Transcription Activator-like Effector Nuclease (TALEN)-mediated Gene Correction in Integration-free β-Thalassemia Induced Pluripotent Stem Cells

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Ning Ma‡§1, Baojian Liao‡§1, Hui Zhang‡§, Linli Wang‡§, Yongli Shan‡§, Yanting Xue‡§, Ke Huang‡§, Shubin Chen‡§, Xiaoxiao Zhou‡§, Yang Chen‡§, Duqing Pei‡§, and Guangjin Pan‡§2

From the ‡Key Laboratory of Regenerative Biology and the §Guangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China and the ¶School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

Significance: Our study extends TALENs for gene correction in patient-specific iPSCs and may have applications in cell therapy.

β-Thalassemia (β-Thal) is a group of life-threatening blood disorders caused by either point mutations or deletions of nucleotides in β-globin gene (HBB). It is estimated that 4.5% of the population in the world carry β-Thal mutants (1), posing a persistent threat to public health. The generation of patient-specific induced pluripotent stem cells (iPSCs) and subsequent correction of the disease-causing mutations offer an ideal therapeutic solution to this problem. However, homologous recombination-based gene correction in human iPSCs remains largely inefficient. Here, we describe a robust process combining efficient generation of integration-free β-Thal iPSCs from the cells of patients and transcription activator-like effector nuclease (TALEN)-based universal correction of HBB mutations in situ. We generated integration-free and gene-corrected iPSC lines from two patients carrying different types of homozygous mutations and showed that these iPSCs are pluripotent and have normal karyotype. We showed that the correction process did not generate TALEN-induced off-targeting mutations by sequencing. More importantly, the gene-corrected β-Thal iPSC cell lines from each patient can be induced to differentiate into hematopoietic progenitor cells and then further to erythroblasts expressing normal β-globin. Our studies provide an efficient and universal strategy to correct different types of β-globin mutations in β-Thal iPSCs for disease modeling and applications.

β-Thalassemia is a group of inherited genetic blood disorders caused by either point mutations or deletions of nucleotides in the β-globin gene. These genetic defects result in reduced, abnormal, or no synthesis of β-globin chains that make up hemoglobin. It is one of the most common genetic diseases in the world, and patients with β-Thal have severe anemia and a shortened life span (2). Hematopoietic stem cell transplantation is the only way to cure β-thalassemia but is challenged by the limited availability of human leukocyte antigen-matched healthy donors. Recently, the development of gene therapy based on viral transduction of a normal HBB gene into a patient’s own hematopoietic stem cells raised hopes for those who do not have access to bone marrow transplantation (3, 4). However, it was still challenged by the concerns of long term safety because of the viral integration. The somatic cells of a patient can be reprogrammed back into pluripotent state as iPSCs that are capable of differentiation into any cells in the body and thus could be potentially used for cell replacement therapy (5–8). Generation of iPSCs from β-Thal patients, correcting mutations, and subsequent differentiation into hematopoietic stem cells raise great hopes for autologous transplantation to treat these inherited diseases (9). Moreover, iPSC cells can undergo indefinite self-renewal without losing the ability to differentiate into all cell types and thus represent an ideal cell population for in situ correction of the disease-causing mutations. However, gene targeting in human pluripotent stem cells by standard homologous recombination is largely inefficient (10) and therefore hampers its extensive application in disease models. Zinc finger nucleases (ZFNs) had been reported to sub-

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1 Both authors contributed equally to this work.
2 To whom correspondence should be addressed: Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Kaiyuan Avenue Science Park, Guangzhou 510530, China. Tel.: 86-020-32015213; E-mail: pan_guangjin@gibh.ac.cn.

3 The abbreviations used are: iPS, induced pluripotent stem; iPSC, iPS cell; β-Thal, β-Thalassemia; ZFN, zinc finger nucleases; HPC, hematopoietic progenitor cell; TALEN, transcription activator-like effector nuclease.
Gene Correction by TALEN

stantially enhance the homologous recombination efficiency by specifically introducing a double-stranded DNA break at the target locus (11–13). Gene targeting aided by ZFN in human iPS or ES cells had been described to be highly efficient (14–16). However, engineering ZFNs for a specific target is quite inefficient and laborious, which largely hampers their widespread adoption. Recently, transcription activator-like effector nucleases (TALENs) had been described to recognize and cleave any given DNA sequences with high efficiency (17–19). The DNA-binding domain of TALEN is unusual and contains multiple units that arranged in tandem (TALE repeats). Each individual unit is composed of 34 amino acids with two highly variable amino acids to determine the unit to recognize one DNA pair in the TALEN recognizing sequence (20). In theory, TALE repeat could be engineered and arranged to specifically recognize any given DNA sequence. TALEN-mediated gene targeting had been described in multiple species, including zebrafish and human iPS, and ES cells (21, 22). Practically, compared with ZFN, TALEN is much more easy and convenient regarding the designing and constructing. Also, TALENs exhibited lower off target effects and reduced nuclease-associated cytotoxocities compared with ZFNs (23–25). In attempt to extend TALEN technology to gene correction for β-Thal, we generated the β-Thal iPS cells through a nonviral approach and developed an efficient process to correct the mutations in β-globin gene by designing and utilizing site-specific TALENs.

EXPERIMENTAL PROCEDURES

iPS Generation—The method of isolating amniotic fluid cells was performed as previously described (26). For reprogramming, an oriP/EBNA1-based pCEP4 episomal vector containing OCT4, SOX2, KLF4, and SV40LT genes (27) and miR-302–367 (28) were co-transfected into amniotic fluid cells via nucleofection (Amaxa). The cells were then plated to Matrigel-coated 6-well plates and cultured with reprogramming medium (mTeSR1). The medium was changed every 2 days and iPS-like colonies were picked onto new Matrigel plate for characterization. Cells of passages from 15 to 40 are used for the following experiments.

TALEN and Donor Vectors for Gene Targeting—TALENs were designed as described (17, 29). The full amino acid sequences of TALENs are given in the supplemental information. For donor DNA, left and right homology arms were amplified from genomic DNA of healthy individual. A loxp-flanked PGK-puromycin cassette or loxp-flanked PGK-neomycin cassette were cloned between two homology arms in the pMD-18T vector. For targeting, 1 × 10⁶ iPS cells were electroporated with 2 μg of donor DNA and 4.5 μg of each TALEN plasmid. Then the cells were plated onto Matrigel-coated 6-well plates in the presence of Y-27632 (10 μM; Sigma) for 1 day. Positive clones were selected by puromycin (0.5 μg/ml) or G418 (100 μg/ml) resistance cassette. Then genomic PCR and Southern blot. All primers used are listed in supplemental Table S1.

GFP Reporter Assay—GFP reporter activation was tested by co-transfecting 293T cells with plasmids carrying TALENs and GFP reporters. 293T cells were seeded into 12-well plates the day before transfection. Approximately 24 h after initial seed-
FBS, and spun onto slides with a cytopin apparatus (TXD-3). Cells were fixed and stained with Wright-Giemsa reagents (BASO Biotech, Auburn, CA).

RESULTS

Derivation and Characterization of iPSCs from Two Patients with Different β-Thal Subtypes—To generate iPS cells, we isolated amniotic fluid cells from two fetuses with informed consents from their mothers. One was diagnosed with β-Thal major of IVS2–654, which carried two homozygous C → T mutations at the second intron of HBB, resulting in the formation of an abnormally spliced mRNA and the deficiency of correctly spliced β-globin transcript (30). The other was diagnosed with β-Thal major of β41–42 (–TCTT), which carried two homozygous TCTT deletions at the second exon caused a frameshift that generates a termination codon (TGA) in the position of the new 59th codon (31). To obtain iPSCs that were more close to clinical grade, we attempted to generate β-Thal iPSCs under nonviral, serum-free, and feeder-free conditions. In detail, we expanded the amniotic fluid cells and delivered oriP/EBNA episomal vectors carrying a combination of reprogramming factors including OCT4, SOX2, SV40LT, and KLF4 (27) and miR-302–367 cluster (28) through electroporation. After ~5 days growth in amniocyte medium, the cells were subsequently plated on Matrigel-coated plate and cultured in defined mTeSR1 medium (32) for further reprogramming (Fig. 1A).

![Figure 1](image-url)
two patients for reprogramming, we usually obtained ~50 alkaline phosphatase-positive iPSC-like colonies. We then picked four colonies that displayed typical iPSC morphology for further expanding and characterization. The clonally expanded β-Thal iPSC cells exhibited typical human ES cell morphology (Fig. 1A) and expressed pluripotent markers such as OCT4 and SSEA4 homogenously (Fig. 1B). By quantitative RT-PCR, we demonstrated that the endogenous pluripotent genes such as OCT4, SOX2, and NANOG were fully activated in β-Thal iPSC cells, as well as that in H1 ES cells (Fig. 1C). More importantly, by using transgene-specific primers to detect transgenes (27), we showed that both of these two β-Thal iPSC cell lines harbor neither the exogenous reprogramming factors such as OCT4, SOX2, and KLF4 nor the genes in episomal vector backbone (Fig. 1D). Moreover, these transgene-free β-Thal iPSC cell lines possessed normal karyotype (Fig. 1E) and could form typical teratomas containing three germ layers upon injection into immunodeficient mice, a well known assay for pluripotency (Fig. 1F). Furthermore, we confirmed that the two transgene-free β-Thal iPSC cell lines carried the homozygous disease-causing mutations as diagnosed (Fig. 1G). Thus, based on their disease subtypes, we named them βThal654_iPS and β(−TCTT)_iPS, respectively (Fig. 1G).

**Construction of Site-specific TALENs for β-Globin Cleavage**—We sought to correct these disease-causing mutations in β-Thal iPSC cells through *in situ* gene targeting using TALENs (33). Based on previous studies (20), we designed a pair of TALENs that could specifically recognize two adjacent 18-bp DNA sequences with a 17-bp spacer that was ~600 bp downstream of the last exon of *HBB* (Fig. 2A). To test the site specificity and cleavage activity of the designed TALENs, we constructed a TALEN-targeting plasmid that harbored a GFP reporter. Two 205-bp duplications of GFP coding sequence that flank each side of TALEN-targeting site were introduced into the middle of GFP coding region (illustrated in Fig. 2A). Once a break was introduced by TALENs cleavage, the duplicated homologous sequence will anneal together and recombine into a full-length GFP and thus can be detected by FACS to evaluate the efficiency and specificity of newly designed TALENs (Fig. 2B). Thus, we transfected the TALEN-targeting GFP reporter together with the pair of TALENs (TALEN-L/R) into 293T cells and showed that the GFP signals were significantly increased compared with that GFP reporter transfected alone or with only one TALEN, demonstrating that the cleavage activity by the TALENS was high (Fig. 2B). To test their site specificity, we introduced different point mutations or deletions in TALEN-binding sequence. We showed that the GFP signals were greatly reduced in reporters with mutated binding sites compared with wild type control reporter (Fig. 2C). These data demonstrated that the nuclease activity of the designed TALENs was highly specific to its targets and can be harnessed for further genome editing.

**Efficient Correction of β-Globin Mutations Using TALEN-mediated Gene Targeting**—To correct the mutations in βThal654_iPS and β(−TCTT)_iPS cells, we constructed a donor template centered on TALENs targeting site. The donor template contains a 2.4-kb 5′ homology arm that harbors the entire wild type β-globin gene and a 1-kb 3′ arm homology to the downstream of β-globin gene (Fig. 2D). Upon cleavage by TALENs and subsequent homology recombination with this donor template, wild type β-globin gene in the donor will replace the mutant one in βThal654_iPS or β(−TCTT)_iPS cells. Thus, we introduced the linearized donor plasmids and TALEN vectors into both βThal654_iPS and β(−TCTT)_iPS cells through electroporation. The positive colonies were selected by puromycin (for βThal654_iPS) or G418 (for β(−TCTT)_iPS). Drug-resistant colonies were manually picked and expanded for further characterization. To identify correctly targeting clones, we designed two pairs of PCR primers as indicated in Fig. 2D and performed genomic PCR with these primers (Fig. 2D). The correctly targeted clones would be positive for both primers but with different sizes. By genomic DNA PCR, we found that the targeting efficiency was remarkable. In the case of βThal654_iPS cells, of 37 picked clones characterized, 25 were double positive for both PCR primer pairs, with an efficiency of 68%. For β(−TCTT)_iPS cells, 4 of 10 picked clones were identified as positive (supplemental Fig. S1). We then randomly expanded two positive clones from each βThal iPSC cell line to confirm the correction of mutant β-globin. First, we confirmed that the correct gene targeting could be detected by genomic PCR with expected size using the designed two primer pairs (Fig. 2E). Second, we performed Southern blot to further analyze the gene targeting. As shown in Fig. 2F, both targeted cell lines showed the expected bands (Fig. 2F). Lastly, through Sanger sequencing, we confirmed that both β-Thal iPSC cell lines were corrected at one allele of mutated β-globin gene (Fig. 2G), indicated as double peaks for βThal654_corrected_iPS and overlapping for β(−TCTT)_corrected_iPS in Sanger sequencing map. Taken together, we showed that TALEN-mediated gene targeting was highly efficient to correct different types of disease-causing mutations in *HBB* gene in β-Thal iPSC cells.

**Characterization of Gene Corrected β-Thal iPSCs**—To further characterize the gene corrected β-Thal iPSC cell lines, we showed that typical pluripotent markers such as OCT4 and SSEA-4 were expressed homogenously in the two corrected βThal654_iPS and β(−TCTT)_iPS cell lines, indicating that they remained pluripotent after correction (Fig. 3A). Furthermore, both of them could differentiate into three germ layer lineages upon embryonic body formation (Fig. 3B). Also, the karyotypes of the corrected iPSC cells remain normal after genetic operation (Fig. 3C). Most importantly, upon injection into immunodeficient mice, the corrected β-Thal iPSC cells could form teratomas that contain all three germ layers (Fig. 3D), demonstrating that the gene corrected β-Thal iPSC cells keep the pluripotency well upon gene targeting. One important concern in using nuclease to aid gene targeting is off target cleavage because it might introduce extra mutations in the genome. To address this question, we blasted TALEN-targeted sequences in the entire human genome and selected six genomic sites that were on the top in homology to the designed TALEN recognition sequence. We designed primers and amplified these regions from the genomic DNA extracted from gene corrected βThal654_iPS or β(−TCTT)_iPS cells and performed Sanger sequencing on those regions. We detected no mutations and deletions on these regions that are most poten-
tial to be recognized and cut by TALENs (Fig. 3E). These data indicated that off target cutting might not be introduced by the designed TALENs in gene targeting. To detect whether the genetic modification process can introduce other mutations affecting the gene function, we amplified the whole \( \beta \)-Hemoglobin gene, and by sequencing we did not find any additional mutations (supplemental information).

**In Situ Gene Correction Restores the Function of HBB Gene in \( \beta \)-Thal- iPSC-derived HPCs and Erythroblasts**—To examine whether the correction of disease-causing mutations in \( \beta \)-Thal iPSCs could restore normal expression of full-length \( \beta \)-globin, we attempted to differentiate these iPSCs into HPCs and then erythroblasts. OP9 mouse bone marrow cells had been shown to efficiently induce hematopoietic differentiation of human pluripotent cells without additional cytokines (34); thus, we employed the OP9 co-culture system to induce the hematopoietic differentiation of \( \beta \)-Thal iPSCs. Upon OP9 co-culture, both \( \beta \)-Thal iPSCs cell lines, no matter whether corrected or uncorrected, could differentiate rapidly and produce HPCs that were detected as CD34\(^{+}\)CD110\(^{+}\)CD31\(^{+}\) (Fig. 4, A and B). These \( \beta \)-Thal iPSC-derived HPCs could further differentiate into various blood lineages upon plating in semisolid culture to form various CFU-Cs, albeit with some efficiency variations between different cell lines and batches of experiments (Fig. 4C). We then

**FIGURE 2.** Site-specific gene correction of the \( \beta \)-thalassemia mutations using TALENs. A, top panel, the recognition sequence of TALENs 600 bp downstream of the HBB gene. Middle panel, different mutation types in the recognition sequence of TALENs. Bottom panel, schematic of the GFP reporter assay. B, fluorescence images of 293T cells transfected with GFP reporter and TALENs. C, specificity of designed TALENs. 48 h after 293T cells transfected with GFP reporter containing different TALEN recognition sequence and TALENs, cells were harvested, and the GFP fluorescence was tested by flow cytometry. The \( p \) values were calculated by one-way analysis of variance. *** indicates \( p < 0.001 \). D, schematic overview of gene targeting strategy for the human HBB locus. The desired recombination event inserts a PGK promoter-puromycin resistance cassette or PGK promoter-neomycin resistance cassette flanked by loxP sites (black triangles) into the position 600 bp downstream of \( \beta \)-globin locus. The Southern blot probe is indicated by an arrow (5' probe), and PCR primers are indicated by arrows (P1, P2, P3, and P4). E, representative PCR analysis of puromycin-resistant clone (\( \beta \)-Thal654_corrected iPSC) and neomycin-resistant clone (\( \beta \)-Thal654_corrected iPSC). P1/P2, 2700 bp; P3/P4, 5000 bp. F, Southern blot of indicated iPSCs using the 5' probe. A HBB allele that has not undergone gene targeting gives a 5-kb band, whereas a targeted allele gives a 6.4-kb band. G, top panel, sequencing results of C \( \rightarrow \) T mutation site in the second intron of HBB in \( \beta \)-Thal654 iPSC and \( \beta \)-Thal654_corrected iPSC cells. A black box indicates the location of the point mutation in the patient. Bottom panel, sequencing results of TCTT deletion site in the second exon of HBB in \( \beta \)-Thal654 iPSC and \( \beta \)-Thal654_corrected iPSC cells.
manually picked the red blood lineage colonies (CFU-E) from the semisolid plate and examined the expression of globin gene \( HBB \) as well as the (fetal type) \( HBG \) gene as a control by quantitative RT-PCR. We showed that the expression of \( HBB \) gene increased 1000-fold more in CFU-E derived from both gene corrected \( \beta \)-Thal iPSCs than that from the uncorrected ones and that the levels were comparable with human ES cell (H1)-derived CFU-E (Fig. 4D). To the contrary, as the control, fetal type globin gene, \( HBG \) expressed at similar levels in CFU-Es derived from either corrected or uncorrected \( \beta \)-Thal iPSCs as well as the human ES cells (Fig. 4D). Lastly, by using conventional RT-PCR and designing primers that could amplify full-length \( HBB \) cDNA (the forward primer was designed in the first exon of \( HBB \), and the reversed primer was designed in the third exon of \( HBB \)), we confirmed that the expressions of full-length \( HBB \) cDNA were successfully restored after gene correction (Fig. 4E). Taken together, our data demonstrated that the \textit{in situ} correction of disease-causing mutations in \( \beta \)-Thal iPSCs restored the function of \( HBB \) gene in the iPSC-derived HPCs and erythroblasts.

**DISCUSSION**

Here, we described an efficient nonintegrating process to generate \( \beta \)-thalassemia iPSCs and subsequently corrected the disease-causing mutations using TALEN-mediated gene targeting. For the purpose of future application, we excluded c-MYC as a reprogramming factor and avoided using serum and mouse feeder cells. The nonviral approach combined with the defined condition employed in our system could be optimized further for generating clinical grade and safe iPSCs. The two \( \beta \)-Thal iPSC cell lines generated in this process remain normal karyotype and stable in maintaining pluripotency. More
importantly, we did not detect any transgene integration in these cell lines, which is essential for their further application.

Gene targeting mediated by traditional homologous recombination in human pluripotent stem cells is particularly difficult and inefficient. The recent developed TALEN technology provides great help to solve this problem by enhancing the gene targeting efficiency. TALEN-mediated gene targeting has been reported to be successful in multiple species (21, 22, 32, 35). A critical point of TALEN application is its specificity to cleave a given DNA sequence. By using a reporter assay, we showed that the cleavage activity of the designed TALENs is highly specific. Moreover, we failed to detect mutations caused by off target cutting by TALENs in corrected \( \beta \)-Thal iPS cells. In sum, TALENs technology provides an efficient and cost-effective way for disease-causing gene correction in human pluripotent stem cells, as we have demonstrated here. Further experiments are needed to investigate genome widely whether TALEN-based gene targeting would cause genomic instability and whether those potential genomic changes would be hazardous to further application.

\( \beta \)-Thal is the most common inherited genetic disease in the world and contains over 200 different types of mutations in \( \beta \)-globin gene that could cause blood disorders. For practical purposes, it is essential to develop an efficient and universal process to correct most of the common type of mutations in \( \beta \)-globin gene. To achieve this goal, we selected 3’ downstream of \( \beta \)-globin as gene targeting site, because any little changes within the \( HBB \) gene region would affect its normal function.
Gene Correction by TALEN

(14). Then, when we constructed the donor template for homology recombination, we included the whole wild type \( HBB \) gene in the 5’ arm. Thus, for correction of mutations in \( \beta \)-thal iPSCs, this template could be used to replace the endogenous gene with mutations or deletions in any site within the \( HBB \) region. As shown here, the gene targeting in \( \beta \)-thal iPSCs with either mutations or deletions was equally efficient, demonstrating that this approach is universal and could be employed for other different types of \( \beta \)-thal iPSCs. Indeed, the in situ correction of disease-causing mutations in two different types of \( \beta \)-thal iPSCs restored the function of the \( HBB \) gene in their derived erythroblasts. Further studies are needed to evaluate whether hematopoietic stem cells differentiated from these corrected \( \beta \)-thal iPSC cells are functional in vivo.

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