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A Universal Approach to Correct Various *HBB* Gene Mutations in Human Stem Cells for Gene Therapy of Beta-Thalassemia and Sickle Cell Disease

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Key Words. Gene therapy • CRISPR/Cas9 • Gene editing • Beta-thalassemia • Hemoglobinopathies • Stem cells

ABSTRACT

Beta-thalassemia is one of the most common recessive genetic diseases, caused by mutations in the *HBB* gene. Over 200 different types of mutations in the *HBB* gene containing three exons have been identified in patients with β-thalassemia (β-thal) whereas a homozygous mutation in exon 1 causes sickle cell disease (SCD). Novel therapeutic strategies to permanently correct the *HBB* mutation in stem cells that are able to expand and differentiate into erythrocytes producing corrected HBB proteins are highly desirable. Genome editing aided by CRISPR/Cas9 and other site-specific engineered nucleases offers promise to precisely correct a genetic mutation in the native genome without alterations in other parts of the human genome. Although making a sequence-specific nuclease to enhance correction of a specific *HBB* mutation by homology-directed repair (HDR) is becoming straightforward, targeting various *HBB* mutations of β-thal is still challenging because individual guide RNA as well as a donor DNA template for HDR of each type of *HBB* gene mutation have to be selected and validated. Using human induced pluripotent stem cells (iPSCs) from two β-thal patients with different *HBB* gene mutations, we devised and tested a universal strategy to achieve targeted insertion of the *HBB* cDNA in exon 1 of *HBB* gene using Cas9 and two validated guide RNAs. We observed that HBB protein production was restored in erythrocytes derived from iPSCs of two patients. This strategy of restoring functional *HBB* gene expression will be able to correct most types of *HBB* gene mutations in β-thal and SCD. STEM CELLS TRANSLATIONAL MEDICINE *2018;7:87–97*

SIGNIFICANCE STATEMENT

A universal strategy to direct a targeted insertion of the *HBB* cDNA at the endogenous *HBB* gene exon 1 using Cas9 and two validated guide RNAs is presented. This strategy is expected to allow correction of most types of *HBB* mutations and to restore functional *HBB* gene expression for treating β-thalassemia and sickle cell disease. It will likely also be applicable to developing gene therapy strategies for treating other types of recessive monogenic diseases.

INTRODUCTION

Beta-thalassemia (β-thal) and sickle cell disease (SCD), two of the most common genetic diseases, are caused by mutations in the *HBB* gene encoding the postnatal form of the beta subunit of hemoglobin. After birth, hemoglobin tetramers contain two alpha subunits and two beta globins coded by the *HBB* gene that is expressed neonatally and after. Before that, beta globins coded by one of the two *HBG* genes that are expressed during the fetal stage and normally silenced after birth. While a point mutation in codon 6 (GAG > GTG, resulting in substitution of glutamic acid to valine amino acid) in the *HBB* gene creates a SCD trait, various mutations in *HBB* gene resulting in reduced or absent of HBB protein cause β-thal starting in early childhood. Over 200 different types of mutations in the *HBB* gene have been identified in patients with β-thal, which could be located anywhere within the ~1,600 basepair (bp) DNA segment containing the three coding exons, splicing sites, and other regulatory elements [1]. Patients with mutations in both *HBB* alleles that significantly reduce the HBB protein production (called β-thal major or Cooley's anemia) suffer from severe anemia and skeletal abnormalities, and have a high level of mortality or shortened life expectancy if left untreated [1]. Similarly, patients carrying both copies of the SCD

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Received March 21, 2017; accepted for publication August 25, 2017; first published November 21, 2017.

http://dx.doi.org/10.1002/sctm.17-0066

HBB mutation, or a heterozygous SCD mutation plus a copy of a severe β-thal mutation will make dysfunctional HBB protein that impedes hemoglobin functions [1].

Although chronic transfusion of red blood cells and some small molecules ameliorate symptoms of β-thal and SCD patients, it is highly desirable to develop a cure for treating these monogenic diseases due to *HBB* gene mutations. Bone marrow transplantation (BMT) using hematopoietic stem cells (HSCs) from an allogeneic donor with the wildtype *HBB* gene has been explored in the past several decades for treating β-thal and SCD. Although successful in some cases, the BMT technology is limited because of graft-versus-host disease and a lack of immunologically matched donors that are unrelated to the treated patients [2]. An alternative approach is to insert a functional copy of the *HBB* gene into the patient's HSCs followed by BMT. In the past decades, scientists have overcome many hurdles in efficient delivery of a functional copy of the *HBB* gene ex vivo into human HSCs, which will home into patient's marrow, differentiate to erythrocytes and express a high-level of the added *HBB* gene [2, 3]. Currently, the best developed approach of gene therapy for treating β-thal and SCD patients relies on using genome-inserting lentiviral vectors that carry the *HBB* or related *HBG* coding sequence (CDS) plus shortened regulatory elements, inserting them permanently into the genome of autologous HSCs [2–4]. Although ongoing clinical trials will ultimately determine the balance of efficacy and risks for treating β-thal and SCD patients, the uncontrollable nature of lentiviral vector insertion that favors coding regions is always a potential risk especially over a long-term [2–7]. In recent years, scientists moved back to achieve precise genome editing via homology-directed repair (HDR) of a *HBB* mutation, which has been explored since 1985 but with a very low efficiency (10^{-6}) [7, 8].

The recent advents of engineered nucleases that make a double-stranded DNA break (DSB) greatly improved our ability to achieve HDR and other forms of DNA repair and recombination in nontransformed human cells. In addition, the availability of immortalized human stem cells harboring *HBB* mutations with ability to differentiate to erythrocytes significantly accelerates the development of functional correction of *HBB* mutations. Since 2008, it became possible to generate human induced pluripotent stem cells (iPSCs) from β-thal and SCD patients that have unique *HBB* mutations [9–12]. During this time, engineered nucleases such as Zinc Finger Nucleases, Transcription Activator Like Effector Nucleases and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas systems have also been developed to enhance HDR and achieve precise genome editing to correct a *HBB* mutation in iPSCs [6–8]. Although the HDR efficiency is still relatively low (<1%) in nontransformed cells, rare clones of iPSCs after HDR-mediated gene correction can be selected, characterized, and expanded extensively. Aided by validated nucleases targeting specific locations of various specific *HBB* mutations, precise genome correction of the SCD point mutation in exon 1, the TCTT deletion in exon 2, or the IVS2-654 mutation in intron 2 has been achieved when a donor DNA template specific for each type *HBB* mutation is also provided [13–23]. The ease and robustness of the CRISPR/Cas9 system has become the preferred choice in recent years for making a specific DSB in the *HBB* locus and achieving HDR to correct a specific *HBB* gene mutation [17–23].

For future clinical applications of correcting various *HBB* mutations, it is highly desirable to develop a universal strategy to correct most if not all of >200 types of *HBB* mutations by using validated CRISPR guide RNAs and one donor DNA template for HDR. To provide a proof-of-principle, we developed a strategy of using two validated guide RNAs (targeting at the *HBB* exon 1 and 3′-un-translated region (UTR)) and a DNA template providing all the *HBB* CDS. In this way, a HDR event near the guide RNA will provide a functional correction of *HBB* mutations not only in exon 1, but also exon 2 and 3 or any downstream sites. We used iPSC lines from two transfusion-dependent β-thal patients with *HBB* mutations in exon 2 and intron 2 as well as an exon 1 mutation to test this new and more universal strategy. To provide a simple readout, we linked a GFP reporter gene downstream to the *HBB* coding cDNA via the 2A self-cleaving peptide so that the GFP reporter expression is indicative of the HBB expression from the same transcript and pro-peptide. Our data provide evidence that this universal approach is able to correct various *HBB* gene mutations and restore HBB protein production. In addition, it provides an experimental system to screen bioactive molecules and to improve HBB protein expression in iPSC-derived erythrocytes based on coexpression of GFP reporter.

Materials and Methods

Human iPSC Generation and Differentiation

The β-thal iPSC lines, BH1 and BH2, were generated by a methodology under feeder-free and xeno-free culture conditions as previously described [24]. Briefly, peripheral blood mononuclear cells (MNCs) were obtained from the two transfusion-dependent, β-thal major patients in the Third Affiliated Hospital of Sun Yat-sen University in China, with approval from the internal review board on research ethics and informed consents. MNCs were cultured to expand and enrich erythroblasts, which were then reprogrammed via transient expression from three improved episomal vectors expressing the four Yamanaka factor plus the *BCL2L1* (BCL-xL) gene [24]. At day 14 of reprogramming, live fluorescent staining of the TRA-1-60 pluripotency marker on emerging iPSC colonies was performed, and the TRA-1-60+ positive colonies were selected and pooled for further iPSC characterization. The established β-thal iPSC lines were cultured in the Essential 8 (E8) medium (Thermo Scientific, Carlsbad, CA, #A1517001) on tissue-culture plates coated with vitronectin (Thermo Scientific, Carlsbad, CA, #A14700) [25]. The previously established normal iPSC line BC1 (as a normal control) and the TNC1 iPSC line from a sickle cell patient were cultured and differentiated similarly [12, 25]. The differentiation of iPSCs was driven via embryoid bodies (EBs) on 96-well plates in the serum-free medium [18, 25]. Fourteen days after EB formation and hematopoietic differentiation, the formed $CD34^+CD45^+$ hematopoietic stem/progenitor cells (HSPCs) released into medium in suspension were harvested and examined. To generate iPSC-erythrocytes, the HSPCs further underwent erythroid differentiation (ED) for 10 days and terminal maturation (TM) for another 8 days consequently, as previously described [18, 25].

Whole Genome Sequencing of β-Thal iPSC Lines

Genomic DNA from the two patient-derived iPSC lines and their original MNCs were isolated using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, #69504). Library preparation using TruSeq DNA PCR-Free Kit (Illumina, San Diego, CA) and Whole Genome Sequencing on HiSeq X using 2 × 150 bp read length, with a ×30 genome coverage on average, were performed at New York Genome Center (NYGC). Initial alignments of reads to GRCh37 (hg19) reference genome as well as variant calling using

Table 1. Human iPSC lines generated from two β-thalassemia major patients.

	Donor information		
iPSC lines	Sex	Transfusion dependence	*HBB* gene mutation
BH1	M	Yes	β17 (AAG>TAG, rs33986703); β-thal654 (c>t, rs34451549)
BH2	F	Yes	β17 (AAG>TAG, rs33986703); β41–42 (-TCTT)

Abbreviation: iPSCs, induced pluripotent stem cells.

GATK were also performed by NYGC. Nonsynonymous variants in *HBB* gene that have been implicated in thalassemia were confirmed by Sanger sequencing. The results are shown in Table 1.

Targeting the *HBB* Gene with a CRISPR/Cas9 Approach

To perform the endogenous *HBB* gene editing, we used the CRISPR/Cas9 system as previously described [18, 26, 27]. A plasmid expressing humanized SpCas9 protein (Addgene, Cambridge, MA, plasmid #41815) and a guide RNA (gR) expression plasmid (Addgene plasmid #41824) were as previously described [26, 27]. We later, we also used pCas9-GFP (Addgene plasmid #447190) that coexpresses SpCas9 and GFP under the control of CAG promoter. The gR-HBB-a specifically targeting HBB exon 1 was described previously [18] and shown below. The second gR-HBB-UTR (5′-AAACTGGGGGATATTATGA-3′) targets on the 3′ UTR of the *HBB* gene. A donor vector providing a required DNA template for HDR is based on a plasmid (Addgene, #31938) as previously published [28], with additional DNA segments inserted by Gibson assembly. Briefly, it contains a left homology arm (560 bp), a right homology arm (880 bp), an *HBB* coding cDNA sequence followed by a P2A linker, an (e)GFP coding DNA segment, and a loxP-flanked PGK-puromycin selection cassette. A synonymous change at HBB codon 14 (Leucine) from **CTG** to TTA is introduced to the HBB cDNA in the donor vector, to prevent from recut by Cas9 complexed with gR-HBB-a that recognizes the target DNA sequence including **CTG** (5′-GTCTGCCGTTACTGCC**CTG**T-3′). The relevant DNA sequences of the donor vector (HBB-GFP-PGK-puro-V2) are provided as Supporting Information Figure S1.

Gene targeting in various human iPSC lines was conducted as we previously described [18, 27]. Briefly, two million iPSCs were resuspended in 100 μl P3 primary cell solution and mixed with 10 μg DNA containing equal amounts of four plasmids (2.5 μg for each of two guide RNAs, pCas9-GFP, and the donor vector), and then underwent electroporation by 4D Nucleofector (Lonza, Allendale, NJ). The locations of homologous recombination were verified by PCR using primer L1-F and L1-R at 5′ terminal and L2-F and L2-R at 3′ terminal. The positive iPSC colonies with an HDR event were identified by genomic PCR using primer gDNA-75-F and gDNA363-R. To excise the loxP-flanked PGK-puromycin selection cassette, human iPSCs were transfected with the plasmid pCAG-Cre-IRES2-GFP (Addgene, #26646) as done previously [18]. After 3 days of transfection, the GFP positive cells were selected by fluorescence-activated cell sorting on FACSAria II (BD Biosciences, San Jose, CA). The cells were plated in low density for clonal selection. Individual clones were picked and screened for excision by genomic PCR using primer L2-F and V4193-R. The positive colonies with excision were further confirmed by Sanger DNA sequencing [18]. The relevant primer sequences are listed in Supporting Information Table S1.

MiSeq-Based Deep Sequencing of Putative Off-Targets by the Second HBB Guide RNA in iPSCs

The specificity of the second guide RNA gR-HBB-UTR that targets the HBB 3′ UTR and possibly other off-target candidate (OTC) sites in the human genome was validated as we did previously with the first guide RNA gR-HBB-a [18, 27], with minor modifications. The list of the sequences of the on-target HBB and top 19 algorithmically predicted OTC loci were listed in Supporting Information Table S2. The MiSeq data of cutting efficiency and specificity in human iPSCs are summarized in Supporting Information Figure S2A. Additional information of the MiSeq is also provided in the Supporting Information.

Immunocytochemistry

At day 14 of reprogramming, the iPSC cultures were directly incubated with fluorescence (R-PE) conjugated antibody human TRA-1-60 (Miltenyi Biotec, Auburn, CA, #130-100-347, 1:10 dilution) in E8 medium for 1 hour [24]. After thorough washing with phosphate-buffered saline (PBS), the live staining was examined by a fluorescence microscope.

Teratoma Formation as an In Vivo Pluripotency Assay

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from Jackson Laboratory. The animal experiments were approved by IACUC at Johns Hopkins University School of Medicine. The care of all experimental animals was in accordance with institutional guidelines. The iPSCs were treated with TrypLE to generate single-cell suspensions before being tested in vivo by teratoma formation [29]. The cells (5×10^6) were mixed with Matrigel (BD Biosciences, 5 mg/ml) in a final volume of 50 μl, and intramuscularly injected into the hind limbs of 8-week-old NSG mice. The mice were sacrificed 8 weeks after iPSC injection. Teratomas were harvested, fixed for 24 hours in 10% buffered formalin and then examined by a routine wax-embedding histological procedure. The paraffin sections of 5 μm thickness were mounted on slides and stained with H&E. The typical morphologies of endoderm, mesoderm, and ectoderm were observed under microscope (×40 magnifications).

Real-Time Quantitative PCR Analysis

Total RNA was isolated using Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, #R1055), including DNase I (RNase free) treatment to eliminate DNA contamination. The reverse transcriptions were undertaken with the SuperScript III First-Strand Synthesis System (Thermo Scientific, #18080-051) following manufacturer's instruction. Real-time quantitative PCR was performed on StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) with SYBR Green PCR Master Mix reagent (Applied Biosystems, #4309155). The expression levels of relevant genes (*OCT4*, *NANOG*, *HBB*, *HBG*) were quantified, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal standard for normalization. The gene-specific PCR primers are also listed in Supporting Information Table S1.

Flow Cytometric Analysis of iPSCs and Differentiated Hematopoietic Cells

Human iPSCs or derived-EBs were dissociated to into a single-cell suspension by TrypLE (Life Technologies, Carlsbad, CA), and washed with a buffer for FACS (PBS plus 1% FBS and 1 mM EDTA). The cells were resuspended in the FACS buffer, and labeled with

fluorochrome-conjugated anti-human CD34-APC (Miltenyi Biotec, #130-090-954), CD45-PE (Miltenyi Biotec, #130-080-201), CD45-Brilliant Violet 605 (BioLegend, San Diego, CA, #304042), CD235a-Pacific Blue (BioLegend, #349108) antibodies. Isotype-matched control antibodies were used to determine the background staining. The erythrocyte enucleation was evaluated by DRAQ5 staining (Thermo Scientific, #62251, 1:2,000 dilution). To characterize human iPSCs, the primary antibodies anti-human TRA-1-60 (Millipore, Billerica, MA, #MAB4360) and SSEA-4 (Millipore, #MAB4303) were used. Their recognitions were detected by respective secondary antibodies anti-mouse IgM-Alexa Fluor 555 (Thermo Scientific, #A21426) and anti-mouse IgG-Alexa Fluor 555 (Thermo Scientific, #A21422). Flow cytometric analysis was performed on a FACSCalibur or LSR II analyzer (BD Biosciences). Data analysis was performed using FlowJo or FCS Express software.

Western Blotting

The iPSCs and erythrocytes at TM day 8 were lysed with RIPA buffer (Sigma, St. Louis, MO, #R0278) in the presence of protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, #5872S). The protein samples were treated and run with NuPAGE Bis-Tris Mini Gel System (Life Technologies) following manufacturer's instruction. The Western blot was performed with Invitrogen iBlot Dry Blotting System with 7 minutes running time in P3 program. To detect HBB protein production, expression, we used a mouse monoclonal IgG1 (Santa Cruz Biotechnology, Dallas, TX, #sc-21757) as a primary antibody, which specifically recognize HBB but not HBG proteins as previously reported [18]. As a loading control, we also probed the blot after using anti-human GAPDH antibodies (Cell Signaling Technology, #5174S, rabbit IgG). For secondary antibodies used for Western blot detection, we used peroxidase labeled anti-mouse IgG(H + L) (Vector Laboratories, Burlingame, CA, #PI-2000) or anti-rabbit IgG(H + L) (Jackson ImmunoResearch Laboratories, West Grove, PA, #711-035-152), respectively. Positive signals were developed by ECL Primer Western Blotting Detection Reagent (GE Healthcare, Pittsburgh, PA, #RPN2232). The protein expression levels were quantified using Photoshop software based on band area and gray scale.

Statistical Analysis

Experiments were repeated three times. The data were subjected to statistical analysis by unpaired Student's two-tailed *t* test. Results were presented as means ± SD. In all comparisons, $p < .05$ was used as the criterion for statistical significance.

Results

Derivation and Characterization of iPSC Lines from Two Beta-Thal Patients

To develop new models and treatment for β-thal major patients, we derived iPSCs from two patients who need chronic blood transfusion (Table 1). Peripheral blood MNCs were obtained with informed consents and expanded to generate erythrocyte populations. They were used to generate human iPSC lines by transient expression of three episomal vectors as previously described [24]. The derived iPSC lines, BH1 and BH2, from these two patients showed typical morphology and markers of human pluripotent stem cells (Fig. 1). The derived iPSC lines BH1 and BH2 also have a normal karyotype (and confirmed XY and XX genotypes), and are pluripotent by in vivo and in vitro assays (data not shown). Whole genome sequencing reveals that BH1 iPSCs has the codon 17 (β17) mutation in exon 1 (**A**AG > **T**AG resulting in a stop codon; rs33986703) and the IVS2-654 splicing mutation located in intron 2 (rs4451549). For BH2, we found the β17 mutation and a β41–42 mutation in exon 2 (TCTT deletion). The same mutations were also found in patient's MNCs, respectively. We did not detect other mutations in BH1 and BH2 that would be deleterious to cell growth or hematopoietic differentiation. The three types of *HBB* mutations detected were among common mutations of β-thal major in south China [1, 30], resulting in premature termination of the HBB protein. The sequencing data are consistent with patients' clinical phenotypes of β-thal major, which are commonly transfusion-dependent. Sanger sequencing was used to confirm and monitor the compound heterozygous *HBB* mutations present in the BH1 and BH2 iPSC lines (Fig. 1D).

Using the standard protocol to generate HSPCs followed by sequential formation of erythroblasts and erythrocytes [18, 25], we examined the capacity of β-thal BH1 and BH2 iPSCs to form erythrocytes (Fig. 2A). They both had similar capacity to form CD34 + CD45+ HSPCs after 14-day differentiation, as compared to the control BC1 iPSCs (Fig. 2B). After ED (10 days) and terminal differentiation (additional 8 days), both BH1 and BH2 also form erythrocytes that are positive for CD235a (glycophorin A) and negative for CD45 (common leukocyte antigen) (Fig. 2C). However, the BH1 and BH2 derived-erythrocytes lacked HBB protein expression (Fig. 2D). These data are consistent with the transfusion-dependent phenotypes of the BH1 and BH2 patients.

Development of a Universal HBB Gene Targeting Approach

While both BH1 and BH2 patients have the β17 mutation, and the *HBB* gene expression could be restored by correcting this point mutation, there are many patients who have either homozygous IVS2-654 (in intron 2) or β41-42 (in exon 2) mutations, or other forms of *HBB* mutations that severely reduce HBB protein production. In order to develop a universal strategy to correct most of *HBB* mutations found in SCD and β-thal, we tested the scheme shown in Figure 3A. Briefly, we designed a strategy to achieve HDR in the exon 1 of the HBB locus with targeted insertion of the HBB coding cDNA, using a validated guide RNA that we previously used to target the SCD point mutation (codon 6) with a high-level of specificity as well as efficiency [18]. In this way, patients with *HBB* mutations in exon 2, intron 2, or exon 3 in both alleles will benefit from the targeted insertion of the *HBB* CDS as those patients with exon 1 mutations in both alleles. To facilitate testing a universal DNA donor template, we further added a GFP reporter gene downstream to the *HBB* CDS (linked by the P2A element). Another feature of this universal donor template, similar to what we and others have used, is a puromycin-resistance expression cassette allowing selection of rare events of HDR at the *HBB* locus [18, 27, 28].

In the initial experiments, we observed abundant HDR events in iPSCs using transient transfection of plasmids expressing SpCas9 and a guide RNA (gR-HBB-a) as we previously used in targeting SCD iPSCs [18]. However, with this new donor vector that contains HBB CDSs identical to the exon 2 and exon 3 of the endogenous HBB locus, we often observed undesirable recombination events between the donor and the endogenous HBB at these homologous regions. To eliminate this aberrant recombination events, we used an additional guide RNA targeting the 3′ UTR immediately downstream to the stop codon in *HBB* exon 3 (Fig. 3A). We first

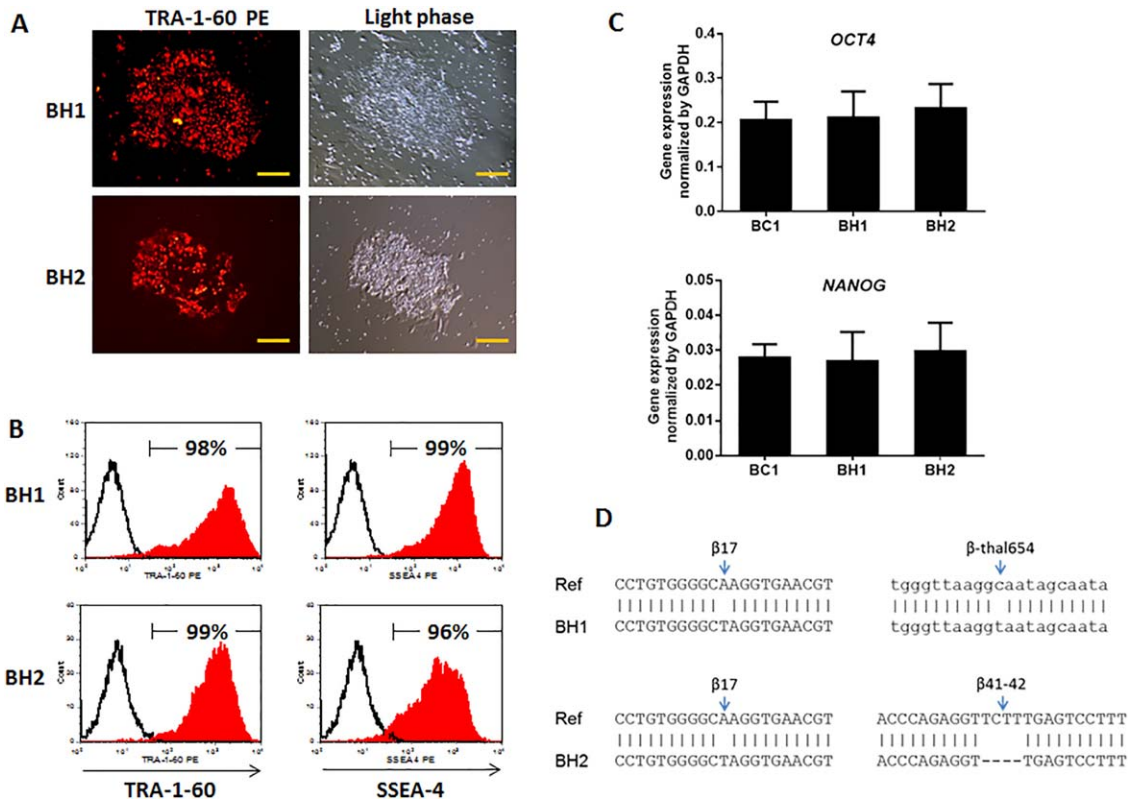

Figure 1. Characterization of BH1 and BH2 induced pluripotent stem cells (iPSCs) generated from beta-thalassemia major patients. **(A):** Live cell staining of iPSCs at reprogramming day 14 under feeder-free and xeno-free culture conditions. The iPSC colonies were incubated with anti-human TRA-1-60-PE in a cell culture incubator for 1 hour. The representative images of live staining were obtained using a fluorescence microscope. The colony morphologies were observed using light phase. Bar size = 100 μm. **(B):** Positive iPS cell colonies of alive TRA-1–60 staining were picked up and pooled to establish BH1 and BH2 human iPS cell lines. The iPSCs were expanded on vitronectin-coated plate in E8 media. The pluripotent stem cell markers, TRA-1-60 and SSEA-4, were measured by flow cytometry and shown in histograms. Black lines represented isotype-matched antibody controls. **(C):** Real-time quantitative PCR analyses of *OCT4* and *NANOG* gene expression in BH1 and BH2 iPSCs. The relative gene expression levels were normalized by housekeeping gene GAPDH. The BC1 iPSCs were used as controls. **(D):** The *HBB* gene mutations in BH1 and BH2 iPSCs were confirmed by Sanger DNA sequencing. β17 and β-thal654 mutations were identified in BH1 iPSCs, while β17 and β41-42 mutations were identified in BH2 iPSCs. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

vigorously tested the efficiency and specificity of this new gR-HBB-UTR in human iPSCs (Supporting Information Fig. S2A), similarly to what we did previously with the gR-HBB-a guide RNA using a transient transfection assay in human iPSCs followed by deep-sequencing [18]. With this improved *HBB*-targeting strategy, we readily obtained clones from both patient-derived iPSC lines and a control iPSC line BC1: either with an insertion (and replacement of the genomic DNA) only at one allele of the *HBB* locus (such as BC1-HBB-GFP #6, BH1-HBB-GFP #2, BH2-HBB-GFP #11) or at both alleles (BC1-HBB-GFP#8), as shown in Figure 3B. These selected iPSC clones were further expanded and characterized to confirm the targeted insertion by standard molecule techniques [13, 18]. The characterizations include the verification of a desirable HDR event in the above-mentioned clones by specific PCR amplification and sequencing at both junctions of the targeted insertion (Supporting Information Fig. S3). We further removed the puromycin-selection cassette flanked by the two loxP sites using a plasmid that transiently expresses Cre in selected iPSC lines (Fig. 3A). The clones of selected iPSC lines with deletion of the puromycin-selection cassette were identified (Fig. 3C). With further characterization of candidates, we identified iPSC clones such as BH1-HBB-GFP-#2 or BH2-HBB-GFP-#11, which have a targeted insertion at a single allele to correct the β17 mutation (Fig. 3D). Similarly, we obtained BC1 iPSC clones with insertion of HBB-GFP sequence at either a single or both alleles of the *HBB* locus (data not shown). In addition, we confirmed that there were no sequence alterations at the 19 possible OTC sites that might be resulted in the gR-HBB-UTR mediated DSB (Supporting Information Fig. S2B).

Characterization of Edited iPSC Clones for HBB and GFP Reporter Gene Expression

We first differentiated the modified BC1 iPSC lines with the HBB-GFP allele to erythrocytes as we did previously [18] with the wild-type BC1 iPSCs (Fig. 4). After the standard three-step differentiation lasting for a total of 32 days, we generated a similar level of erythrocytes that expressed CD235a (>84%). By FACS analysis, the BC1-HBB-GFP #6C2 iPSC line that has a single copy of the HBB-GFP allele showed GFP+ cells at 24%, while the BC1-HBB-GFP-#8C8 clone that have two copies the HBB-GFP allele showed GFP+ cells at 35% (Fig. 4A). Analysis of sorted cell populations based on expression of GFP, which is designed to serve as a reporter for HBB expression, demonstrated that the GFP+ population is enriched for *HBB* gene expression (Fig. 4B). In contrast, GFP– cells are enriched for HBG gene expression. Nearly all the GFP+ cells expressed CD235a but lacked CD45 leukocyte antigen.

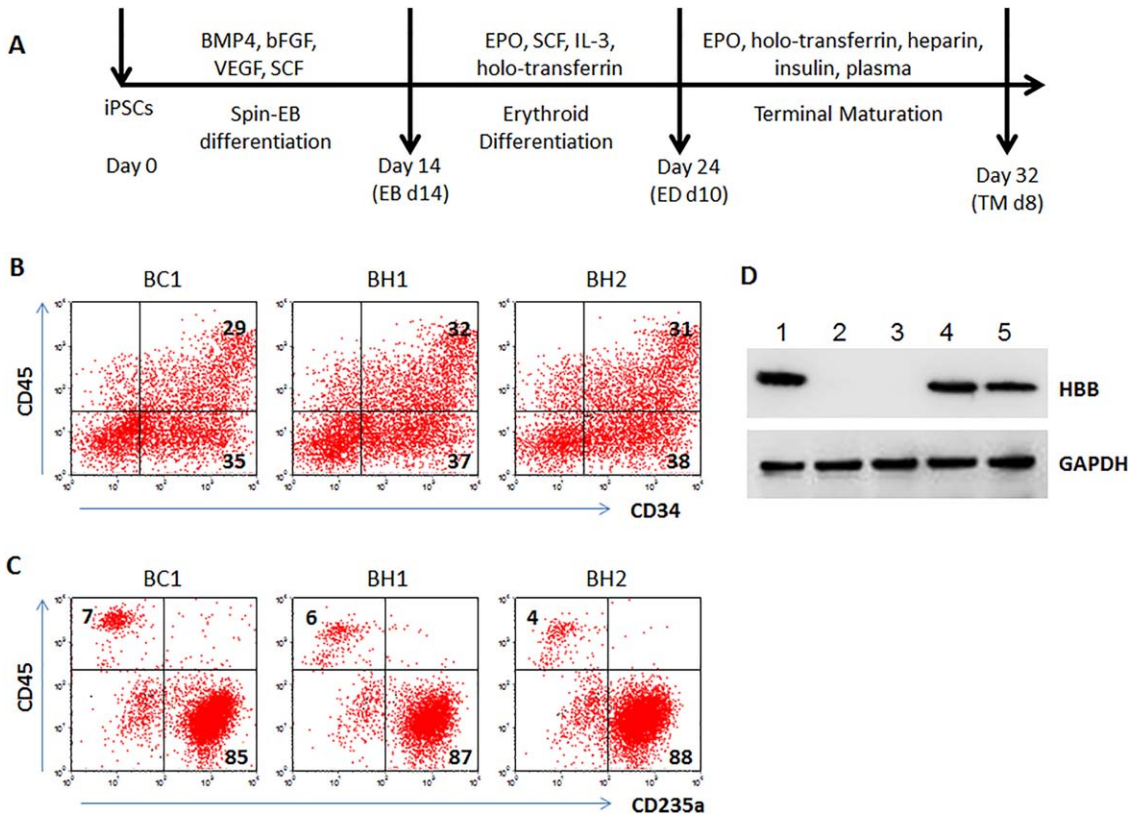

Figure 2. Erythrocyte differentiation of BH1 and BH2 iPSCs. **(A):** Diagrammatic sketch of differentiation procedure. To generate red blood cells, the iPSCs underwent three stages of spin-EB differentiation, erythroid differentiation, and erythrocyte terminal maturation (TM) in feeder-free and xeno-free culture conditions. **(B):** Flow cytometric analyses of hematopoietic stem/progenitor cells ($CD34^+CD45^+$) cells at EB day 14. **(C):** Flow cytometric analyses of erythrocytes ($CD235a^+CD45^-$) at TM day 8. The differentiation derivatives from BC1 iPSCs were used as controls. **(D):** Western blot to detect HBB proteins of erythrocytes from various iPSCs after terminal differentiation. GAPDH was used as loading control. Lane 1: BC1; Lane 2: BH1; Lane 3: BH2; Lanes 4, 5: two other control iPSC lines. Abbreviations: EB, embryoid bodies; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iPSCs, induced pluripotent stem cells.

In addition, most of the GFP+ cells have much reduced DNA staining reflecting an active enucleation process. These data show that iPSCs with the engineered *HBB* (cDNA) allele coupled with a GFP gene indeed rendered GFP expression in terminally differentiated erythrocytes as designed.

We also analyzed the selected clones of BH1 and BH2 iPSCs that have a single copy of the HBB-GFP sequence replacing a mutated β-thal allele. After the terminal differentiation (TM) to generate erythrocytes, we observed cell populations that expressed CD235a at a high level (Fig. 5A). We analyzed the HBB protein directly by Western blotting, using levels of the housekeeping GAPDH protein in various samples as a loading control (Fig. 5B). The differentiated erythrocytes from the parental BH1 and BH2 iPSCs derived from two β-thal major patients did not show HBB protein production even after using the same TM procedure (Fig. 5B, 5C). However, HBB protein production is restored in the corrected BH1 and BH2 cells (with the HBB-GFP allele).

Discussion

In this report, we describe a universal strategy to correct *HBB* mutations in human iPSC lines that are derived from transfusion-dependent β-thal patients, by achieving a targeted insertion at the 5′ end of the first exon and express a full-length of *HBB* cDNA. This universal strategy enables correction of not only HBB mutations in first exon (such as SCD and β17 mutations) but also downstream mutations in exon 2, intron 2, and exon 3 found in β-thal patients. In this study, we used the validated guide RNA that we previously employed to correct the SCD point mutation at codon 6 [18], so that this universal strategy should also be applicable to patients with a single or double SCD mutations. With a guide RNA targeting a further upstream *HBB* DNA sequence in exon 1 (including 5′ UTR) or even at the intragenic sequence 5′ to exon 1, this strategy will be applicable to most if not all the mutations found in β-thal and SCD patients. Although we focused on β-thal iPSCs from two such patients in this study, we also achieved similar result using the same donor DNA and guide RNAs in TNC1 iPSCs derived from a SCD patient [12, 18] (data not shown).

One can envision that human iPSCs and derived hematopoietic progeny may provide an unlimited cell source for autologous cell therapy for β-thal and SCD patients by two distinct strategies. In the first strategy, we will need to generate transplantable HSPCs from gene corrected iPSCs, which are able to generate erythrocytes in marrow for a long-term and produce functional HBB proteins in circulating erythrocytes. Despite extensive studies in the past two decades, however, a robust and reproducible method is still elusive for generating long-term transplantable HSPCs from human iPSCs as well as embryonic stem cells [31–33]. This is at least partially due to the lack of culture condition to expand extensively transplantable HSPCs, whether they are from postnatal cord blood and adult marrow or human iPSCs [33]. The second strategy

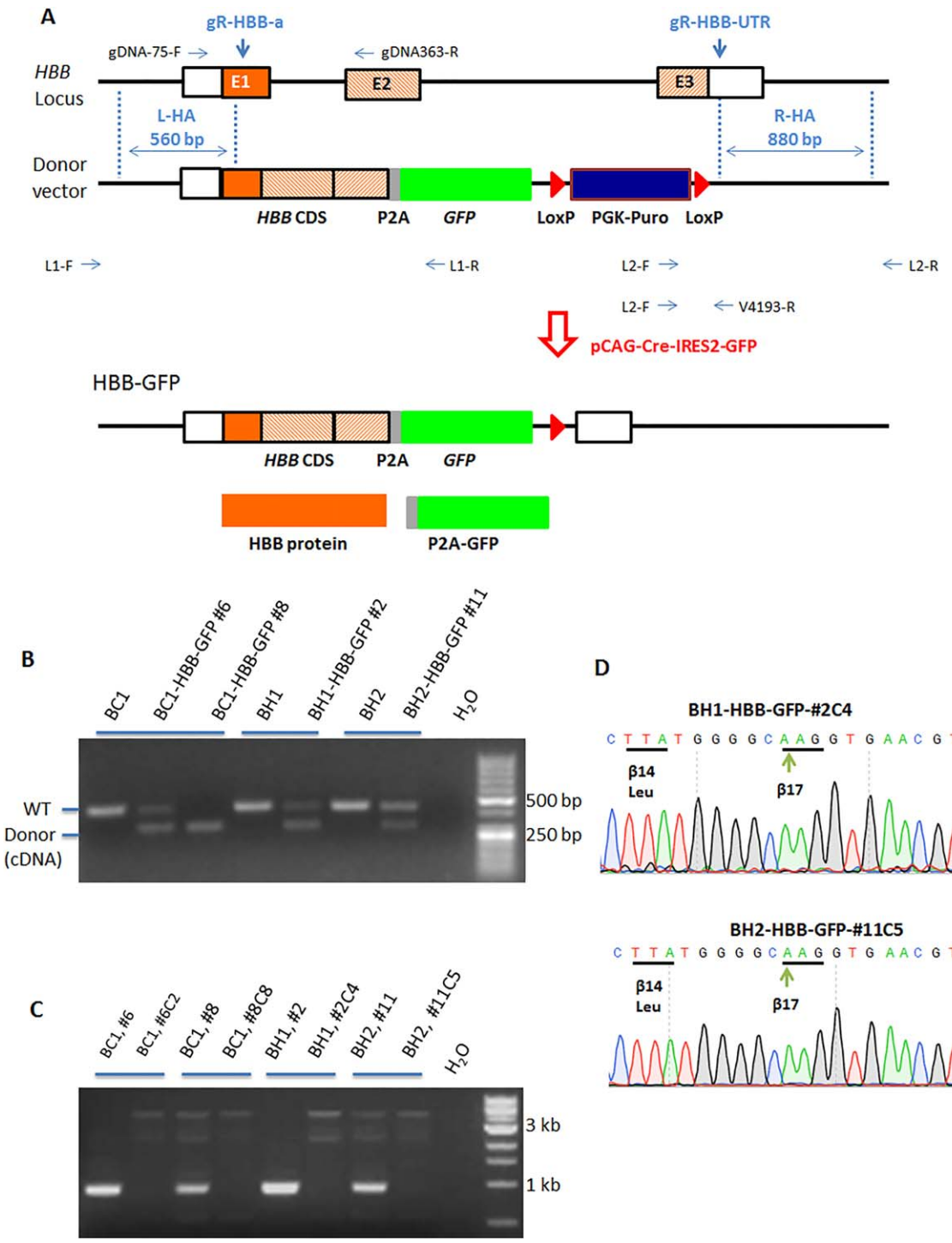

Figure 3. CRISPR/Cas9 mediated genome editing of the human *HBB* gene by its cDNA. **(A):** A diagram of a strategy to replace the *HBB* genomic DNA by its CDS linked by a P2A self-cleaving peptide with the GFP reporter gene. The two guide RNAs, gR-HBB-a and gR-HBB-UTR, were designed to target on exon 1 and 3′ UTR of HBB gene, respectively. The donor vector containing HBB CDS, a L-HA and a R-HA was transfected into BH1, BH2, or BC1 induced pluripotent stem cells (iPSCs) along with guide-RNA vectors and Cas9 vector. The positive colonies with homology-directed repair (HDR) were identified by genomic PCR using indicated primers in Supporting Information Figure S3. Next, the edited iPSCs were transfected with plasmid pCAG-Cre-IRES2-GFP to excise the loxP-flanked PGK-puromycin expression cassette. The positive colonies with anticipated excision were identified by genomic PCR using primer L2-F and V4193-R. **(B):** Genomic PCR screening for HDR positive (either homozygous or heterozygous) colonies using primer gDNA-75-F and gDNA363-R. The wildtype *HBB* locus gave rise to a 438 bp PCR product (WT) whereas the corrected edited locus would be a 306 bp PCR product due to the lack of the intron (132 bp) in the donor cDNA. **(C):** Genomic PCR screening for excision of the PGK-Puro cassette using primer L2-F and V4193-R. The targeted colonies without excision (such as BC1, #6) gave rise to a 952 bp PCR product whereas the positive colonies with excision did not give rise to specific PCR product. **(D):** Sanger sequencing to confirm *HBB* gene corrections in BH1 and BH2 iPSCs. The synonymous change of β14 (Leucine) from CTG to TTA, in addition to the corrected β17 (AAG), was observed in selected clones of BH1 and BH2 iPSCs. Abbreviations: bp, basepair; CDS, coding sequence; L-HA, left homology arm; R-HA, right homology arm; WT, wild type.

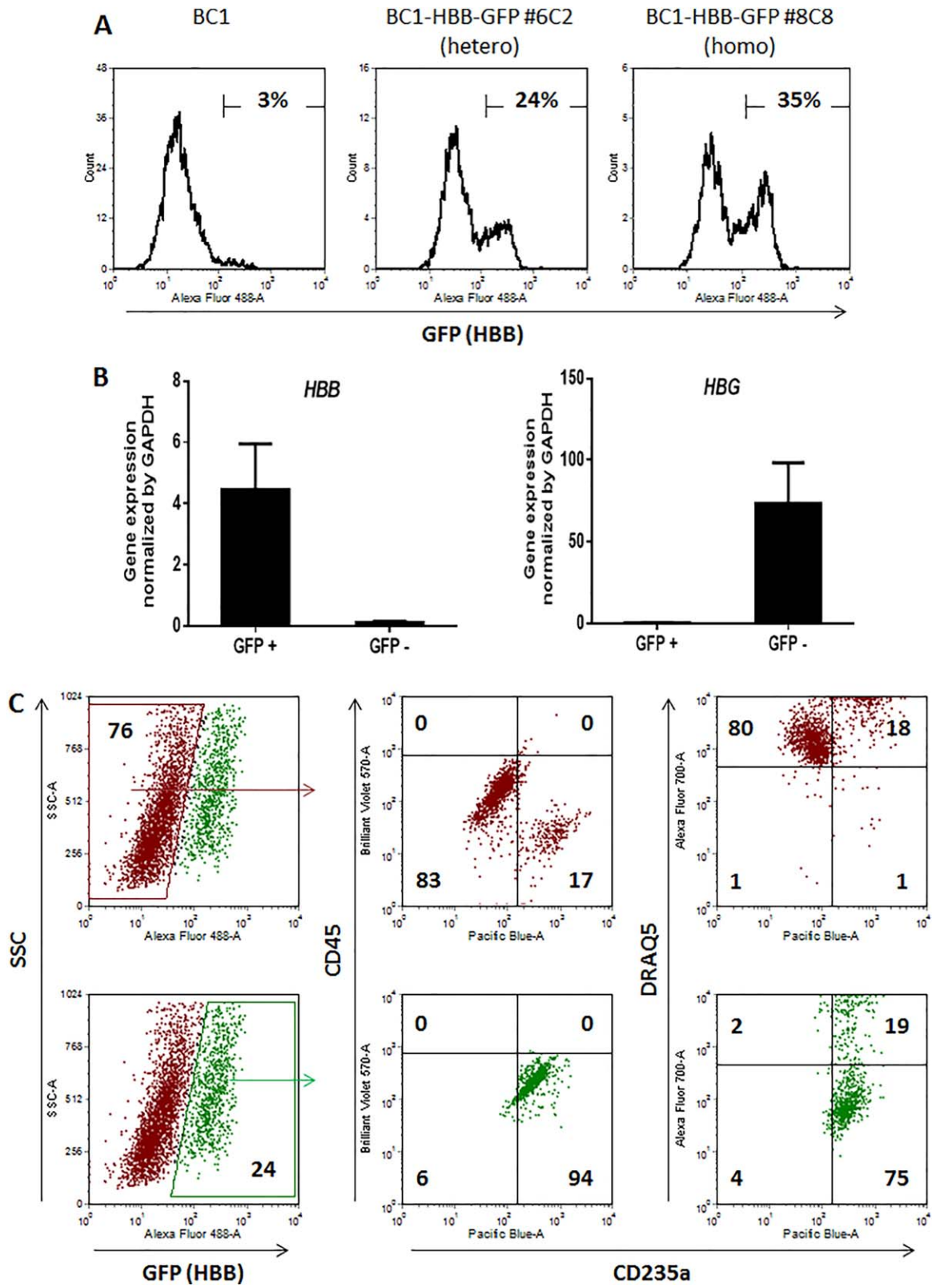

Figure 4. The GFP expression under the control of the *HBB* locus from induced pluripotent stem cell (iPSC)-derived erythrocytes after genome editing. **(A):** Histogram of flow cytometric analyses at terminally differentiated erythrocytes from genome edited BC1 iPSCs. BC1 cells with a heterozygous integration of the HBB-GFP allele (#6C2) or homozygous integration (#8C8) were used, together with parental BC1 iPSCs. **(B):** Real-time quantitative PCR analyses of *HBB* and *HBG* gene expression. GFP positive and negative cells were isolated by fluorescence-activated cell sorting at day 8 after terminal differentiation. The relative gene expression levels were normalized by housekeeping gene GAPDH. **(C):** Flow cytometric analyses of other marker expression in the GFP positive versus negative cells at day 8 of terminal differentiation of #6C2 iPSCs. CD235a and CD45 were used to characterize erythrocytes. A DNA-staining fluorescent dye DRAQ5 was used to evaluate enucleation of erythrocytes lacking nuclear DNA. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

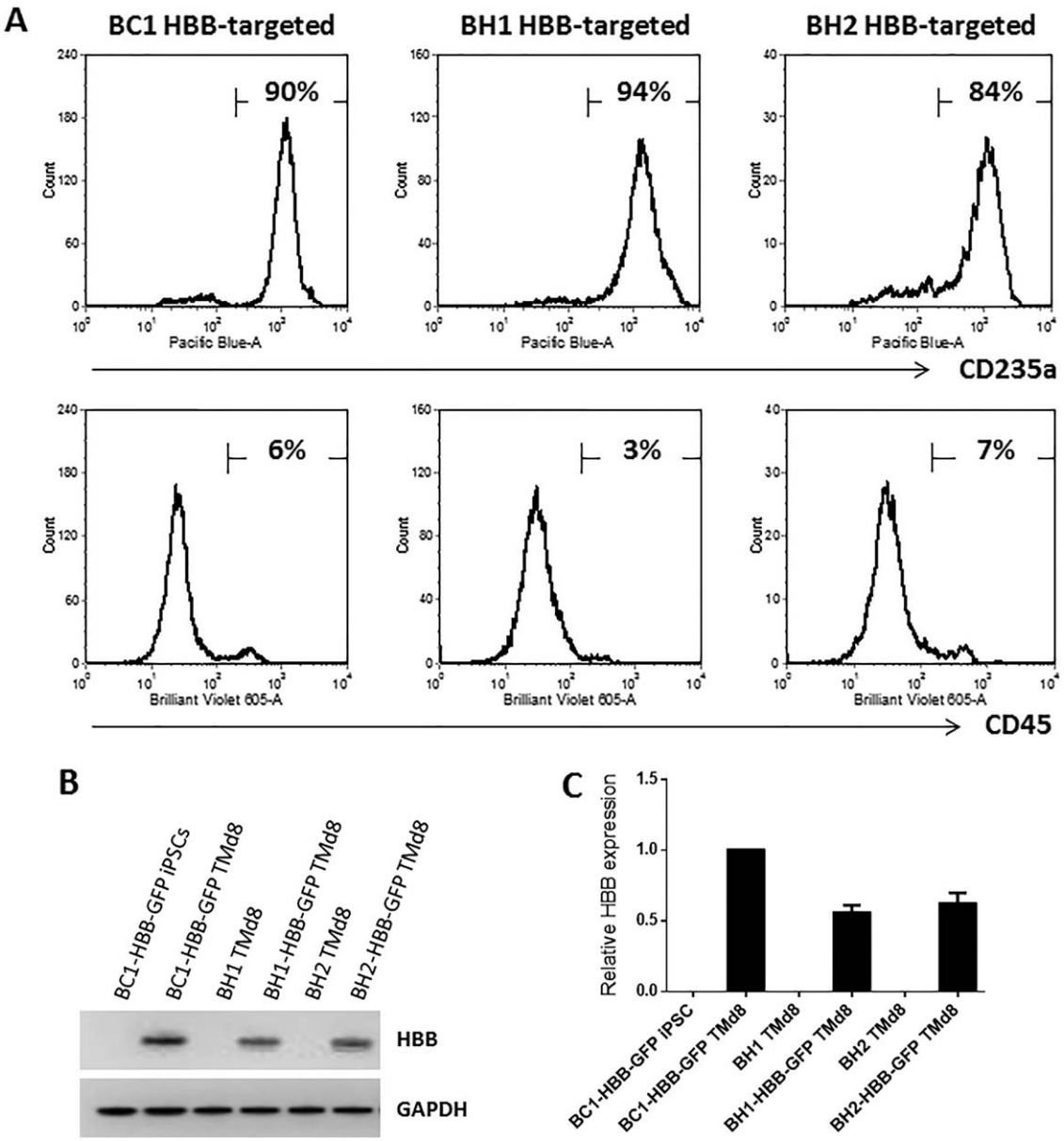

Figure 5. Expression of HBB protein in erythrocytes from the *HBB*-corrected BH1 and BH2 iPSCs. **(A):** Histogram of flow cytometric analyses of erythrocytes from *HBB* gene targeted BH1, BH2, and BC1 iPSCs at TM day 8. Efficient erythrocyte generation was demonstrated by a high percentage of cells expressing CD235a and a low percentage of cells expressing CD45. **(B):** Western blot of HBB expression in erythrocytes at TM day 8. The defective beta hemoglobin expression in erythrocytes from beta thalassemia patient iPSCs was rescued by mono allelic *HBB* gene targeting (heterozygous). The undifferentiated iPSCs of each line were used as negative controls. **(C):** Quantification (mean ± SEM) of HBB protein expression based on Western blot data of multiple experiments ($n = 3$). Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iPSC, induced pluripotent stem cells; TM, terminal maturation.

is to produce erythrocytes ex vivo from autologous human iPSCs, and then infuse to patients who are transfusion dependent [34–36]. Despite of recent progress to increase efficiencies of enucleation and HBB production from iPSC-derived erythrocytes [18, 37, 38], further improvements are needed for this approach to be clinically relevant [34–36]. Currently, the level of HBB expression in iPSC-derived erythrocytes is approximately at 10% of the endogenous HBG expression, or of that in erythrocytes from postnatal HSPCs after terminal differentiation [18, 37–40]. The gene-targeting system developed in this study may also help us to screen for bioactive molecules or culture conditions that increase the HBB protein, by simply monitoring the co-expressed GFP level. This strategy of using a reporter gene expression under the control of *HBB* or *HBG* locus was previously used in K562 cell line (that expresses HBG but not HBB proteins) [41]. By this approach, several small molecules have been identified, which stimulated *HBG* and *HBB* gene expression. However, our current study provides the first case that functional human erythrocytes expressing both HBB and genetically linked GFP reporter (albeit at a relatively a low level) can be produced consistently and in large numbers. We plan to use the HBB-GFP marked erythrocytes to determine if previously identified small molecules [41] could improve HBB production and enucleation by iPSC-derived erythrocytes.

The universal strategy we describe here for *HBB* gene correction could also be adapted to correct various *HBB* gene mutations in primary human postnatal HSPCs, followed by BMT. Historically, it has been very difficult to achieve HDR in human CD34+ HSPCs because the DNA delivery efficiency is low. The inability to expand extensively transplantable postnatal HSPCs further limits our capability to select and expand targeted HSPCs as we did with human iPSCs [33]. Recently, several papers described approaches to improve HDR efficiency in primary human CD34+ HSPCs and correct the SCD point mutation [42–44]. The reported improved efficiency was achieved at least in part by delivering the required Cas9 protein and guide RNA as a preassembled complex, and by delivering a donor template as an oligonucleotide or recombinant AAV6 viruses into CD34+ HSPCs. For this approach to be successfully used for treating β-thal patients with various *HBB* gene mutations, one still has to design a universal template that can be efficiently delivered into postnatal HSPCs. It is likely that we or others will be able to combine the strength of this new delivery approach with our described strategy to correct most types of various *HBB* mutations using a universal donor DNA template. This strategy of inserting a cDNA (or mini-gene) in a 5′ exon with a validate guide RNA to achieve targeted insertion and functional restoration of gene expression/function will likely be applicable to many other forms of human monogenic diseases.

Conclusion

The advent of efficient and precise human genome editing aided by improved tools such as CRISPR/Cas9 significantly broadens our horizons to treat monogenic diseases such as β-thal and SCD. While other scientists are working on developing a universal approach of genome editing in postnatal CD34+ HSPCs to derepress the expression of HBG in erythrocytes (i.e., able to substitute the missed HBB in β-thal or to counter-balance the SCD HBB proteins) [45, 46], our described approach is to directly restore functional HBB proteins after genome editing at the *HBB* locus. Future investigations will determine if one or both approaches will be clinically effective for permanently curing β-thal patients with various *HBB* mutations using an optimized gene and cell therapy.

Acknowledgments

We thank Drs. Gabsang Lee and Yohan Oh for discussions of using gene targeting vectors. This work was supported in part by research grants from National Natural Sciences Foundation of China (81170533) and Guangdong Natural Science Foundation (S2012040006785) to Dr. Cai and her team in Guangzhou, NIH (R01-HL130676) to Dr. Cheng, Maryland stem cell fund (2016-MSCRFE-2739) to Dr. Mahairaki, and a summer intern fellowship from the Foundation for Advanced Research in the Medical Sciences to RL Pan. Professor Cheng is also supported by Edythe Harris Lucas and Clara Lucas Lynn Chair in Hematology of Johns Hopkins University.

Author Contributions

L.C. and Y.W. provided and processed blood samples from β-thal patients in China. L.C. also performed experiments in China and US. H.B. conducted research, collected and analyzed data, and wrote the manuscript. V.M. made and tested target vectors. Y.G., C.H., Y.C.J., Y.W., R.L.P., and A.Q. performed or assisted research. Z.Y. designed research and target vectors, analyzed and interpreted data, wrote the manuscript. L.C. designed research, analyzed and interpreted data, wrote the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Note Added in Proof

This article was published online on 21 November 2017. Minor edits have been made that do not affect data. This notice is included in the online and print versions to indicate that both have been corrected 28 December 2017.

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