Engineered zinc-finger nuclease to generate site-directed modification in the KLF1 gene for fetal hemoglobin induction

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Abstract
Elevation of hemoglobin F (HbF) ameliorates symptoms of β-thalassemia, as a common autosomal recessive disorder. In this study, the ability of an engineered zinc-finger nuclease (ZFN) system was assessed to disrupt the KLF1 gene to inhibit the γ to β hemoglobin switching in K562 cells. This study was performed using a second generation integration-deficient lentiviral vector assigned to transient gene targeting. The sequences coding for zinc finger protein arrays were designed and subcloned in TDH plus as a transfer vector. Transduction of K562 cells was performed with the integrase minus lentivirus containing ZFN. The indel percentage of the transduced cells with lentivirus containing ZFN was about 29%. Differentiation of K562 cell line into erythroid cell lineage was induced with cisplatin concentration of 15 µg/mL. After differentiation, γ-globin and HbF expression were evaluated using real-time reverse-transcription polymerase chain reaction and hemoglobin electrophoresis methods. The levels of γ-globin messenger RNA were nine-fold higher in the ZFN treated cells compared with untreated cells 5 days after differentiation. Hemoglobin electrophoresis method showed the same results for HbF level measurement. Application of the ZFN tool to induce KLF1 gene mutation in adult erythroid progenitors might be a candidate to stimulate HbF expression in β-thalassemia patients.

KEYWORDS
hereditary persistence of HbF, integrase minus lentivirus, KLF1, zinc-finger nuclease, γ-globin
INTRODUCTION

Hemoglobinopathies are a collection of inherited disorders categorized according to abnormalities of structure, function, or globin chains synthesis. Blood transfusion as a common treatment modality has its own complications including iron overload even when iron-chelation regimens are administered. Allogeneic hematopoietic cell transplantation is considered another therapeutic option. Gene or cell therapy have proposed promises in hemoglobinopathies treatment but with numerous challenges yet to be addressed. Some of these state-of-the art strategies focus on epigenetic approaches, which involved in the reactivation of γ-globin gene expression. Increased hemoglobin F (HbF) production has been shown to be related to reduced severity of γ-globin gene expression. Hemoglobinopathies are a collection of inherited disorders categorized according to abnormalities of structure, function, or globin chains synthesis. Blood transfusion as a common treatment modality has its own complications including iron overload even when iron-chelation regimens are administered. Allogeneic hematopoietic cell transplantation is considered another therapeutic option. Gene or cell therapy have proposed promises in hemoglobinopathies treatment but with numerous challenges yet to be addressed. Some of these state-of-the art strategies focus on epigenetic approaches, which involved in the reactivation of γ-globin gene expression. Increased hemoglobin F (HbF) production has been shown to be related to reduced severity of the symptoms in some patients. On the contrary, coinheritence of heterozygosity for sickle cell disease (SCD) and hereditary persistence of HbF (HPFH) mutations reported to lead to normal hematologic phenotype. In some regions of Saudi Arabia and India, SCD patients who also have an unusual high level of HbF would mostly remain asymptomatic. Moreover, children with SCD reported to develop the thalassemia trait with increasing age, which was attributed to higher HbF level in early childhood. These and other similar observations were also confirmed with large-scale epidemiological research, indicated that elevated HbF levels could significantly reduce the clinical severity of SCD and β-thalassemia.

The fetal-to-adult hemoglobin switching is regulated primarily at the molecular level with some regulators including BCL11A, SOX6, KLF1, MYB, microRNA 15-A/a/16-a, and HDAC1/2. KLF1 as one of the regulatory molecules proved to have a dual effect in Hb switching using functional assays. In addition to its recognized role in adult β-globin expression, KLF1 is a critical activator of the BCL11A gene, encoding a suppressor of γ-globin gene expression. HPFH is characterized by persistent high levels of fetal hemoglobin (HbF) expression in adults. A nonsense mutation in the KLF1 gene, p.K288X, results in ablating the DNA binding domain of this crucial erythroid transcriptional regulator. Downregulation of KLF1 target genes in HPFH samples has been revealed. The gene editing tools (GET) seem to be a suitable system to induce insertion/deletion (indel) mutation on KLF1 gene based on KLF1 dual role in Hb switching.

GETs are engineered endonucleases suggesting highly sequence-specific gene-targeting tools to induce double-strand breaks (DSBs) in a wide range of DNA sites. The DSBs lead to activation of cellular DNA repair systems, which induce homologous recombination (HR) phenomenon subsequently in the presence of a donor DNA. While, in the absence of any DNA template, Nonhomologous end joining (NHEJ) is recruited to repair the DNA lesions. NHEJ is an error-prone mechanism, offering Indel mutations at the DSB site, which may culminates in the open reading frame (ORF) or termination of gene translation.

In our previous study, this mutation was induced by CRISPR and considerable results were achieved to increase γ-globin expression. The present investigation was launched to decrease the great number of off-targets and improve the problem of random integration of the vectors into the genome using ZFN, which is replaced CRISPR.

Optimal function of GETs could be achieved recruiting ZFNs in a protein-based designation using cell-penetrating peptides (CPPs). By accurately designing ZFN with minimum off-targets, although ZFN is costly and suffers from some designing limitations. The present study was performed to study the ability of an engineered ZFN, to make disruption in KLF1 and obstruct the process of switching to γ-globin elevation.

MATERIALS AND METHODS

2.1 Design and construction of zinc-finger nuclease cassette

The protein coding sequences for the left and right zinc finger protein (ZFP) arrays, each containing three fingers, were designed with zinc-finger tools software (http://www.scripps.edu/barbas/zfdesign/zfdesignhome.php). The left and right ZFP arrays target two sites flanking the codon 288 on the KLF1 gene (Figure 1). Subsequently, the nucleic acid sequence of ZFP was deduced from the protein sequence using the SMS website (http://www.bioinformatics.org/sms2/). The FOKI KKR and ELDs linked to the left and right ZFN arrays-linker peptide coding sequences, respectively. ZFPs and the FOKI cleavage domains joined by the linker peptide “TGEKFP.” The nucleic acid sequences of ATG-HIS tag-NLS-GLY SER GLY-ZF left-FOK1 KKR-IRES-ATG-HA tag-NLS-GLY SER GLY-ZF Right- FOK1 ELD were codon optimized based on codon frequencies in human and ordered to the GeneCust company for synthesis and subcloning in TDH101PA-G (Addgene) vector (TDHplus) (Figure 2).

2.2 The cell lines

2.2.1 The HEK293T cell line

The HEK293T cell line was obtained from Pasteur Institute of Iran (Tehran, Iran). The cells were retained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, MI) containing 100 U/mL of
FiguRe 1 The sites on KLF1 targeted with the left and right ZFN arrays. The KLF1 gene contains three exons. A nonsense mutation was identified at codon 288 in the end of exon 2 in individuals affected with HPFH in a Maltese family. We designed two ZF arrays containing three fingers, attach to left and right side of codon 288 and one of which sitting on the left side of codon 288 while the other is positioned on the right side. HPFH, hereditary persistence of HbF; ZF, Zinc-Finger; ZFN, Zinc-finger nuclease.

2.2.2 The K562 cell line

The human myeloid leukemia cell line, K562, was obtained from Pasteur Institute of Iran. The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) medium supplemented with 50 IU/mL of penicillin, 50 mg/mL of streptomycin, and 10% FBS (Sigma-Aldrich) in 5% CO2 humidified atmosphere. BCR-ABL presence was confirmed in K562 cells as described previously.16

2.3 Western blot analysis

HEK293T cell line was transfected with TDH plus vector to evaluate the ZFN expression (left and right arrays). After overnight incubation, the cells were then assessed for the expression of green fluorescent protein (GFP) using fluorescence microscopy (Nikon Inverted Microscope; Nikon, Tokyo, Japan). Cell lysis was performed after 48 hours of transfection, using radiolmmunoprecipitation assay buffer (RIPA Lysis Buffer System: sc-24948) following the manufacturer’s instructions. Detection of right and left ZFN arrays done using Western blot analysis with anti-HA antibody (Cell Signaling Technology, Danvers, MA) and anti-His antibody (Sigma-Aldrich), respectively.

2.4 ZFN-lentivirus packaging

In the first step, the HEK293T cell line was transfected with the PMD2G, psPAX2 integrase minus17 and TDH plus vector containing ZFN (transfer plasmid) simultaneously using polyfect reagent (Qiagen, Venlo, Netherlands). The expression of GFP was assessed in HEK293T cells after overnight incubation using fluorescence microscopy. The media was replaced with fresh DMEM and incubated at 37°C and 5% CO2. After 24, 48, and 72 hours, the cell culture media were harvested and kept at 4°C. The medium was centrifuged at 2151g for 5 minutes to pellet any HEK-293T cells that were collected during harvesting. The harvested virus was concentrated by high-speed ultracentrifugation (Sigma Sartorius-3KC; Sigma-Aldrich) at 58 000g for 2 hours and assessed for viral titer.18,19 The manipulated lentivirus was named ZFN-lentivirus.

2.5 Transduction of the K562 cell line

Approximately 3 × 10^5 K562 cells were cultured in a 12-well plate. After overnight incubation, the medium was changed with 0.5 mL RPMI-1640 medium containing polybrene (Hexadimethrine bromide; #H9268; Sigma-Aldrich), and then 0.5 mL of ZFN-lentivirus was added. The cells were incubated for 18 to 20 hours, then medium was replaced with 1 mL of RPMI-1640 from Day 4. The medium was changed every 2 to 3 days until GFP expression started. Cells were spun and resuspended in cold PBS for flow cytometry analysis to record GFP positive cells percentage.

2.6 IDT surveyor mutation detection kits for on-target analysis

K562 cells were harvested partially and genomic DNA was isolated from ZFN-lentivirus transduced cells using DNA extraction Kit (Promega, Madison, WI) according to the manufacturer’s instructions. The untransduced K562 cells served as a control. Polymerase chain reaction (PCR) was performed for 35 cycles to amplify endogenous loci with forward primer: 5'-TCCTTCCTGAGTTGTTTGG-3' and reverse primer: 5'-TGGGACTAGGATGAACAAAG-3') and the fragments were purified using gel extract kit (Bioneer, Oakland, South Korea) according to the manufacturer’s instructions.

About 1000 ng of purified PCR product was denatured and reannealed in complete KCl reaction buffer (Cat. No.
102006; Bioron; Germany) using a thermocycler with the following protocol: 95°C for 10 minutes; 95 to 85°C at −2°C/s for each cycle; 85°C for 1 minute, 85 to 75°C at −0.3°C/s; 75°C for 1 minute, 75 to 65°C at −0.3°C/s; 65°C, for 1 minute, 65 to 55°C at −0.3°C/s; ....... 25°C for 1 minute, hold at 4°C. Hybridized PCR products were then treated with 1 μL of surveyor nuclease S, 1 μL of surveyor enhancer S and 1.2 μL of MgCl₂ (0.15 M) at 42°C for 60 minutes in a reaction volume of 17 μL. Stop solution was added after incubation and the product was run on a 2.5% agarose gel.

The percentage of indel formation were obtained using the following formula: % indel formation = 100 × [1−(1−fraction cleaved)1/2]; fraction cleaved = 100 × sum of the cleavage product peak/(cleavage product + parent peak).

### 2.7 Erythroid differentiation and validation of K562 cells

For induction of K562 cell differentiation into erythroid cell lineage, another group of K562 cells were incubated for 7 days in RPMI-1640 medium containing 10% FBS and 15 µg/mL cisplatin. The expression of the erythroid-specific surface antigen CD235a was examined using direct immunofluorescent staining with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody against human CD235a (Biolegend, San Diego, CA). FITC-conjugated mouse IgG1 was used as the isotype antibody (Biolegend) to confirm antibody specificity. Flow cytometric analysis was performed using FACSCalibur Flow Cytometry (BD Bioscience) by accumulating up to 100,000 events per tube.

### 2.8 Relative quantitation of γ-globin expression

To measure γ-globin mRNA levels, real-time reverse-transcription (RT-PCR) method was developed and β actin was used as the endogenous control. The RNA extraction and cDNA synthesis were performed on the cells treated with ZFN system as well as on those with no treatment. cDNAs were used as template in real-time PCR. Real-time PCR was performed as described previously.

### 2.9 Hemoglobin analysis

Hemoglobin analysis was performed as described previously with slight modifications. Approximately, 4 × 10⁷ to 5 × 10⁷ cells of erythroid culture in differentiation phase were lysed in 50 mL of hemolysate reagent (Helena Laboratories, Beaumont, TX) and refrigerated overnight. Subsequently, cells were centrifuged at 14,000 rpm for 15 minutes at 4°C to remove cellular debris. Hemoglobin electrophoresis by cellulose acetate was used for characterization of hemoglobin production in cleared supernatant.

### 2.10 Data analysis

The results are shown as mean ± SD and were analyzed using nonparametric Mann-Whitney U test. Data analysis was performed using the SPSS 16 software (SPSS, version 16; SPSS Inc, Chicago, IL). Graphs were drawn using MS Office Excel 2007 (Microsoft Office Excel 2007; Microsoft Inc).

### 3 RESULTS

#### 3.1 Western blot analysis

We performed Western blot analysis to investigate the ZFN expression. Western blot analysis illustrated the capture of 65 kDa protein band in both of left and right arrays (Figure 3).

FIGURE 3 Western blot analysis. Western blot analysis was performed to investigate the left and right array expression of ZFN in K562 cells transduced with lentivirus containing ZFN. A, Western blot analysis to assess the expression of left array ZFN using anti-His Ab. (1) Protein ladder, (2) positive control for His tag, (3) 65 kDa protein band related to left array ZFN. B, Western blot analysis to assess the expression of right array ZFN using anti-HA Ab. (1) 65 kDa protein band related to left array ZFN, (2) protein ladder. ZFN, zinc-finger nuclease
3.2 | ZFN-lentivirus packaging and the K562 cell line transduction

The titer of the viral vector was evaluated by National RNAi Core Facility (NRC) protocol using transduction of HEK293T cells at different dilutions \((10^2-10^6)\) in a six-well plate. The percentage of GFP-positive cells were assessed using FACSCallibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ). According to the formula stated,\(^{18,19}\) the ZFN-lentivirus titer obtained by the GFP expression assay was approximately \(3.2 \times 10^5\) U/mL.

The GFP expression was detected in 52% of the K562 cells which were successfully transduced with ZFN-lentivirus (Figure 4).

3.3 | IDT surveyor mutation detection kits for on-target analysis

The on-target cleavage on the \(KLF1\) gene was evaluated by the IDT Surveyor Mutation Detection Kit (Skokie, IL). The induced indel mutation percentage by ZFN around codon 288 on the \(KLF1\) gene was assayed. The ZFN cleaved the target and had an obvious indel mutation rate. After on-target analysis, the amplicon was digested into 165 basepairs (bp) and 213 bp fragments in the gene mutated with ZFN, as expected (Figure 5). The transduced cells with ZFN-lentivirus showed indel mutation percentage of about 29% based on the above formula.

3.4 | Erythroid differentiation and validation of K562 cells

Flow cytometry analysis was performed to evaluate the differentiation rate of transduced K562 cells into erythroid cell lineage. About 85% of the K562 cells showed to express erythroid CD235a marker on their surface that confirmed erythroid differentiation (Figure 6).

3.5 | Relative quantitation of \(\gamma\)-globin expression

We examined the disruption efficiency of the ZFN at the \(KLF1\) target sites. After mutation confirmation, \(\gamma\)-globin expression was analyzed by real-time RT-PCR. Notably, the level of \(\gamma\)-globin mRNA on day 5 of differentiation was elevated to nine-fold in the cells treated with ZFN compared with untreated cells (Figure 7). Therefore, as expected, an indel mutation was induced in the \(KLF1\) gene, which reduced the inhibitory effect of KLF1 on the \(\gamma\)-globin gene and increased \(\gamma\)-globin mRNA levels.

3.6 | Hemoglobin analysis

Mature HbF level was measured by hemoglobin electrophoresis (Figure 8). Induction of indel mutation in the
KLF1 gene resulted in upregulation of HbF to 7% when compared with 0.5% HbF expression in K562 control cells.

4 | DISCUSSION

In the present research, we showed the ability of ZFNs to generate indel mutations in the KLF1 gene to increase HbF levels. This strategy could be used in the future for the treatment of β-thalassemia patients. Thus far, many studies have been carried out for β-thalassemia gene therapy.

Mutation in β-globin gene has been corrected in induced pluripotent stem (iPS) cells which were later differentiated into hematopoietic stem cells (HSCs) and returned to the patient. The elimination of the transcription factors, which are no longer needed after induction and reestablishment of correct reprogramming for inhibition of iPS cells to develop into tumors are the most urgent issues to be addressed in this strategy.23 Another therapeutic approach is transferring a working copy of β-globin gene in which handling large quantities of genetic material and the possibility of positional effect need to be taken into account.24 Thus, the γ-globin reactivation in patients with β-thalassemia or SCD would be an effective cure based on increasing number of studies.25

Chemotherapeutic agents such as 5-azacytidine, hydroxyurea, myleran, and butyrate, capable of stimulating HbF synthesis, have long been applied for β-thalassemia treatment; although their clinical use has been negatively affected by their growth-inhibitory effect, cytotoxicity and possible carcinogenic effect while only modestly induce HbF activity.25

KLF1 is a major contributor engaged in the γ- to β-globin gene switching. Previous studies involved knockdown of the KLF1 and BCL11A genes using short hairpin RNA (shRNA) emerged acceptable results.26 However, shRNA has a number of direct and indirect side effects including off-target repression of mRNAs, induction of an interferon response27 and competition with native RNAs for access to RNAi processing machinery,28 which have limited shRNA application.

In this study, we successfully applied the ZFN technology to knock out the KLF1 gene through induction of targeted genome deletions. We predicted that targeted induced mutation in the coding region of
the \( KLF1 \) gene culminates with reduction of \( \beta \)-globin chain synthesis and a decrease of \( BCL11A \) expression, leading to the removal of its inhibitory effect on \( \gamma \)-globin gene expression. Conventional mutagenesis in coding regions could make truncated proteins or frameshift mutations. The increase in activity of mutated proteins or gain-of-function phenotypes are also introduced as the rare mutagenesis methods consequences.\(^{29}\) ZFN was first used to induce frameshift mutations at the CCR5 locus and yield CCR5 deficient T cells phenotype which are resistant to HIV-1 infection in animal models.\(^{30}\) To date, many genes in various organisms have been successfully mutated with ZFNs by various companies such as Sangamo Biosciences. However, the high price of this service may make this system too expensive for most academic users.\(^{31}\) Furthermore, OPEN ZFNs have been used.\(^{32}\) In comparison, precharacterized modular assembly of ZFNs using standard recombinant DNA technology offer faster and more convenient approach.\(^{33}\)

Applying gene editing tools for \( \beta \)-thalassemia treatment was first performed by Xu et al\(^{14}\), using TALENs and CRISPR/Cas9 for the \( HBB \) intron2 IVS2-654 C > T mutation which led to efficient HR. In another study, Wienert et al\(^{35}\) engineered another mutation (\( −175T > C \)) leading to the HPFH phenotype using TALEN as a gene editing tools and obtained significant increased expression of \( \gamma \)-globin. In our previous study, induced mutation in \( KLF1 \) gene was performed using CRISPR and we reported a significant increase in \( \gamma \)-globin expression (8.1-fold) and induction of HPFH phenotype.\(^{21}\)

In this investigation, ZFN was applied to target codon 288 (exon 2) of the \( KLF1 \) gene to induce site-specific cleavage of the gene. The level of \( \gamma \)-globin expression was significantly (nine-fold) elevated in differentiated K562 cells treated with ZFN in comparison with differentiated untreated cell line based on the quantitative RT-PCR results. HbF levels were assessed to be 5.8-fold higher in differentiated-treated K562 cells as compared with the control cells (0.5%). In a study by Xu et al\(^{22}\) silencing of \( SOX6 \) and \( BCL11A \) genes by shRNA in primary human CD34+ cells reported to increase \( \gamma \)-globin expression in \( BCL11A, SOX6, \) and \( BCL11A-SOX6 \) silenced cells (about five, two, and eight-fold, respectively). Basically, this strategy can avoid the side effects of using chemotherapeutic agents such as cytotoxicity, growth-inhibitory effect, and their possible carcinogenic effect. In the last studies, \( SOX6 \) gene disruption using ZFN and CRISPR/Cas9 was performed and effectively increased \( \gamma \)-globin expression.\(^{17,36}\)

One of the main problems of \( \beta \)-thalassemia patients is ineffective erythropoiesis; the cells get involved in the compensatory synthesis of \( \beta \)-globin chain which is defective due to mutation.\(^{37}\) Although, \( \gamma \) to \( \beta \) switching would stop in case of the \( KLF1 \) gene knocking out, ineffective erythropoiesis will cease in addition to gamma reactivation. Moreover, the Maltese HPFH family reported to show increased HbF, whereas the function of other organs of the body have no problem and this fact reduces our concern in \( KLF1 \) gene manipulation.

In this study, lentiviral vectors were generated to deliver ZFN to the target cell line. These vectors may still be restricted in efficacy by transgene silencing due to DNA methylation and heterochromatinization through chromosomal positional effects which negatively affect the consistency and stability of the transgenic expression in lentiviral-based gene therapy.\(^{38}\) In the present study, integrase-defective lentiviral vector was constructed to decrease disadvantages of lentiviruses such as random integration.

Although ZFN is more costly and suffers from some designing limitations,\(^{15}\) it has advantages including minimum off-targets and could be applied in a protein-based format. Moreover, there are some criticism due to high frequency of off-targets due to the CRISPR/Cas9 technology which is stated repeatedly in other previous studies.
Since vector-based delivery is associated with more abundant off-targets due to difficulty in their expression control, use of optimum amounts of gene editing tools (GET) in either RNA or protein-based constructs with no associated vectors should be considered for gene therapy purpose. GETs could be optimally used by accurately designing ZFN with minimum off-targets and by using CPPs. Furthermore, the problems of random integration of the vectors into the genome and a great number of off-targets are omitted using GETs.

5 | CONCLUSIONS

In summary, we were able to increase γ-globin expression by manipulating the KLF1 gene using ZFN. As one of the main therapeutic approaches, β-globin gene mutations can be corrected via ZFNs in iPS or HSCs and reestablishment of engineered cell to the patient. Geneticists and molecular biologists could apply ZFNs to delete gene clusters or induce indels in the genes of interest.

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