this study. These mutation pairs were selected based on their compatibility for concurrent correction using the same base editor in engineered HUDEP-2 cells harboring a spectrum of β-thalassemia mutations. We achieved simultaneous correction of CD15 (TGG>TAG) and HbE/CD26 (GAG>AAG) with 87% and 78% base conversions, respectively, with less than 15% bystander editing at both sites, using ABE8e (Figure S2.2A). Similarly, the correction of initiation CD (T>C) and IVS2-849 (A>G) mutation showed 30%–40% on-target conversion with 12% or less bystander editing by CBE (Figure S2.2B).

These results demonstrate that the multiplex base editing approach efficiently reverses various mutations using both ABE8e and CBE variants. Hence this can be used in β-thalassemia patients carrying compound heterozygous mutation for the therapeutic correction and more effective restoration of β-globin production.

Creation of a human erythroid cellular model of β-thalassemia/HbE using base editor and Cas9

To functionally validate BE efficacy at pathogenic variants in their native locus and determine its effect on the restoration of β-chain synthesis, we created two different HUDEP-2 cell lines harboring β^0-thalassemia mutations at the coding (initiation codon (ATG>ACG)) and non-coding (IVS2- 849(A>G)) region of the endogenous *HBB* gene. We separately employed CRISPR-Cas9 HDR for knockin of these pathogenic variants. Both approaches were compared in modeling severe β^0-thalassemia mutations. To generate these mutations, we delivered ABE8e mRNA with sgRNA for base editing (Figure S3.1A), and the Cas9 protein along with sgRNA and single-stranded oligodeoxynucleotide (ssODN) donor template for HDR into HUDEP-2 cells by nucleofection.

The base editing approach generated a higher proportion of cells with *HBB* initiation codon (T>C) mutation with relatively fewer undesired alleles with bystander edits compared with the HDR approach that yielded lower on-target efficiency and more undesired alleles with indels (Figure 3A (a-b)). In creating the IVS2-849 (A>G) mutation, both base editing and HDR demonstrated similar efficiency, but HDR led to more undesired indel outcomes relative to fewer undesired outcomes with bystander edits generated using base editing (Figure 3B (a-b)). Although undesired bystander editing was observed at both the target sites using BE, single-cell sorting yielded clones with mono and bi-allelic edits that lacked any bystander edit (Figures S3.1C-D and S3.2A-B(a)). Genotype analysis showed the retention of intron-2 in *HBB* mRNA in cells harboring a homozygous IVS2-849 (A>G) mutation and production of non-functional protein from alternative shorter ORFs in cells harboring homozygous initiation-codon mutations,[9,32] suggesting that the generation of functional β-globin protein was adversely affected in both the cellular models after erythroid differentiation (Figures 3A-3B(c) and S3.2A-B(b-c)). This results in the minimal HbA0 tetramer formation with a compensatory elevation of HbF level (Figures 3A-3B(d)), which is insufficient to exhibit normal phenotype, as observed in β-thalassemia patients.

For generating an HbE/CD26 (G>A) cellular model, we adopted Cas9/HDR approach as a suitable PAM site with an efficient editing window was not available for BE (Figure S3.1B). We resorted to a two-step HDR approach as the low editing efficiency after the first treatment did not yield any homozygous clones (Figures 3C(a-b), S3.1E, and S3.2C(a)). Mutation at the CD26 (G>A) has been reported to activate a cryptic splice site at CD25 which could cause aberrant intron splicing and decrease the *HBB* expression.[35,36]

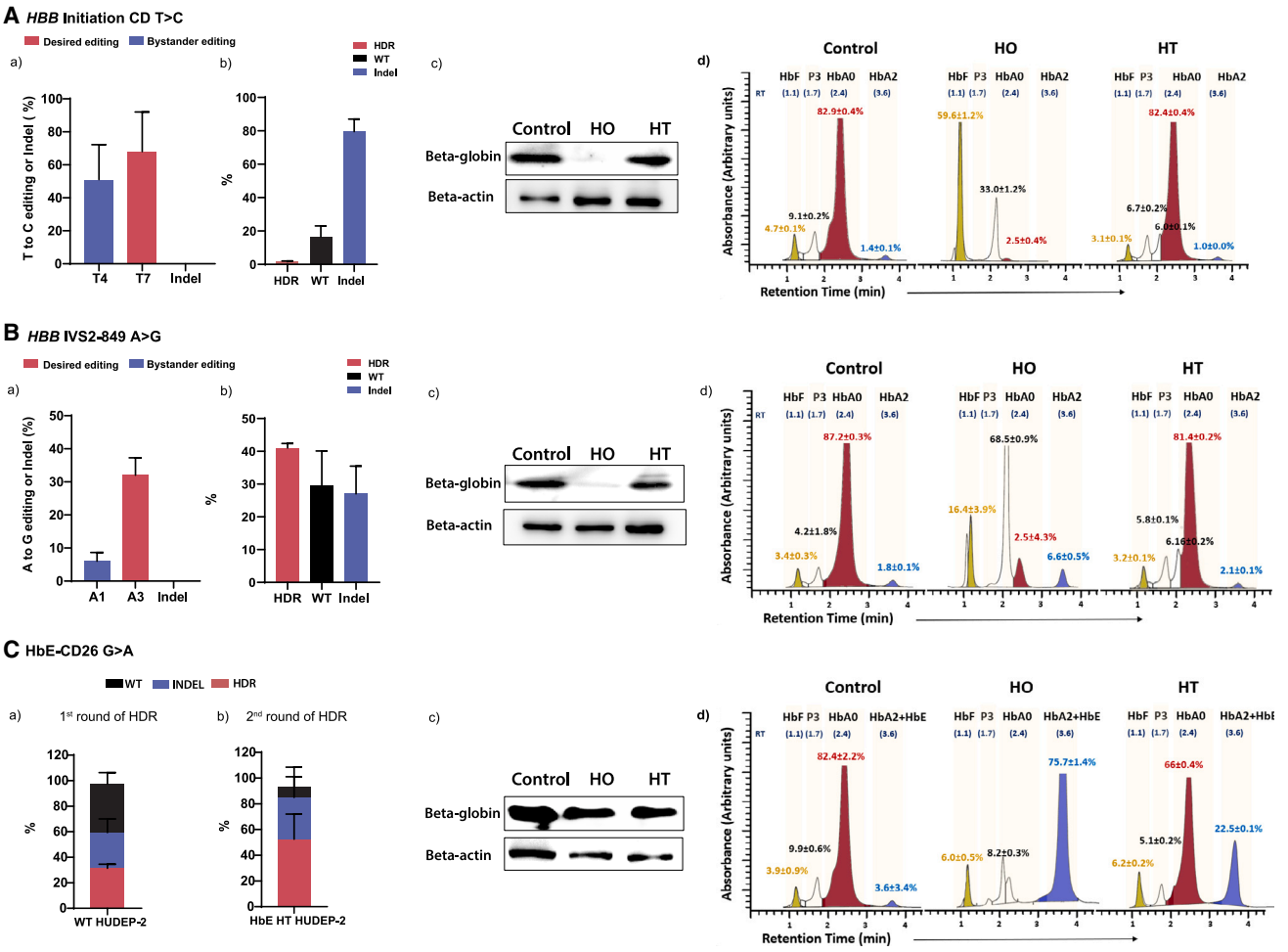


Figure 3. Creation of initiation codon (T>C), IVS2-849 (A>G), and HbE/CD26 (G>A) pathogenic mutations in human erythroid cells using a base editor and Cas9

(A-C) Genotypic and phenotypic characterization of the created individual cellular model of initiation codon (T>C) (A), IVS2-849(A>G) (B), and HbE/CD26(G>A) (C) mutation. The editing efficiency for the creation of desired mutations using base editor (a) and CRISPR-Cas9 mediated HDR (b) for (initiation CD (T>C) and IVS2-849 (A>G)) were evaluated by Sanger sequencing. Similarly editing efficiency for two rounds of HDR for creation of HbE/CD 26 (G>A) (C(a-b)), was evaluated by Sanger sequencing. The homozygous and heterozygous mutant clones with the desired mutation were pooled and then validated by western blot for β-globin protein expression (c) and hemoglobin variant analysis using HPLC (RT-Retention Time) (d). HO-Homozygous, HT-Heterozygous. Data are expressed as mean ± SEM from triplicates.

However, Sanger sequencing of the cDNA from homozygous clones for the CD26 (G>A) mutation revealed normal splicing of exons without any marked difference in β-globin chain expression after erythroid differentiation (Figures 3C(c) and S3.2C(b-c)). Further, cells with either mono- or bi-allelic CD26 (G>A) mutations produced the HbE variant, with no change in the level of HbF, unlike the other two cellular models of β-thalassemia (Figure 3C(d)). These data suggest that pathogenesis associated with HbE/CD26 (G>A) mutation is more likely due to functional change of HbE rather than impaired β-globin expression.

To further assess the efficacy of the base editor across different cell lines, we established a β^0-thalassemia disease model (initiation codon (T>C) and IVS2-849 (A>G)) in K562 cells. Following the editing of K562 cells, we observed a similar editing pattern to that of HUDEP-2 cells (Figures S3.1F-G), suggesting that the base editor has the potential to effectively edit various cell lines.

Together, these results suggest that BEs are highly efficient in the precise creation of cellular disease models for β-thalassemia mutation compared to the HDR-based approach in human erythroid cells but might be of limited application at certain sites due to the lack of availability of suitable editing window. The created cellular models with homozygous and heterozygous mutations can provide molecular insight into the pathophysiology of β-thalassemia. Furthermore, these β-thalassemia cellular models can now be used for the optimization of genome editors mediated correction followed by functional validation of HbA0 tetramer (α2β2) restoration.

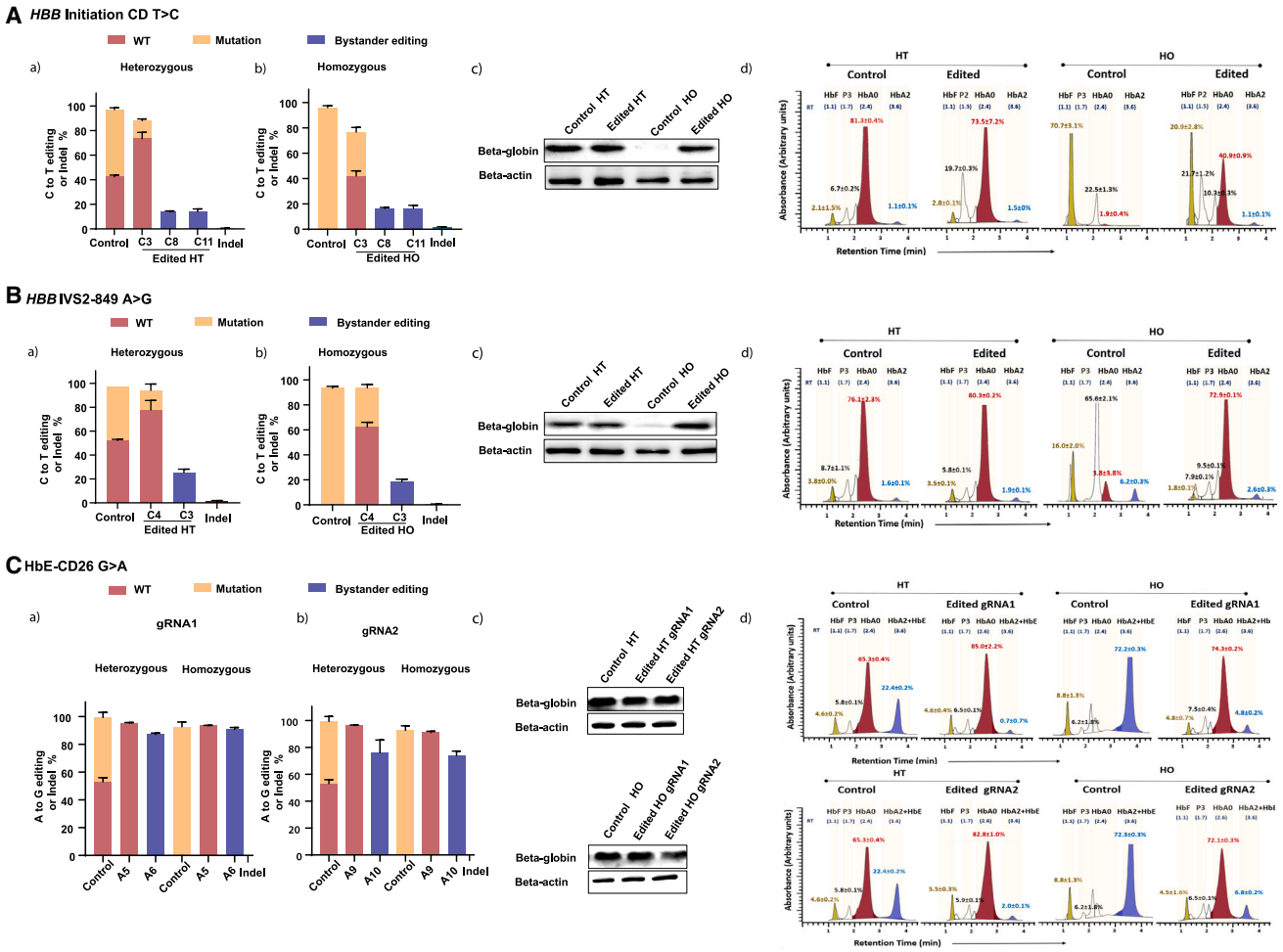


Figure 4. Correction of initiation codon (T>C), IVS2-849 (A>G), and HbE/CD26 (G>A) pathogenic mutations in human erythroid cells using base editors

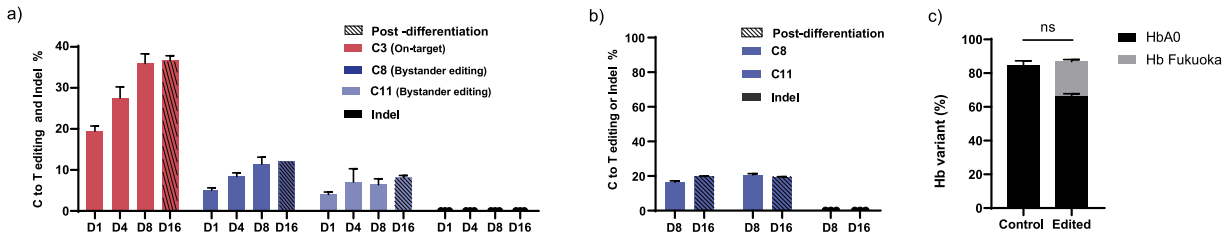
(A–C) Genotypic and phenotypic characterization for the individual cellular models of initiation codon (T>C) (A), IVS2-849 (A>G) (B), and HbE/CD26 (G>A) (C) after correction of relevant point mutation with the base editor. The efficient correction of point mutations (red) along with undesired bystander edits (blue) in heterozygous (a) and homozygous (b) cellular disease models are represented. Immunoblot shows the expression of the β-globin chain (c). Chromatograms represent the functional restoration of adult hemoglobin (RT-Retention Time) (d) in the relevant cellular models after erythroid differentiation. Control-AAVS1 transduced cells, HO-Homozygous cellular model, HT-Heterozygous cellular model. Data are expressed as mean ± SEM from triplicates.

Correction of mutations in human β-thalassemia/HbE cellular models using BEs

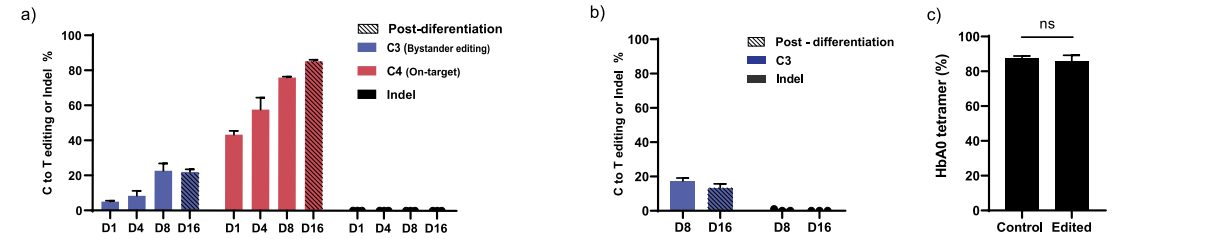
After creating the cellular disease model, we evaluated the BE potential in the native locus for correction of these point mutations and restoration of β-globin synthesis. The correction potential for initiation codon (T>C) point mutation was evaluated by lentiviral delivery of CBE along with respective sgRNA (Figure S4A(a)). Consistent with the preliminary screening, we observed 33% and 42% conversion in heterozygous and homozygous models for initiation codon mutation, respectively, with minimal bystander edits at the adjacent codons and no indels detected (Figure 4A(a-b)). Additionally, the formation of the corrected *HBB* mRNA transcription was detected by sequencing the cDNA (Figure S4A(b)). In contrast to the truncated β -globin generated in the initiation codon mutated cells, the efficient correction of the mutation enabled the synthesis of wild type *HBB* protein after erythroid differentiation[32] (Figures 4A(c) and S4A(c)). Despite the possibility that the observed bystander edits could result in Hb-Fukuoka and a silent mutation, we observed an average of 39% increase in the HbA0 restoration with a corresponding decrease in the expression of HbF in the edited homozygous cells compared to control which could be therapeutically sufficient to alleviate the severity of the disease (Figure 4A(d)). Additionally, we observed an increase in the peak (P2) both in edited heterozygous and homozygous cells in comparison to control cells, which we expect corresponds to Hb-Fukuoka.

In parallel, we performed CBE-mediated correction of *HBB* IVS2-849 (A>G) point mutation in the generated cellular model by transduction with respective sgRNA (Figure S4B(a)). The efficient editing

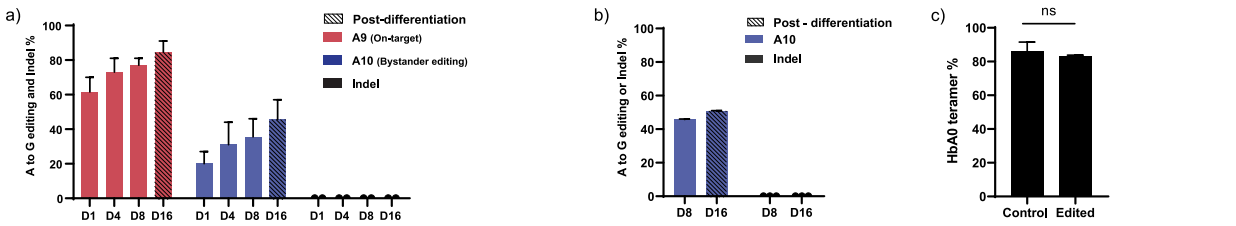
A ***HBB*** **Initiation CD T>C**



B ***HBB*** **IVS2-849 A>G**

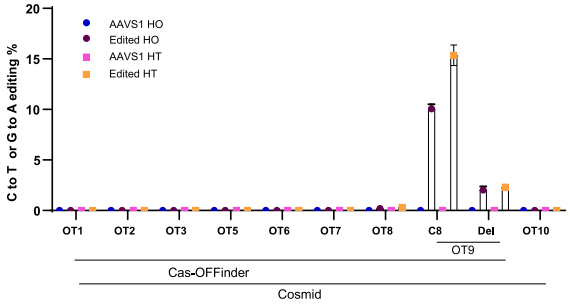


C **HbE-CD26 G>A**

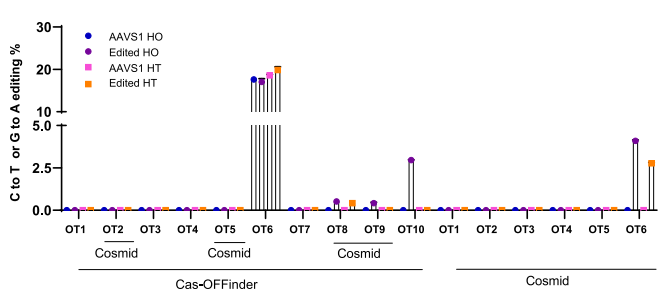


D **Evaluation of Off-target**

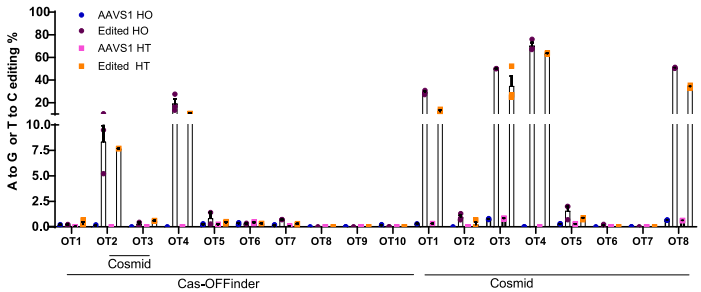
a) ***HBB*** **Initiation Codon T>C**



b) ***HBB*** **IVS2-849 A>G**



c) **HbE-CD26 G>A**



(legend on next page)

window allowed for cytosine deamination at both the targeted base "C" at IVS2-849 and the adjacent non-targeted base at the IVS2-850 position. Surprisingly, we observed a higher on-target conversion while editing this region in the native locus than what was noted in the designed *HBB* construct, with a minimal bystander impact (Figure 4B(a-b)). Although the bystander effect accounted for roughly 18% of total editing that could influence the consensus sequence at the splice junction ("GT-AA"), the remaining edited cells with the desired sequence ("GT-AG") could have contributed to efficient translation into the normal β-globin chain, without the retention of intron-2 (Figures 4B(c) and S4B(b-c)). The precise correction of splice site mutation led to 73% restoration of the HbA0 expression in homozygous condition (Figure 4B(d)). These results suggest that the higher on-target correction of desired mutation compared to bystander edit at splice junction could be sufficient in clinical settings to correct disease phenotypes.

Further, we also attempted to reverse the HbE genotype in the HUDEP-2 (ABE8e stable) cells carrying the *HBB*/CD26 (G>A) mutation by transduction with suitable sgRNAs (Figure S4C(a)). Due to the presence of two adjacent adenine bases at *HBB* CD26, we designed two different sgRNAs based on the position of the PAM that altered the position of target and bystander conversion-sgRNA1 (A5, A6) and sgRNA2 (A9, A10). Interestingly, a minimal reduction in bystander conversion was observed with the alternative sgRNA2 in both homozygous and heterozygous population, with a mild reduction in efficiency of correction at the HbE mutation (Figures 4C(a-b) and S4C(b)). Moreover, the produced bystander edits resulted in Hb-Aubenas variants (*HBB* CD26 (Glu>Gly)) which is reported to exhibit a normal phenotype when heterozygous.[32] In addition, the level of β-globin chain expression was not altered after the correction of a structural HbE/CD26 (G>A) mutation in erythroid differentiated cells, which is consistent with our previous results (Figures 4C(c) and S4C(c)). However, evaluation of edited cells through hemoglobin variant analysis showed a significant reduction in HbE variant levels with a concomitant increase in Hb-Aubenas variant expression as expected (Figure 4C(d)). These results suggest that alteration of the target base position within the editing window by selecting the suitable sgRNA has the potential to reduce the undesired conversions mediated by the BEs during the correction of HbE mutation.

Altogether, by using β-thalassemia cellular models that mimic the genotype of actual patient samples, we successfully demonstrated the BE-mediated correction of β-thalassemia and HbE mutations at coding and non-coding region of *HBB*.

The effect of bystander edits on *HBB* gene expression

As bystander edits were present in all base edited samples, we sought to evaluate the functional consequences of these bystander variants. Upon analyzing the editing pattern over several days, we observed that no pathological variants were generated in any of the samples, except for the sgRNA targeting IVS2-849. In these samples, we noticed a gradual increase in the frequency of bystander edits and an increase in on-target editing during the correction of all three mutations (Figure 5A(a)-C(a) and S5A(a-c)). The persistence of bystander variants until the end of differentiation suggests that there are no survival disadvantages associated with these mutations.

Although the generated mutations are mostly non-pathogenic, and their proportion is relatively low, we conducted further evaluation to determine if these variants had any effect on β-globin production. To address this, we introduced bystander edits in wildtype HUDEP-2 cells at frequencies comparable to those generated after editing our HUDEP-2 disease models, and then analyzed the effect on HbA0 formation (Figure S5B(a-c)). As expected, two mutations were generated during the correction of initiation codon (T>C) mutation, CD2- Hb-Fukuoka and CD3-silent mutation (Figures 5A(b) and S2.1C). This resulted in a slight decrease in HbA0 levels and a concomitant increase in unknown hemoglobin variant (P2 peak) (Figures 5A(c) and S5C). To confirm if the resulting unknown hemoglobin variant is indeed Hb-Fukuoka, we obtained single-cell clones with individual mutations (CD2- Hb-Fukuoka and CD3-silent mutation) and evaluated the produced hemoglobin variants. The resulting chromatogram confirmed that the additional hemoglobin variant (P2 peak) corresponds to Hb-Fukuoka but its frequency relatively low in the pool of edited cells (Figures S5C and S5D). Surprisingly, the IVS 2–850 mutation, created by the gRNA targeting IVS2-849 (A>G) was expected to affect mRNA splicing, but did not show any significant alteration in adult hemoglobin production, possibly due to its low frequency (∼17%) (Figures 5B(b-c) and S5E). Subsequently, the Hb-Aubenas variant generated by bystander editing with gRNA targeting HbE mutation could not be discerned due to its similar retention time with HbA0[37] (Figures 5C(b-c) and S5F). Considering that the bystander edits are produced at a lesser frequency than the on-target conversion and that the generated hemoglobin variants are non-pathogenic

Figure 5. Evaluation of the effects of bystander edits on *HBB* expression and off-target editing associated with base editor during the correction of various β-thalassemia/HbE mutations in human erythroid cells

Genotypic and phenotypic characterization upon the creation of bystander edits with the respective sgRNAs for initiation codon (T>C) (A), IVS2-849 (A>G) (B) and HbE/CD26 (G>A) mutations (C). Bar plot represents the efficiency of bystander conversion along with the correction of on-target point mutations on different days of pre- and post-differentiation in homozygous cellular model using base editors (a). Base conversion percentage of bystander edits generated by each of the sgRNAs targeting β-globin gene in wild type HUDEP-2 cells expressing base editors evaluated by Sanger sequencing (b) and its effect on hemoglobin production analyzed by HPLC on day 8 of HUDEP-2 cell differentiation (c) (unpaired t test). (D) The percentage of base conversions at the top-10 in silico predicated Cas9-dependent DNA off-target sites during the correction of initiation codon (T>C) (a), IVS2-849 (A>G) (b) and HbE/CD26 (G>A) (c) mutations analyzed using targeted amplicon sequencing. Control-AAVS1 transduced cells; HO, Homozygous cellular model; HT, Heterozygous cellular model. Data are expressed as mean ± SEM from triplicates.

variants, our results support that the BE is a viable approach for the therapeutic correction of β-thalassaemia mutations.

Analysis of off-target effects and chromosomal deletions between *HBB* and *HBD* in β-thalassemia/HbE cellular models after correction using BEs

Although BEs reduce genotoxicity induced by DSB, sgRNA-dependent off-target editing is inevitable. Hence, investigating the off-target editing sites for the sgRNAs employed in correcting initiation codon (T>C), IVS2-849 (A>G), and HbE/CD26 is required before proceeding to clinical settings. We used two *in silico* prediction tools, Cas-OFFinder and Cosmid with default settings including up to three mismatches, to identify the top 10 off-target sites in the human genome (Tables S4 and S5). Between the two computational tools, few of the top nominated off-target sites overlapped. We performed targeted amplicon sequencing to assess base conversion at the off-target sites in the genomic DNA from edited cells. Among the predicted off-target sites for the sgRNAs used in the correction of initiation codon (T>C), we observed 15% editing at the *HBD* region (OT9), due to high homology with *HBB* gene (Figure 5D(a)). In case of an IVS2-849 targeting sgRNA, we observed ∼ 2%–4.5% editing in the pseudo gene (Cas-OFFinder OT10 and Cosmid OT6). HbE-sgRNA2 displayed unexpected edits in the pseudogenes (Cosmid OT3 and OT8), intergenic (Cosmid OT1) and intronic regions of GDF6 (Cas-OFFinder OT2), SALC25A48 (Cas-OFFinder OT4) and TBL1X (Cosmid OT4) genes respectively (Figure 5D(b-c)). Notably, no alterations in coding sequence were observed except for the off-target editing in *HBD*, which is generally not highly expressed relative to the other adult β-globin gene *HBB*. Therefore, the sgRNAs used for editing the initiation codon (T>C), IVS2-849 (A>G) and HbE/CD26 mutation have no clear detrimental off-target editing propensities. Further we speculate that sgRNA-independent off-target editing using ABE7.10, ABE8e (A-to-I conversion) and CBE (C-to-U conversion) edited cells would be minimal as previously reported.[38–40]

Due to the high homology between *HBB* and *HBD* genes,[41,42] we further evaluated the editing and deletion events in the *HBD* gene within β-thalassemia/HbE cellular models after the correction of corresponding mutations (Figure 6A). We hypothesized that simultaneous nicking by BEs at both loci might result in the deletion of *HBD* (∼7.4 kb), similar to what occurs at the highly homologous *HBG* locus. Interestingly, our findings revealed that following BE correction of the initiation codon T>C variant, *HBD* editing (determined by Sanger sequencing) and deletion (determined by the presence of a PCR product only formed after deletion between the target and off-target sites in *HBB* and *HBD*) had occurred (Figures 6B(a-b) and 5D(a)). Conversely, no *HBD* editing or deletion was observed for the IVS2-849 (A>G) mutation, possibly due to the consecutive presence of three mismatches in the *HBD* target site (Figure 6C(a-b)). When assessing the HbE (G>A) cellular model, we observed *HBD* gene editing and deletion following the correction of the mutation using sgRNA1 and sgRNA2 (Figure 6D(a-b)). These findings shed light on the potential implications of base editor mediated editing and deletion events within the highly homologous *HBB* and *HBD* genes in β-thalassemia/HbE cellular models.

Therapeutic correction of HbE/CD26 (G>A) mutation in severe β^0-thalassemia patient cells using adenosine base editor

To determine the therapeutic potential of BE to correct the HbE/CD26-mutation, two severe, plerixafor-mobilized, β^0-thalassemia patient's cells with the heterozygous genotypes (β^0/β^E) (HbE (G>A)/IVS1-5 (G>C)) were edited independently. These patient cells were nucleofected with ABE8e-mRNA and sgRNA2. After five days of nucleofection, we noticed a reduction in HbE alleles for both the patient samples (Figure 7A). To our surprise, we noticed a significant reduction in the proportion of bystander edits in patient cells compared to erythroid cell line, which might be due to the transient delivery of BE components.[43]

Even though the presence of the HbE variant alone does not result in a major clinical phenotype, its coexistence with other β-thalassemia mutations leads to increased disease severity, possibly due to the inability of the produced structural variant (HbE) to compensate for the loss of β-globin caused by the mutation in the other allele. Therefore, we assessed the concomitant reversal of phenotypic manifestations after the correction of HbE variant in patient cells that were *in vitro* differentiated into erythroblasts. We first evaluated the erythroid maturation and enucleation potential of the base edited cells. The expression of the terminal erythroid-differentiation markers and the percentage of enucleation was higher in HbE-corrected cells compared to the control (AAVS1 edited cells) (Figures 7B and 7C), suggesting that erythroid differentiation is stabilized after the correction of HbE variant. We noticed a decrease in the mean fluorescence intensity (MFI) of DCF in the edited samples, demonstrating a reduction in ROS production (Figure 7D). Furthermore, HPLC analysis showed a remarkable shift in the expression of hemoglobin from HbE to HbA0 in both patients without any alteration in HbF levels. The average production of HbA0 in these patients was around 13.2% (average from both patients) before base editing, which after correction increased to about 31%, accompanying the reduction of HbE (Figure 7E). During the correction of the HbE G>A mutation in patient HSPCs, we noticed editing in the *HBD* gene, accompanied by a slight decrease in *HBD* gene content, which mirrors our observations in the cellular models (Figures S6A and S6B); however, the editing frequency was low because of mismatch in gRNA sequence due to absence of mutation in *HBD* region. Overall, our data suggest that the BE-mediated precise correction of HbE/CD26 using the optimized sgRNA and BE variant in severe $\beta^0\beta^E$ patient cells efficiently restores the production of HbA0 to a therapeutically relevant level.

DISCUSSION

Genome editing approaches have become an attractive strategy for the treatment of β-thalassemia as even partial correction of disease-causing mutations can be sufficient to restore functional hemoglobin production and ameliorate disease severity. Most of the current studies rely on Cas9-dependent HDR-mediated direct correction of

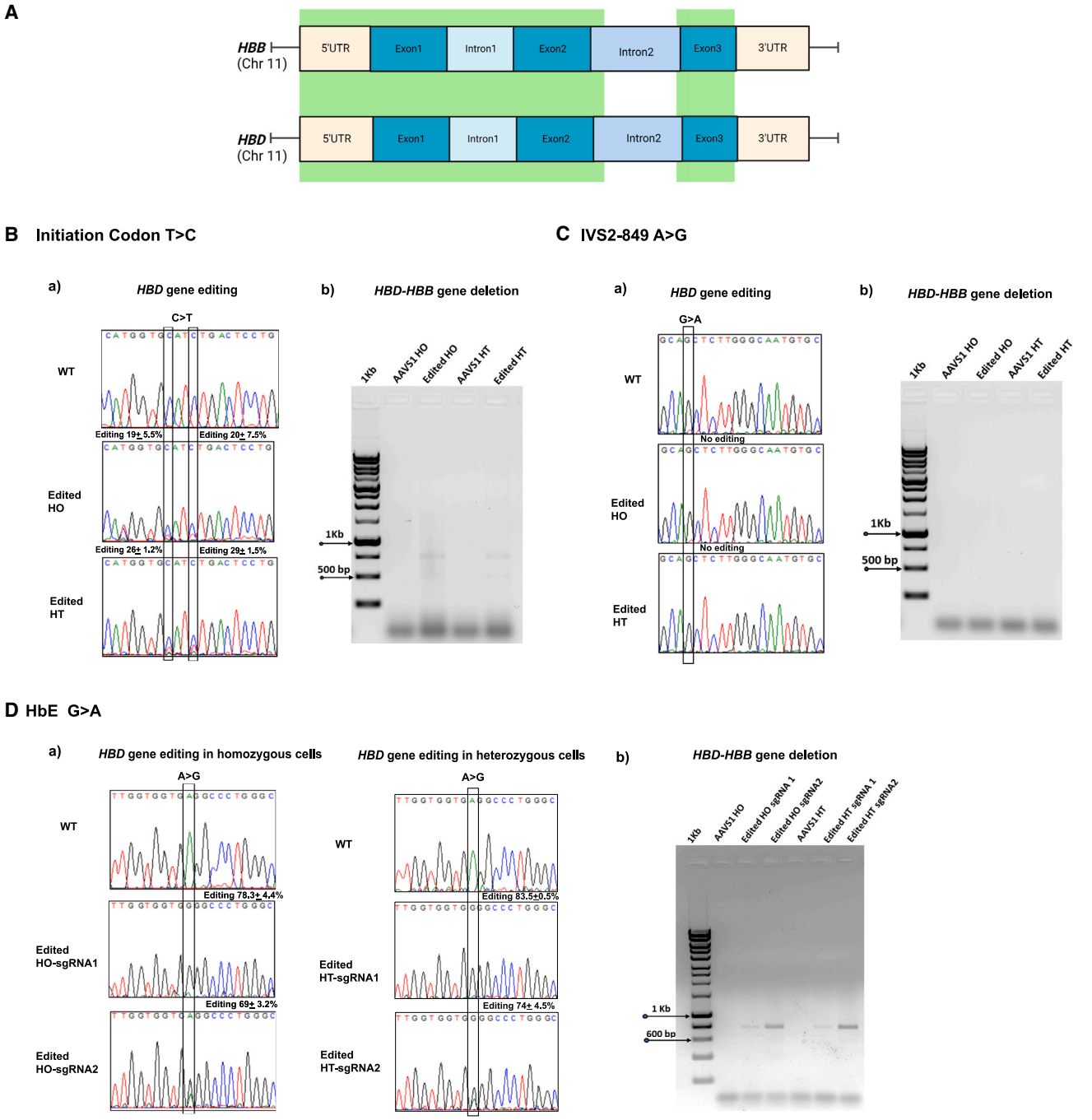


Figure 6. Validation of *HBD-HBB* gene integrity in the β-thalassemia/HbE cellular model after base editor meditated correction of relevant point mutations

(A) Schematic illustration of the highly homologous *HBB* and *HBD* gene (highlights in green color represents the homologous regions between two genes). Chromatogram representing the editing of *HBD* gene obtained by Sanger sequencing (a) and agarose gel electrophoretogram showing the deletion of *HBD-HBB* gene confirmed by PCR amplification (b) in the cellular models of initiation-codon (B), IVS2-849 (C), and HbE/CD26 (D); the presence of band at 788 bp indicates the deletion of the *HBD* gene. HO, Homozygous cellular model; HT, Heterozygous cellular model. Data are expressed as mean ± SEM from triplicates.

various β-thalassemia mutations.[10–12,44,45] The major drawback of the CRISPR-Cas9 HDR system for disease correction is its relative inefficiency in quiescent cells and the creation of large unintended deletions and chromosomal-level alterations due to the DSBs.[19] Moreover, indels at the coding region of the β-globin locus might lead to severe β^0-thalassemia phenotypes.

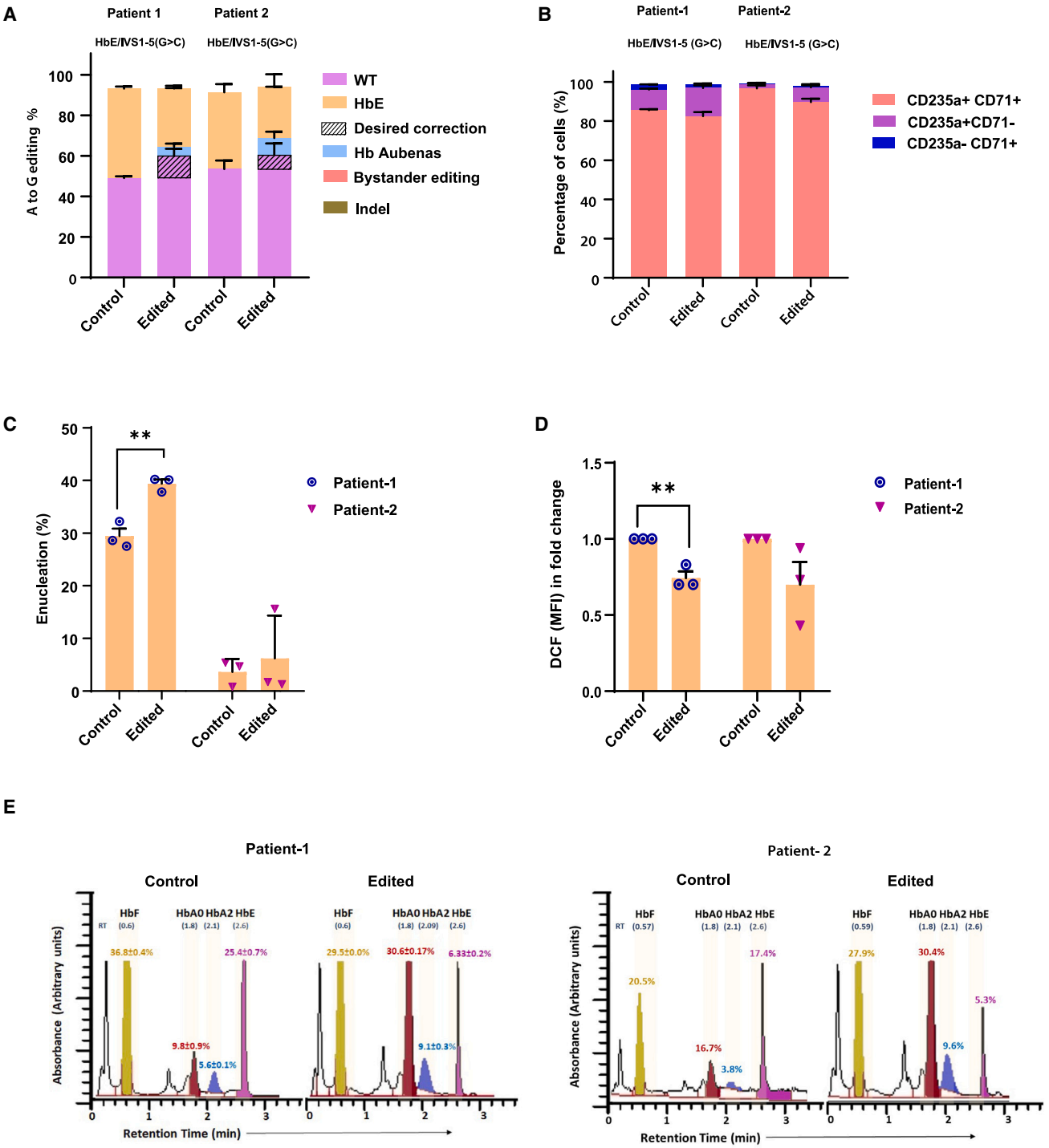


Figure 7. Therapeutic correction of HbE/CD26 (G>A) point mutation in severe β^0/β^E -thalassemia patient cells using adenosine base editor

(A) The base correction efficiency for HbE mutation in edited patient cells after 5 days of nucleofection analyzed by NGS (Patient-1 $n = 3$; Patient-2 $n = 3$). (B) Erythroid-differentiation (CD71+/CD235a+) and (C) maturation (NucRed-CD235a+) potential of edited cells analyzed by flow cytometry on the final day of erythroid differentiation (unpaired t test; nonparametric t test). (D) Change in ROS production was determined by the fold change in the DCF mean fluorescence intensity (MFI) in HbE mutation-corrected erythroblasts (unpaired t test). (E) Chromatograms representing the restoration of the functional adult hemoglobin variant in erythroblast derived from base edited patient cells analyzed using HPLC (Patient-1 $n = 3$; Patient-2 $n = 1$) (RT-Retention Time). Data are expressed as mean ± SEM from triplicates. Asterisks indicate levels of statistical significance (**$p < 0.01$).

Previous studies have demonstrated the use of BEs to create or correct specific mutations in different genomic loci in various genetic disorders.[46–49] Most studies in β-hemoglobinopathies have demonstrated the therapeutic up-regulation of HbF expression by introducing hereditary persistence of fetal hemoglobin (HPFH)-like mutations or by disruption of HbF repressors using BEs to compensate for the deficient β-globin.[38] Despite this, only a few attempts have been made to address the correction of β-thalassemia point mutations using base editing strategy. The most common *HBB*−28(A>G) promoter mutation that decreases the production of β-globin has been extensively studied for correction using different BE variants in various cell types.[46,50] Additionally, while the manuscript was in preparation, two other articles have shown the direct correction of IVS1-110 and IVS2-654 mutations using adenosine base editor.[51,52]

In our present study, we extended the use of BEs for direct correction of eight β-thalassaemia point mutations across the proximal promoter, exons, and introns of the *HBB* gene for the DSB free editing at the target site. We achieved highly efficient correction of disease-causing point mutations along with the restoration of HbA0 in the β-thalassaemia cellular model and in patients HSPCs, which confirms the potential of base editing for therapeutic gene correction. The selection of BEs offers several advantages over conventional gene editing approaches. First, the BEs enable the generation of point mutations without introducing undesired indels, which is especially advantageous in correcting mutations in the coding regions where the sequence integrity is critical. Second, highly efficient variants like ABE8e enable more than 50% editing in almost all of the evaluated target sites. Further, the disadvantages associated with DNA DSB, such as deletions, inversion, translocations, p53 activation etc could be largely circumvented using BEs.[18,19] Base edited HSPCs have been demonstrated to engraft and persist in animal models over time,[46,47] which will be required for an effective therapeutic applications.

We used an innovative strategy of introducing an engineered *HBB* construct harboring diverse β-thalassemia mutations into human erythroid cells for screening and optimizing BE components. This eliminated the requirement for patient samples or patient-derived cell models such as iPSCs with specific mutations for the validation studies. The acquired editing frequency using this approach was comparable to that of the human β-thalassemia cellular model, indicating that this method is suitable and could be applied for other genetic disorders with a diverse mutation spectrum. We validated editing of eight β-thalassemia mutations (−88 C>T; −28 A>G; initiation CD T>C; CD 15 G>A; IVSI-5 G>A; IVSI-110 G>A; IVS2- 849 G>A; HbE/CD26 G>A). While modeling editing of this lentiviral transgene is a convenient way to assess many targets in one cell line, the outcomes may not perfectly match editing at the endogenous locus because the transgene has an uncontrolled chromosomal integration and may have different epigenetic features relative to the endogenous locus. Thus, preclinical tests at native variants are still important to verify the activity that can be achieved in a clinical context.

Therefore, we demonstrated a simple and efficient approach for generating the human erythroid cellular model harboring specific β-thalassemia point mutations at the endogenous *HBB* locus using BEs. In these models, we validated our optimized editing strategies and achieved the desired correction at a significantly higher frequency than bystander edits in almost all the sites. We demonstrated that these editing strategies led to the production of functional HbA0 hemoglobin both in our HUDEP-2 disease models and in patient (β^0/β^E) samples.

One important consideration when designing BE strategies to correct disease variants is the possibility of bystander editing at other nucleotides within the editing window. Careful selection of BE variants and designing of sgRNA with alternative editing windows can help to overcome this limitation.[53] The *HBB* CD15(G>A) correction illustrates this point where precise 65% on-target editing was obtained with ABE7.10, while the use of the same sgRNA with ABE8e resulted in around 90% editing with bystander editing, which might be due to the expanded editing window or higher processivity. Although we achieved substantial correction of two specific mutations in the *HBB* promoter and three *HBB* mutations in intronic regions, these edits co-occurred with bystander conversion, and they were not reported to cause any disease phenotype other than IVS2-849. Further, efficient base conversion at three exonic mutations was also accompanied by bystander edits. Remarkably, most of these undesired conversions were reported to display normal phenotypes in heterozygous conditions. In this study, we observed off-target editing at the homologous *HBD* site and the presence of deletions between the targeted *HBB* site and the off-target *HBD* site. These *HBB-HBD* deletions result in the loss of part of the *HBD* coding region and the entire *HBB* promoter. The generated hybrid fusion may be expressed at a lower level since it is expressed under the control of the *HBD* promoter which is much less active than *HBB*. However, the level of deletion observed was low and we do not expect this would substantially reduce the effectiveness of a treatment based on these editing strategies.

In conclusion, our study provides compelling evidence for using BEs to create and correct various β-thalassemia mutations in human erythroid cells with high efficiency and precision, thus establishing a promising therapeutic framework to address hereditary monogenic disorders caused by point mutations. As BE-mediated approaches are entering clinical trials, we anticipate that this developed approach could benefit β-thalassemia patients in the near future.

MATERIALS AND METHODS

Cloning of sgRNA and *HBB*-mutant fragment

All sgRNAs (Tables S2 and S3) were designed using SnapGene software and either obtained as synthetic-gRNA (Synthego) or cloned into the lentiviral backbone (Addgene#57822/57823).[38] The *HBB* gene sequence (Gene ID 3043) was retrived from the NCBI website and the details of various β-thalassemia point mutations was obtained from Ithagenes and globin gene server. Among multiple point mutations, we selected the mutations to be incorporated based

on their prevalence and their suitability for correction using BEs. A synthetic gene fragment of *HBB* was designed by incorporating these point mutations at appropriate locations along with suitable restriction site overhangs required for cloning into the plasmid (Table S7) and synthesized by IDT, which was then cloned into Addgene-plasmid#57822 via Gibson-Assembly using HiFi DNA-assembly master-mix (NEB).

Cell culture and flow cytometry analysis

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1× penicillin-streptomycin-glutamine (Gibco). K562 cells were cultured in RPMI (Hyclone) supplemented with 10% FBS and 1× Pen-Strep. HUDEP-2 cells were cultured and differentiated toward erythroid lineage as previously described.[29] β-thalassemia patient blood samples were obtained with informed consent as per clinical guidelines authorized by the Christian Medical College, Vellore Intuitional Review Board. Peripheral blood mononuclear cells (PBMNCs) were isolated from G-CSF mobilized β-thalassemia patient blood samples by density gradient centrifugation (Lymphoprep, STEMCELL Technologies) and directly subjected to three-phase erythroid-differentiation protocol as previously described,[54,55] due to the lower yield. HbF+ cells were estimated as per the previously described protocol.[56] Enucleation of edited patient HSPCs was determined in the differentiated cells by staining with CD235a (BD) and NucRed (Invitrogen). ROS levels in patients HSPCs were estimated by acquiring DCF(2′,7′-dichlorofluorescein) (Inivitrogen) stained cells after 30 min of incubation at 37^0C by flow cytometry. AnnexinV (Invitrogen) and 7AAD (Invitrogen) were used to stain the percentage of early and late apoptotic cells (Table S9). All experiments were performed in BC CytoFLEX-LX and BD FACSCelesta cell analyzer. Single-cell sorting was performed using BD-FACSAriaIII.

Lentivirus production and transduction

The second-generation packaging constructs (Addgene-plasmid #12259/12260) were used for lentivirus production for sgRNAs as well as stable preparation (ABE7.10 (Addgene-plasmid#112675), CBE (Addgene-plasmid#110841), and pLenti-ABE8e-puro vector constructed from ABE8e (Addgene-plasmid#138495)). HUDEP-2 cells were transduced with produced lentivirus as previously described.[38] After 48 hrs, the transduction efficiency of all sgRNAs was measured using flow cytometry for GFP/RFP expression. For stable cellline preparation, the transduced cells were selected using puromycin (1 μg/mL (Sigma-Aldrich)) for 10 days.

Nucleofection

To generate engineered HUDEP-2 cells with a spectrum of β-thalassemia mutations, endogenous *HBB* gene was deleted from HUDEP-2 cells by RNP-complex with two synthego sgRNAs (Table S2) (EnGen Cas9 NLS (M0646M) Cas9:40 pmol; each sgRNA:100 pmol) electroporated into 1×10^6 HUDEP-2 cells using Lonza-4D electroporator (pulse-code: EN138). To generate cellular models for HbE, initiation codon and IVS2-849 using an HDR-based approach, we electroporated an RNP-complex (Cas9: 50 pmole; sgRNA: 100 pmol) along with donor template (Table S8) (100 pmol; IDT) in 1×10^6 HUDEP-2 cells using Lonza-4D electroporator (pulse-code: CA137). For multiplex editing, we simultaneously electroporated two different sgRNAs (50 pmol each sgRNA) targeting β-thalassemia point mutations into 0.6×10^6 engineered HUDEP-2 stable cells harboring spectrum of β-thalassemia point mutations using Lonza-4D electroporator (pulse-code: CA137). For the creation of β-thalassemia mutation cellular models (initiation codon and IVS2-849) and correction of HbE point mutation in patient samples, ABE8e-mRNA was prepared using T7-mScriptStandard-mRNA Production System (CELLSCRIPTTM) using Addgene#138495 as a template. *HBB* initiation codon and IVS2-849 cellular models were generated similarly using respective sgRNA (100 pmole) and ABE8e-mRNA (5 μg) (pulse-code: EN138). The correction of HbE mutation in patient samples was performed in unsorted PBMNCs that were differentiated to erythroid precursors, with HbE-sgRNA2 (100 pmole) and ABE8e-mRNA (5 μg) using Maxcyte-electroporator (pulse-code: HSC-3).

Editing outcomes and off-target analysis

Genomic DNA was isolated using DNA isolation kit (Nucleospin Blood-Macherey-Nagel). HUDEP-2 cells were collected on day 6 of erythroid differentiation for RNA isolation using NucleoSpin RNA kit (Macherey-Nagel) and reverse transcribed to cDNA by using Primescript RT-mastermix kit (Takara) as per the manufacturer's protocol. Genomic DNA/cDNA was amplified with respective primers (Table S6) and subjected to Sanger sequencing. The data were analyzed for base editing efficiency by EditR and indels by Inference of CRISPR-Edits (ICE-Synthego).[57,58] The deletion of *HBB* was confirmed using gel electrophoresis after amplification from genomic DNA with primers using PrimeSTARGXL DNA-Polymerase (Takara) and quantified by qRT-PCR[59] using SYBR-green (Takara). *HBB*-deletion and *HBB*-mutant fragment incorporation was reconfirmed by performing MLPA using SALSA MLPA probe mix P102 *HBB* (MRC Holland). *HBD* gene deletion was confirmed by gel electrophoresis after amplification from genomic DNA with primers (Table S6) using Hot-Start GoTaqDNA polymerase (Promega). The top 10 sgRNA-dependent off-targets identified from Cas-OFFinder and Cosmid were deep sequenced by MiSeq System (Illumina)[32] (Table S4, S5, S10, and S11). The data were analyzed using CRISPResso-2.[60]

Hemoglobin chain and variant analysis

For the globin-chain analysis, 25 μg of lysate from HUDEP-2 cells in erythroid differentiation was used for western-blots analysis using anti-hemoglobin β (1:1,000) and anti-actin (1:1,500) (Santacruz) with anti-mouse IgG HRP-secondary antibody (Invitrogen) (1:5,000). For the total hemoglobin estimation, samples were prepared on the final day of erythroid-differentiation as per published protocols[61] and quantified by VARIANT-II Hemoglobin-Testing System (Bio-Rad) (HUDEP-2 cells) or G11-90SL HPLC analyzer (Tosoh-Bioscience, HLC-723G11) (HbE-patient cells and HUDEP-2 cells).

Statistics

GraphPad-Prism 8.1 was used for all the statistical tests. An unpaired t test (nonparametric t test) was performed to evaluate the statistical significance, and p value < 0.05 was considered significant.

DATA AND CODE AVAILABILITY

NGS data (submission number: PRJNA910509) have been submitted to NCBI-SRA. The entirety of the data can be found either in the main text or the supplemental information. The supporting data for this study can be accessed from the corresponding author upon a reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102205.

ACKNOWLEDGMENTS

We sincerely acknowledge CSCR (a unit of inStem Bengaluru, CMC Campus, Vellore, India) for providing startup funds. Kirti Prasad is supported by Senior Research Fellowship from Indian Council of Medical Research India. Anila George and Nithin Sam Ravi are supported by Senior Research Fellowship from the Council of Scientific & Industrial Research India. Vignesh Rajendiran is supported by Senior Research Fellowship from DBT India. We thank Mrs Sumithra, Mr Neelagandan and Dr Eunice Sindhuvi at the Department of Hematology, CMC, for helping us with HPLC variants; we thank Mr Dhiyaneshwaran.S for helping us in making figures using BioRender.com; Mrs. Sandhiya. G. for technical assistance and Dr. Muthuraman. N. for proofreading the manuscript. We also acknowledge CSCR Core for all instrumentation facilities and support while performing experiments.

The research reported in this work was supported by SERB grant: CRG/2022/006064 (Science and Engineering Research Board, New Delhi, India), DBT grant: BT/PR38392/GET/119/301/2020 and NAHD grant: BT/PR17316/MED/31/326/2015 (Department of Biotechnology, New Delhi, India).

AUTHOR CONTRIBUTIONS

M.K.M. conceptualized and supervised the study; K.P. performed most of the experiment under the guidance of M.K.M.; K.P. and M.K.M. analyzed the data; D.N., A.G., N.S.R., J.P., V.R., and A.A.P. provided experimental support; K.L. and D.N. assisted in screening most prevalent β-thalassaemia mutations; G.M. and Y.P. under the guidance of S.M. assisted in ABE8e mRNA preparation; V.V. under the guidance of S.T. assisted in performing HDR; Y.N. and RK provided HUDEP-2 cells; G.A.N. assisted in predicting off-target sites. A.S. provided patient samples; K.P., L.P., D.N., N.S.R., and M.K.M. prepared all figures; K.P., A.G., D.N., and M.K.M. wrote the manuscript; A.S., R.V.S., S.T., S.M., G.A.N., and P.B. provided intellectual insight and feedback on the data and manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Thein, S.L. (2013). The Molecular Basis of β-Thalassemia. Cold Spring Harb. Perspect. Med. *3*, a011700. https://doi.org/10.1101/cshperspect.a011700.
2. Sanchez-Villalobos, M., Blanquer, M., Moraleda, J.M., Salido, E.J., and Perez-Oliva, A.B. (2022). New Insights Into Pathophysiology of β-Thalassemia. Front. Med. *9*, 880752. https://doi.org/10.3389/fmed.2022.880752.
3. Musallam, K.M., Rivella, S., Vichinsky, E., and Rachmilewitz, E.A. (2013). Non-transfusion-dependent thalassemias. Haematologica *98*, 833–844. https://doi.org/10.3324/haematol.2012.066845.
4. Kountouris, P., Lederer, C.W., Fanis, P., Feleki, X., Old, J., and Kleanthous, M. (2014). IthaGenes: An Interactive Database for Haemoglobin Variations and Epidemiology. de Brevern AG. PLoS One *24*, 9. https://doi.org/10.1371/journal.pone.0103020.
5. Thein, S.L. (2018). Molecular basis of β thalassemia and potential therapeutic targets. Blood Cells Mol. Dis. *70*, 54–65. https://doi.org/10.1016/j.bcmd.2017.06.001.
6. Fucharoen, S., and Weatherall, D.J. (2012). The Hemoglobin E Thalassemias. Cold Spring Harb. Perspect. Med. *2*, a011734. https://doi.org/10.1101/cshperspect.a011734.
7. Spritz, R.A., Jagadeeswaran, P., Choudary, P.V., Biro, P.A., Elder, J.T., deRiel, J.K., Manley, J.L., Gefter, M.L., Forget, B.G., and Weissman, S.M. (1981). Base substitution in an intervening sequence of a beta+-thalassemic human globin gene. Proc. Natl. Acad. Sci. USA *78*, 2455–2459.
8. Hardouin, G., Antoniou, P., Martinucci, P., Felix, T., Manceau, S., Joseph, L., Masson, C., Scaramuzza, S., Ferrari, G., Cavazzana, M., and Miccio, A. (2023). Adenine base editor-mediated correction of the common and severe IVS1-110 (G>A) β-thalassemia mutation. Blood, blood *141*, 1169–1179. https://doi.org/10.1182/blood.2022016629.
9. Atweh, G.F., Anagnou, N.P., Shearin, J., Forget, B.G., and Kaufman, R.E. (1985). Beta-thalassemia resulting from a single nucleotide substitution in an acceptor splice site. Nucleic Acids Res. *13*, 777–790.
10. Wattanapanitch, M. (2021). Correction of Hemoglobin E/Beta-Thalassemia Patient-Derived iPSCs Using CRISPR/Cas9. Methods Mol. Biol. *2211*, 193–211. https://doi.org/10.1007/978-1-0716-0943-9_14.
11. Liu, Y., Yang, Y., Kang, X., Lin, B., Yu, Q., Song, B., Gao, G., Chen, Y., Sun, X., Li, X., et al. (2017). One-Step Biallelic and Scarless Correction of a β-Thalassemia Mutation in Patient-Specific iPSCs without Drug Selection. Mol. Ther. Nucleic Acids *6*, 57–67. https://doi.org/10.1016/j.omtn.2016.11.010.
12. Wattanapanitch, M., Damkham, N., Potirat, P., Trakarnsanga, K., Janan, M., U-Pratya, Y., Kheolamai, P., Klincumhom, N., and Issaragrisil, S. (2018). One-step genetic correction of hemoglobin E/beta-thalassemia patient-derived iPSCs by the CRISPR/Cas9 system. Stem Cell Res. Ther. *9*, 46. https://doi.org/10.1186/s13287-018-0779-3.
13. Dever, D.P., Bak, R.O., Reinisch, A., Camarena, J., Washington, G., Nicolas, C.E., Pavel-Dinu, M., Saxena, N., Wilkens, A.B., Mantri, S., et al. (2016). CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. Nature *539*, 384–389. https://doi.org/10.1038/nature20134.
14. Pattabhi, S., Lotti, S.N., Berger, M.P., Singh, S., Lux, C.T., Jacoby, K., Lee, C., Negre, O., Scharenberg, A.M., and Rawlings, D.J. (2019). In Vivo Outcome of Homology-Directed Repair at the HBB Gene in HSC Using Alternative Donor Template Delivery Methods. Mol. Ther. Nucleic Acids *17*, 277–288. https://doi.org/10.1016/j.omtn.2019.05.025.
15. DeWitt, M.A., Magis, W., Bray, N.L., Wang, T., Berman, J.R., Urbinati, F., Heo, S.-J., Mitros, T., Muñoz, D.P., Boffelli, D., et al. (2016). Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. Sci. Transl. Med. *8*, 360ra134. https://doi.org/10.1126/scitranslmed.aaf9336.
16. Patsali, P., Turchiano, G., Papasavva, P., Romito, M., Loucari, C.C., Stephanou, C., Christou, S., Sitarou, M., Mussolino, C., Cornu, T.I., et al. (2019). Correction of IVS I-110(G>A) β-thalassemia by CRISPR/Cas-and TALEN-mediated disruption of aberrant regulatory elements in human hematopoietic stem and progenitor cells. Haematologica *104*, e497–e501. https://doi.org/10.3324/haematol.2018.215178.

17. Xu, S., Luk, K., Yao, Q., Shen, A.H., Zeng, J., Wu, Y., Luo, H.-Y., Brendel, C., Pinello, L., Chui, D.H.K., et al. (2019). Editing aberrant splice sites efficiently restores β-globin expression in β-thalassemia. Blood *133*, 2255–2262. https://doi.org/10.1182/blood-2019-01-895094.

18. Leibowitz, M.L., Papathanasiou, S., Doerfler, P.A., Blaine, L.J., Sun, L., Yao, Y., Zhang, C.Z., Weiss, M.J., and Pellman, D. (2021). Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nat. Genet. *53*, 895–905. https://doi.org/10.1038/s41588-021-00838-7.

19. Kosicki, M., Tomberg, K., and Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nat. Biotechnol. *36*, 765–771. https://doi.org/10.1038/nbt.4192.

20. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature *533*, 420–424. https://doi.org/10.1038/nature17946.

21. Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I., and Liu, D.R. (2017). Programmable base editing of A·T to G·C in genomic DNA without DNA cleavage. Nature *551*, 464–471. https://doi.org/10.1038/nature24644.

22. Prasad, K., George, A., Ravi, N.S., and Mohankumar, K.M. (2021). CRISPR/Cas based gene editing: marking a new era in medical science. Mol. Biol. Rep. *48*, 4879–4895. https://doi.org/10.1007/s11033-021-06479-7.

23. Liu, Z., Chen, S., Shan, H., Jia, Y., Chen, M., Song, Y., Lai, L., and Li, Z. (2020). Efficient base editing with high precision in rabbits using YFE-BE4max. Cell Death Dis. *11*, 36. https://doi.org/10.1038/s41419-020-2244-3.

24. Kim, Y.B., Komor, A.C., Levy, J.M., Packer, M.S., Zhao, K.T., and Liu, D.R. (2017). Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat. Biotechnol. *35*, 371–376. https://doi.org/10.1038/nbt.3803.

25. Huang, C.Y., Liu, C.L., Ting, C.Y., Chiu, Y.T., Cheng, Y.C., Nicholson, M.W., and Hsieh, P.C.H. (2019). Human iPSC banking: barriers and opportunities. J. Biomed. Sci. *26*, 87. https://doi.org/10.1186/s12929-019-0578-x.

26. Kuang, Y.-L., Munoz, A., Nalula, G., Santostefano, K.E., Sanghez, V., Sanchez, G., Terada, N., Mattis, A.N., Iacovino, M., Iribarren, C., et al. (2019). Evaluation of commonly used ectoderm markers in iPSC trilineage differentiation. Stem Cell Res. *37*, 101434. https://doi.org/10.1016/j.scr.2019.101434.

27. Liu, Z., Lu, Z., Yang, G., Huang, S., Li, G., Feng, S., Liu, Y., Li, J., Yu, W., Zhang, Y., et al. (2018). Efficient generation of mouse models of human diseases via ABE- and BE-mediated base editing. Nat. Commun. *9*, 2338. https://doi.org/10.1038/s41467-018-04768-7.

28. Qi, T., Wu, F., Xie, Y., Gao, S., Li, M., Pu, J., Li, D., Lan, F., and Wang, Y. (2020). Base Editing Mediated Generation of Point Mutations into Human Pluripotent Stem Cells for Modeling Disease. Front. Cell Dev. Biol. *8*, 590581. https://doi.org/10.3389/fcell.2020.590581.

29. Kurita, R., Suda, N., Sudo, K., Miharada, K., Hiroyama, T., Miyoshi, H., Tani, K., and Nakamura, Y. (2013). Establishment of Immortalized Human Erythroid Progenitor Cell Lines Able to Produce Enucleated Red Blood Cells. PLoS One *8*, e59890. https://doi.org/10.1371/journal.pone.0059890.

30. Topfer, S.K., Feng, R., Huang, P., Ly, L.C., Martyn, G.E., Blobel, G.A., Weiss, M.J., Quinlan, K.G.R., and Crossley, M. (2022). Disrupting the adult globin promoter alleviates promoter competition and reactivates fetal globin gene expression. Blood *139*, 2107–2118. https://doi.org/10.1182/blood.2021014205.

31. Martyn, G.E., Wienert, B., Kurita, R., Nakamura, Y., Quinlan, K.G.R., and Crossley, M. (2019). A natural regulatory mutation in the proximal promoter elevates fetal globin expression by creating a de novo GATA1 site. Blood *133*, 852–856. https://doi.org/10.1182/blood-2018-07-863951.

32. Giardine, B., Borg, J., Viennas, E., Pavlidis, C., Moradkhani, K., Joly, P., Bartsakoulia, M., Riemer, C., Miller, W., Tzimas, G., et al. (2014). Updates of the HbVar database of human hemoglobin variants and thalassemia mutations. Nucleic Acids Res. *42*, D1063–D1069. https://doi.org/10.1093/nar/gkt911.

33. El-Beshlawy, A., Mostafa, A., Youssry, I., Gabr, H., Mansour, I.M., El-Tablawy, M., Aziz, M., and Hussein, I.R. (2008). Correction of Aberrant Pre-mRNA Splicing by Antisense Oligonucleotides in β-Thalassemia Egyptian Patients With IVSI-110 Mutation. J. Pediatr. Hematol. Oncol. *30*, 281–284. https://doi.org/10.1097/MPH.0b013e3181639afe.

34. Cai, L., Bai, H., Mahairaki, V., Gao, Y., He, C., Wen, Y., Jin, Y.C., Wang, Y., Pan, R.L., Qasba, A., et al. (2018). A Universal Approach to Correct Various HBB Gene Mutations in Human Stem Cells for Gene Therapy of Beta-Thalassemia and Sickle Cell Disease. Stem Cells Transl. Med. *7*, 87–97. https://doi.org/10.1002/sctm.17-0066.

35. Orkin, S.H., Kazazian, H.H., Antonarakis, S.E., Ostrer, H., Goff, S.C., and Sexton, J.P. (1982). Abnormal RNA processing due to the exon mutation of beta E-globin gene. Nature *300*, 768–769. https://doi.org/10.1038/300768a0.

36. Hirsch, R.E., Sibmooh, N., Fucharoen, S., and Friedman, J.M. (2017). HbE/β-Thalassemia and Oxidative Stress: The Key to Pathophysiological Mechanisms and Novel Therapeutics. Antioxidants Redox Signal. *26*, 794–813. https://doi.org/10.1089/ars.2016.6806.

37. Badat, M., Ejaz, A., Hua, P., Rice, S., Zhang, W., Hentges, L.D., Fisher, C.A., Denny, N., Schwessinger, R., Yasara, N., et al. (2023). Direct correction of haemoglobin E β-thalassaemia using base editors. Nat. Commun. *14*, 2238. https://doi.org/10.1038/s41467-023-37604-8.

38. Ravi, N.S., Wienert, B., Wyman, S.K., Bell, H.W., George, A., Mahalingam, G., Vu, J.T., Prasad, K., Bandlamudi, B.P., Devaraju, N., et al. (2022). Identification of novel HPFH-like mutations by CRISPR base editing that elevate the expression of fetal hemoglobin. Elife *11*, e65421. https://doi.org/10.7554/eLife.65421.

39. Zuo, E., Sun, Y., Wei, W., Yuan, T., Ying, W., Sun, H., Yuan, L., Steinmetz, L.M., Li, Y., and Yang, H. (2019). Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. Science *364*, 289–292. https://doi.org/10.1126/science.aav9973.

40. Jin, S., Zong, Y., Gao, Q., Zhu, Z., Wang, Y., Qin, P., Liang, C., Wang, D., Qiu, J.-L., Zhang, F., and Gao, C. (2019). Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. Science *364*, 292–295. https://doi.org/10.1126/science.aaw7166.

41. Steinberg, M.H., and Adams, J.G. (1991). Hemoglobin A2: Origin, Evolution, and Aftermath. Blood *78*, 2165–2177. https://doi.org/10.1182/blood.V78.9.2165.2165.

42. Moleirinho, A., Seixas, S., Lopes, A.M., Bento, C., Prata, M.J., and Amorim, A. (2013). Evolutionary Constraints in the β-Globin Cluster: The Signature of Purifying Selection at the δ-Globin (HBD) Locus and Its Role in Developmental Gene Regulation. Genome Biol. Evol. *5*, 559–571. https://doi.org/10.1093/gbe/evt029.

43. Zhao, D., Jiang, G., Li, J., Chen, X., Li, S., Wang, J., Zhou, Z., Pu, S., Dai, Z., Ma, Y., et al. (2022). Imperfect guide-RNA (igRNA) enables CRISPR single-base editing with ABE and CBE. Nucleic Acids Res. *50*, 4161–4170. https://doi.org/10.1093/nar/gkac201.

44. Xiong, Z., Xie, Y., Yang, Y., Xue, Y., Wang, D., Lin, S., Chen, D., Lu, D., He, L., Song, B., et al. (2019). Efficient gene correction of an aberrant splice site in β-thalassaemia iPSCs by CRISPR/Cas9 and single-strand oligodeoxynucleotides. J. Cell Mol. Med. *23*, 8046–8057. https://doi.org/10.1111/jcmm.14669.

45. Servatian, N., Abroun, S., Shahzadeh Fazeli, S.A., and Soleimani, M. (2023). Investigating The Correction of IVS II-1 (G> A) Mutation in HBB Gene in TLS-12 Cell Line Using CRISPR/Cas9 System. Cell J. *25*, 176–183. https://doi.org/10.22074/cellj.2022.560725.1118.

46. Zeng, J., Wu, Y., Ren, C., Bonanno, J., Shen, A.H., Shea, D., Gehrke, J.M., Clement, K., Luk, K., Yao, Q., et al. (2020). Therapeutic base editing of human hematopoietic stem cells. Nat. Med. *26*, 535–541. https://doi.org/10.1038/s41591-020-0790-y.

47. Newby, G.A., Yen, J.S., Woodard, K.J., Mayuranathan, T., Lazzarotto, C.R., Li, Y., Sheppard-Tillman, H., Porter, S.N., Yao, Y., Mayberry, K., et al. (2021). Base editing of haematopoietic stem cells rescues sickle cell disease in mice. Nature *595*, 295–302. https://doi.org/10.1038/s41586-021-03609-w.

48. Koblan, L.W., Erdos, M.R., Wilson, C., Cabral, W.A., Levy, J.M., Xiong, Z.-M., Tavarez, U.L., Davison, L.M., Gete, Y.G., Mao, X., et al. (2021). In vivo base editing rescues Hutchinson-Gilford progeria syndrome in mice. Nature *589*, 608–614. https://doi.org/10.1038/s41586-020-03086-7.

49. Ma, S., Jiang, W., Liu, X., Lu, W.-J., Qi, T., Wei, J., Wu, F., Chang, Y., Zhang, S., Song, Y., et al. (2021). Efficient Correction of a Hypertrophic Cardiomyopathy Mutation by ABEmax-NG. Circ. Res. *129*, 895–908. https://doi.org/10.1161/CIRCRESAHA.120.318674.

50. Liang, P., Ding, C., Sun, H., Xie, X., Xu, Y., Zhang, X., Sun, Y., Xiong, Y., Ma, W., Liu, Y., et al. (2017). Correction of β-thalassemia mutant by base editor in human embryos. Protein Cell *8*, 811–822. https://doi.org/10.1007/s13238-017-0475-6.

51. Gaudelli, N.M., Lam, D.K., Rees, H.A., Solá-Esteves, N.M., Barrera, L.A., Born, D.A., Edwards, A., Gehrke, J.M., Lee, S.-J., Liquori, A.J., et al. (2020). Directed evolution of adenine base editors with increased activity and therapeutic application. Nat. Biotechnol. *38*, 892–900. https://doi.org/10.1038/s41587-020-0491-6.

52. Liao, J., Chen, S., Hsiao, S., Jiang, Y., Yang, Y., Zhang, Y., Wang, X., Lai, Y., Bauer, D.E., and Wu, Y. (2023). Therapeutic adenine base editing of human hematopoietic stem cells. Nat. Commun. *14*, 207. https://doi.org/10.1038/s41467-022-35508-7.

53. Antoniou, P., Miccio, A., and Brusson, M. (2021). Base and Prime Editing Technologies for Blood Disorders. Front Genome ed. *3*, 618406. https://doi.org/10.3389/fgeed.2021.618406.

54. Devaraju, N., Rajendiran, V., Ravi, N.S., and Mohankumar, K.M. (2022). Genome Engineering of Hematopoietic Stem Cells Using CRISPR/Cas9 System. Methods Mol. Biol. *2429*, 307–331. https://doi.org/10.1007/978-1-0716-1979-7_20.

55. Psatha, N., Reik, A., Phelps, S., Zhou, Y., Dalas, D., Yannaki, E., Levasseur, D.N., Urnov, F.D., Holmes, M.C., and Papayannopoulou, T. (2018). Disruption of the BCL11A Erythroid Enhancer Reactivates Fetal Hemoglobin in Erythroid Cells of Patients with β-Thalassemia Major. Mol. Ther. Methods Clin. Dev. *10*, 313–326. https://doi.org/10.1016/j.omtm.2018.08.003.

56. Canver, M.C., Smith, E.C., Sher, F., Pinello, L., Sanjana, N.E., Shalem, O., Chen, D.D., Schupp, P.G., Vinjamur, D.S., Garcia, S.P., et al. (2015). BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. Nature *527*, 192–197. https://doi.org/10.1038/nature15521.

57. Kluesner, M.G., Nedveck, D.A., Lahr, W.S., Garbe, J.R., Abrahante, J.E., Webber, B.R., and Moriarity, B.S. (2018). EditR: A Method to Quantify Base Editing from Sanger Sequencing. CRISPR J. *1*, 239–250. https://doi.org/10.1089/crispr.2018.0014.

58. Conant, D., Hsiau, T., Rossi, N., Oki, J., Maures, T., Waite, K., Yang, J., Joshi, S., Kelso, R., Holden, K., et al. (2022). Inference of CRISPR Edits from Sanger Trace Data. CRISPR J. *5*, 123–130. https://doi.org/10.1089/crispr.2021.0113.

59. Li, C., Psatha, N., Sova, P., Gil, S., Wang, H., Kim, J., Kulkarni, C., Valensisi, C., Hawkins, R.D., Stamatoyannopoulos, G., and Lieber, A. (2018). Reactivation of γ-globin in adult β-YAC mice after ex vivo and in vivo hematopoietic stem cell genome editing. Blood *131*, 2915–2928. https://doi.org/10.1182/blood-2018-03-838540.

60. Clement, K., Rees, H., Canver, M.C., Gehrke, J.M., Farouni, R., Hsu, J.Y., Cole, M.A., Liu, D.R., Joung, J.K., Bauer, D.E., and Pinello, L. (2019). CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat. Biotechnol. *37*, 224–226. https://doi.org/10.1038/s41587-019-0032-3.

61. Loucari, C.C., Patsali, P., van Dijk, T.B., Stephanou, C., Papasavva, P., Zanti, M., Kurita, R., Nakamura, Y., Christou, S., Sitarou, M., et al. (2018). Rapid and Sensitive Assessment of Globin Chains for Gene and Cell Therapy of Hemoglobinopathies. Hum. Gene Ther. Methods *29*, 60–74. https://doi.org/10.1089/hgtb.2017.190.