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ORIGINAL ARTICLE

Inhibition of γ/β Globin Gene Switching in CD 34$^+$ Derived Erythroid Cells by BCL11A RNA Silencing

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Abstract The induction of fetal haemoglobin (Hb F), due to the sustained clinical effects, is one of the most promising methods for the treatment of β hemoglobinopathies, such as thalassemia major and sickle cell disease (SCD). Inhibition of γ-globin gene silencing, possibly is a suitable strategy to induce HbF expression in these patients. In this study, the possibility of increasing HbF in the CD34$^+$ derived erythroid cells was investigated by BCL11A inhibition using specific small-interfering RNAs (siRNAs). Human peripheral blood-derived hematopoietic stem cells were isolated and differentiated to erythroid cells. Erythroid maturation was investigated using cell morphology parameters and flow cytometry analysis of CD235a expression On day 20, siRNA complementary to BCL11A was transfected to differentiating cells via electroporation. BCL11A expression was evaluated through real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme linked immunosorbant assay (ELISA). β actin was used as the reference gene to confirm the relative expression level of BCL11A gene mRNA. 48 hours after transfection, BCL11A siRNA significantly reduced BCL11A mRNA levels and consequently led to 2.0 fold decrease in corresponding protein. On the 28th day, haemoglobin electrophoresis results showed that Hb F levels in transfected erythroid cells increased 3.3-fold when compared with non transfected cells. In this study we showed that BCL11A inhibition in erythroid cells could increase fetal hemoglobin, and this strategy can be the basis for designing a γ globin expressing cellular system to increase Hb F in patients with thalassemia and SCD.

Keywords Erythroid cells · Gene silencing · BCL11A · SiRNA

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Introduction

β hemoglobinopathies, such as β thalassemia, and SCD are two of the most common genetic disorders in the world. These disorders are autosomal recessive and as a result of the gene mutation of the β globin chain [1, 2].

In thalassemia, there is a reduction or non-synthesis of the β globin chain. The main pathophysiology of which is the extra uncoupled alpha chain in the red blood cells in the peripheral blood and its precursors in the bone marrow, which causes hemolysis and ineffective erythropoiesis, respectively. β thalassemia major is a severe form of disease associated with severe anemia [3].

In SCD, the replacement of hydrophobic valine instead of the negatively charged glutamic acid at position 6 results in the synthesis of a defective β chain of adult hemoglobin (HbS, βGlu6Val) which is polymerized in deoxygenated conditions and causes crises of vascular obstruction and hemolysis [4, 5]. Regular blood transfusion is used for the treatment of these patients which is supportive and acts as a temporary treatment but is associated with severe complications.

Definitive treatment for both disorders is a bone marrow allograft transplant, and this is only successful if there is a suitable HLA for the patient. The most important limitation is the lack of suitable donors, resulting in acute and chronic graft-versus-host reactions (GVHD) [1, 6]. New approaches are considered for the treatment of inherited hemoglobin disorders such as gene therapy and alteration of the γ globin gene expression and reactivation of this gene in adult erythroid cells [7]. It seems that preventing the extinction of γ globin gene has reduced the severity of the disease and improved clinical symptoms, and so far various drugs and methods have been used to achieve this goal [2].

Three quantitative trait loci (QTL) are responsible for changes in hemoglobin F levels in patients with β thalassemia and sickle cell anemia. One of these loci XMN1-HBG2 is associated with the β gene cluster, and the other two are HBS1L-MYB on the chromosomes 6q23 and BCL11A on the chromosome 2P16. It has been shown that BCL11A has the highest association with elevated levels of hemoglobin F [8–11]. BCL11A is a Zinc finger transcription factor, which is related to the developmental stage of erythroid cells, so that primitive and fetal liver erythroid progenitors expressing high levels of γ globin, have low expression and a smaller variant of this factor, but in adult erythroid cells, the level of this factor increases [12]. This factor is linked to the β gene cluster control loci (LCR) and an intragenic sequence between δ and γ globin genes and acts as the main regulator of γ/β switch during erythroid development [9, 13].

BCL11A inhibition, unlike other factors involved in regulating β globin gene expression, does not cause erythroid maturation defects, and pharmacological inducers which increase hemoglobin F such as histone deacetylase and methyltransferase inhibitors are better off in the absence of BCL11A [14, 15].

In this study, it has been attempted to inhibit BCL11A by siRNA in CD34 $^{+}$ derived erythroid cells, resulting in increased γ globin expression for gene therapy purposes.

Materials and Methods

CD34^{+} Isolation

Of healthy volunteers, 500 ml of whole blood was collected in citrate phosphate dextrose (CPD) anticoagulant. Peripheral blood mononuclear cells (PBMNCs) were isolated using ficoll, according to the manufacturer's instructions (Lymphodex, Inno-Train, Germany). In short, the peripheral blood diluted 1: 3 with phosphate buffered saline (PBS) and slowly layered on a ficoll solution. After centrifugation, the layer of PBMNCs was harvested. CD34^{+} hematopoietic stem cells were isolated by CD34 MicroBead Kit according to the manufacturer's instructions (Miltenyi Biotec, Germany). Briefly, PBMNCs washed in PBS buffer, then for every 10^{8} cells, 300 µl buffer, 100 µl of FCR blocking and 100 µl of CD 34 MicroBead were added and the mixture was incubated for 30 min at 2–8° C. After centrifuging, cell pellet was suspended in 500 µl buffer and inserted into the MACS column. Finally, by placing the MACS column in the magnetic field, CD34^{+} cells were isolated.

Erythroid Differentiation

Erythroid progenitors were differentiated from CD34^{+} HSCs using one-phase liquid medium culture system in a 6-well culture plate at a density of 6×10^{6} cells per ml of Iscove's Modified Dulbecco's Medium (IMDM, Caisson, USA) containing 30% Not heat-inactivated fetal bovine serum (Gibco, USA), 1% bovine serum albumin, β-mercaptoethanol (10^{-5} M, Sigma, USA), human holo-transferrin (0.3 mg/mL, Sigma, USA), dexamethasone (10^{-6} M, Sigma, USA) and StemSpan^{TM} Erythroid Expansion supplement which premixed recombinant human cytokines (stem cell factor (SCF), interleukin-3 (IL-3) and erythropoietin (EPO))(STEMCELL Technologies, Canada). Cells were incubated at 37 °C with 5% CO2 for 28 days and the differentiation medium was refreshed every 3 days. Cells were monitored for the formation of erythroid colonies on days 3, 7, 10 and 18 by inverted microscope and prepared for morphology assessment (Wright staining) on days 10 and 18.

Flow Cytometry

Erythroid differentiation was assessed by the percentage of the erythroid-specific surface marker; CD235a (Glycophorin A) positive cells. Cells were collected, transferred to a 1.5 ml microtube and spun down at 500 xg for 5 min. In order to avoid interfering with pre-existing red blood cells (RBCs), the cell pellet was washed in 1 ml of RBC lysing solution and incubated with 5 µl of monoclonal anti-Glycophorin A-phycoerythrin (PE) (Dako, Denmark). The cells were washed in PBS and the single cell suspension prepared in 500 µl PBS, and it was subjected to a flow cytometer (BD FACSCalibur, USA) versus PE-labeled isotype control. Data were analyzed using the FlowJo 7.6 software (Three Star Inc., USA).

siRNA Transfection

BCL11A siRNA was purchased from Santa Cruz Biotechnology, Inc. (sc-43578) along with control siRNA (Fluorescein conjugated) (SC-36869). Control siRNA was used to monitor transfection efficiency by fluorescence

Table 1 Primer sequences used for RT-qPCR (Quantitative reverse transcription polymerase chain reaction)

Gene	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
BCL11A	GTCTCGCCGCAAGCAAGG	GCCGTGGTCTGGTTCATCATCT
γ-globin	TTCACAGAGGAGGACAAGGCTAC	GCAGAGGCAGAGGACAGGTT
β-globin	CTGAGGAGAAGTCTGCCGTTA	AACAGCATCAGGAGTGGACA
β-actin	ATCGTGCGTGACATTAAGGAG	GAAGGAAGGCTGGAAGAGTG

microscopy and flow cytometry. On day 20, siRNA was transfected into cells using electroporation. One day before transfection, cells were incubated in an antibiotic-free IMDM. 2×10^6 cells were added in 4 mm cuvettes with 10 µl siRNA (or Fluorescein conjugated control). Optimized parameters of electroporation were used; voltage 270 V and capacitance 850 µF (Gene Pulser Xcell electroporation System, Bio-Rad, USA). After electroporation, cells were incubated 5 min on ice and then replaced into the medium.

Transfection Efficiency

Erythroid cells which transfected with Fluorescein conjugated control were detected with flow cytometry and fluorescent microscopy on the day of transfection. Cells were initially harvested and washed in PBS, and then assessed by BD FACSCalibur flow cytometer. Also, a number of cells were evaluated by Fluorescence Microscope Axiostar Plus (Gottingen, Germany).

Quantitative Reverse Transcription Polymerase Chain Reaction

Within three consecutive days after transfection (24, 48 and 72 h after transfection), cells were collected and TRIzol reagent was used for total RNA extraction (Life Technologies, USA) and quantified using the NanoDrop spectrophotometer. Subsequently, DNaseI treated RNA was reverse transcribed to complementary DNA (cDNA) by using the one-step SYBR PrimeScript RT Reagent Kit (TaKaRa, Japan). Relative quantification of β globin, γ globin, and BCL11A genes expression was assessed using specific primers and SYBR Green PCR master mix (TaKaRa, Japan) (Table 1). β actin was used as the reference gene to confirm the relative expression level of these gene mRNAs in both transfected and non-transfected cells. All reactions were undertaken in duplicate.

ELISA; Enzyme-Linked Immunosorbent Assay

ELISA method was used to measure BCL11A levels in erythroid cells. (ABIN420201, Germany). After 72 h of transfection (day 23), cell lysate was made from transfected and non-transfected cells. Briefly, cells were washed three times in cold PBS and cell lysate was prepared using three freeze-thawing cycles at − 20 °C, and debris was removed by centrifugation (1500×*g* for 10 min at 2–8 °C). Absorption spectroscopy was used to determine the total protein concentration. The supernatant was used for ELISA measurement. The BCL11A protein levels were measured as triplicate.

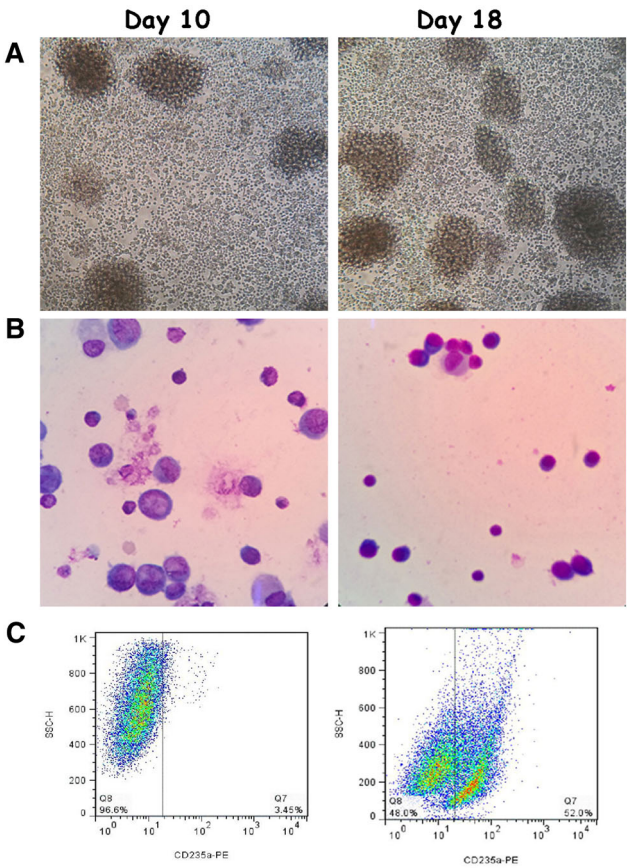

Fig. 1 Examination of erythroid differentiation by inverted (× 40), light (× 100) microscopic assays and expression analysis of CD235a by flow cytometry. **a** Erythroid colonies on day 10 and 18. **b** Wright's stain of differentiated erythroid cells on day 10 and 18, cells displayed pronormoblast and basophilic normoblast morphology on day 10 and mostly polychromatophilic normoblast on day 18. **c** CD235a expression in differentiated cells on day 18 with 52% CD235a positive cells (Right) compared with Negative (isotype) control for CD235a (left)

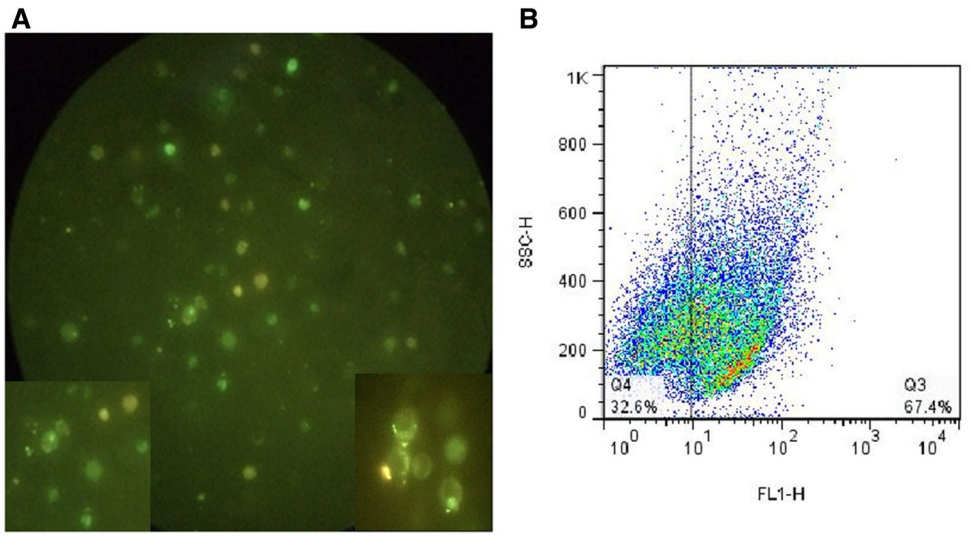

Fig. 2 Transfection efficiency in erythroid progenitor cells (EPCs). **a** Transfection efficiency was assessed through fluorescent microscope. **b** Flow cytometry post-transfection, the dot-plot histogram represents fluorosin conjugated scramble control siRNA uptake by about 67% of cells

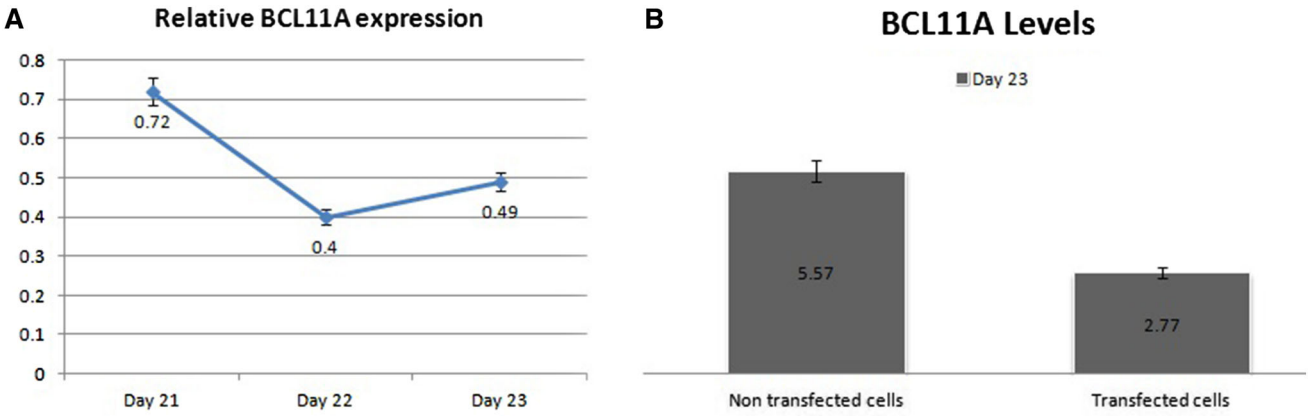

Fig. 3 Effect of BCL11A siRNAs on the BCL11A expression. **a** Relative expression of BCL11A transcript in transfected cells was significantly reduced compared to non-transfected cells, with the highest reduction occurring 48 h after transfection ($p < 0.05$). **b** BCL11A protein levels by ELISA which showed twofold decrease in the treated cells compared to non-treated cells (2.77 ng/ml vs. 5.57 ng/ml) ($p < 0.05$)

High-Performance Liquid Chromatography

At the end of the 28-day course of differentiation, cell lysates were prepared from transfected erythroid cells. Additionally, non-treated erythroid cells on day 28 of differentiation were collected and hemolysates were prepared. The levels of HbF in hemolysate were analyzed by HPLC (Knauer Liquid Chromatography System, Germany).

Statistical Analysis

Data are presented as mean ± SD based on replicate or triplicate experiments. To compare unpaired groups, independent *t* test was used. The results with $p < 0.05$ was considered as statistically significant.

Result

$CD34^+$ Isolation and Erythroid Differentiation

$CD34^+$ cells were successfully isolated from peripheral blood mononuclear cells. Flow cytometric study with anti-CD34 showed a high percentage of cell purity (88.8%). $CD34^+$ cells were differentiated into erythroid cells. Cells were examined in culture medium every day by inverted microscope. The early formation of erythroid colonies was observed from the third day of culture initiation. Erythrocyte colonies continued to grow and a re-examination of erythroid colonies was performed between days 10 and 18 (Fig. 1a). Cells displayed the successful differentiation of the erythroid lineage with Wright's stain, which showed that the cells at these stages were most pronormoblast,

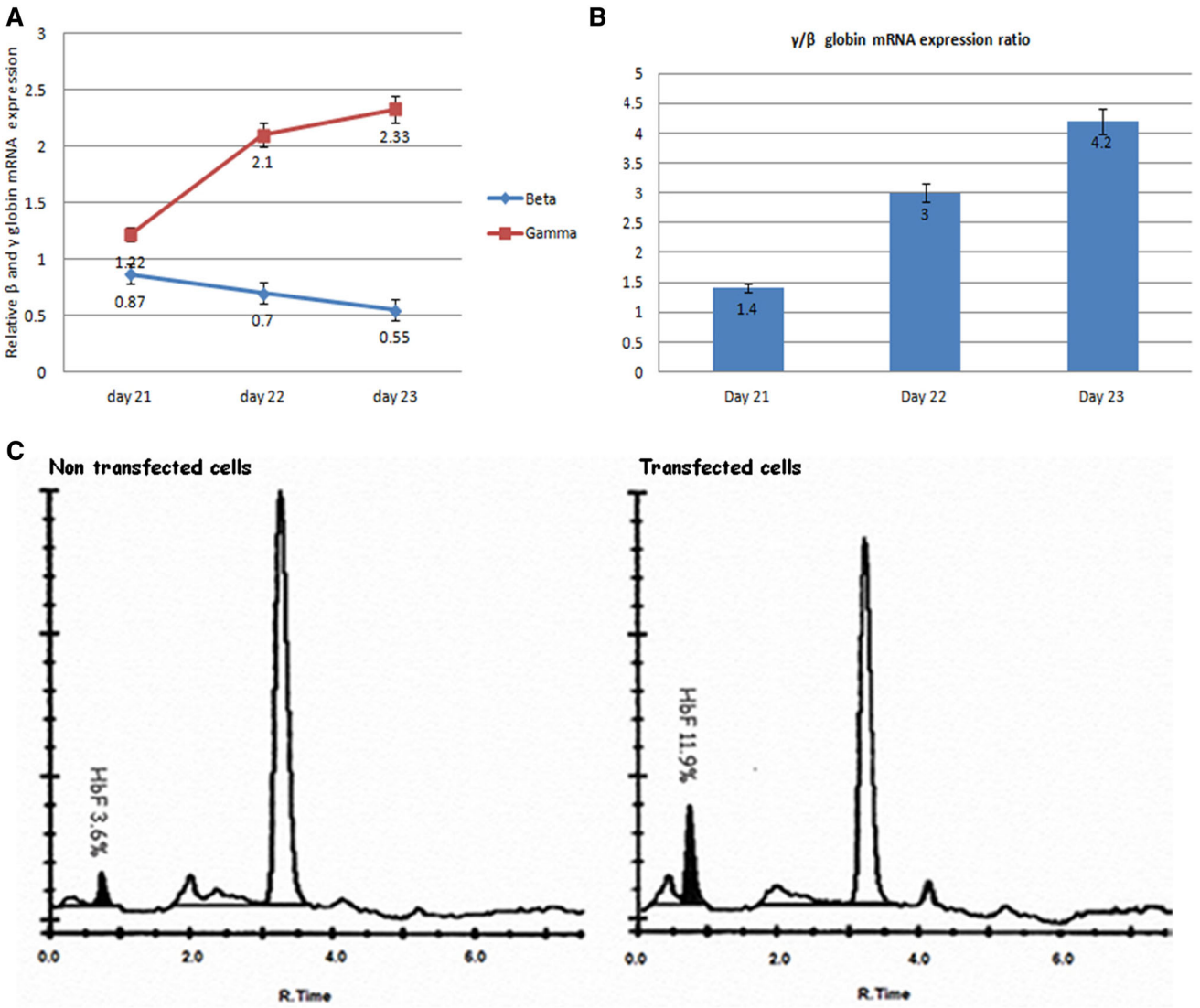

Fig. 4 Expression analysis of β-globin, γ-globin, γ/β-globin mRNA ratio and fetal hemoglobin. **a** Relative expressions of β globin in transfected cells on days 21, 22 and 23, were decreased by 0.87, 0.7 and 0.55 respectively ($p < 0.05$) and, the relative expressions of γ globin increased by 1.22, 2.1 and 2.33, respectively ($p < 0.05$). **b** The γ/β expression ratios on days 21 to 23 were 1.4, 3.0 and 4.2, respectively ($p < 0.05$). **c** Hb F measurement by HPLC represented by 3.3 fold increase 11.9% in transfected cells vs. 3.6% in non-transfected cells ($p < 0.05$)

basophilic normoblast and polychromatophilic normoblast (Fig. 1b). A flow cytometric study was performed on day 18 using CD235A antibody and it was found that about 52% of cells have this specific marker of erythroid lineage (Fig. 1c).

Transfection Efficiency

On day 20, transfection of differentiated erythroid cells with BCL11A siRNA was performed by the electroporation method. Fluorosin conjugated scramble control was used for the assessment of transfection efficiency. Green fluorescent signal visualized from fluorosin in cells showed that the cells were successfully transfected (Fig. 2a). A flow cytometric study showed that the transfection efficiency in these cells was about 67% (Fig. 2b), which showed that transfection of differentiated erythroid cells with BCL11A siRNA has been successfully performed.

Effect of BCL11A siRNAs on the BCL11A Expression

In order to evaluate the effect of siRNA treatment on erythroid cells, the expression of BCL11A at the mRNA level was evaluated using qRT-PCR according to the $2^{-\Delta\Delta CT} \times 100\%$ formula. It was found that the expression of BCL11A was significantly reduced compared to non-treated cells, with the highest reduction occurring 48 h

after transfection. Relative expression percentage of BCL11A at 24, 48 and 72 h after transfection were 72, 40 and 49%, respectively ($p < 0.05$) (Fig. 3a). ELISA was used to investigate the effects of siRNA treatment on BCL11A protein expression. After 72 h of transfection, the level of BCL11A protein was measured in transfected and non-transfected cell lysate which showed a decrease of twofold in the treated cells compared to non-treated cells. On the third day following transfection, the level of BCL11A protein was 2.77 ng/ml, while being 5.57 ng/ml in control cells ($p < 0.05$) (Fig. 3b). All data have shown three independent experiments. These findings showed that specific siRNA against BCL11A could reduce the expression of mRNA and decrease the twofold protein content of BCL11A and the goals we followed in the study were achieved.

The Expression Level of γ/β-Globin and Fetal Hemoglobin Following BCL11A siRNA Transfection

In three consecutive days after transfection, the relative level of β and γ globin expression in transfected and non-transfected cells was evaluated using qRT-PCR. A decreasing trend was observed in the relative expression rates of β globin, being 0.87, 0.7 and 0.55 on days 21, 22 and 23, respectively ($p < 0.05$). On the contrary, the relative expression rates of γ globin had an increasing trend of 1.22, 2.1 and 2.33, respectively ($p < 0.05$) (Fig. 4a). The γ/β expression ratios on days 21 to 23 were 1.4, 3.0 and 4.2, respectively ($p < 0.05$) (Fig. 4b). These findings showed that following the reduction of BCL11A expression by siRNA, the γ globin expression significantly increased and β globin expression decreased. Hemoglobin electrophoresis with HPLC at the differentiation endpoint (day28) showed that the amount of fetal hemoglobin in erythroid cells which previously transfected with BCL11A siRNA was 3.3 fold greater in comparison with non-transfected erythroid cells (11.9 vs. 3.6, $p < 0.05$) (Fig. 4c). In sum, these findings showed that BCL11A siRNAs were effective in reducing the expression of BCL11A mRNA and protein. They were also showed that BCL11A inhibition in $CD34^+$ derived erythroid cells; induce fetal hemoglobin production which is the goal of therapeutic strategies for thalassemia and SCD.

Discussion

Manipulation of the fetal hemoglobin to adult hemoglobin switching via induction of silencing of HbF repressors may improve clinical manifestations of β hemoglobinopathies. BCL11A, one of the most effective regulators of HbF switching, was until recently considered a potential clinical target.

In 2008, Uda et al. showed that BCL11A levels are related to the inherited continuation of fetal hemoglobin and reduce the severity of clinical symptoms in β thalassemia patients [16]. In 2011, Xu J et al. showed that, by inhibiting BCL11A and interfering with γ globin expression, the increase in F-hemoglobin results in the improvement of SCD in mice [14]. In another study by Wilber using shRNA-BCL11A, an increase in F-hemoglobin levels in erythroid progenitors derived from $CD34^+$ cells was shown in thalassemic patients [17].

Small interfering RNA or siRNA is the most commonly observed RNA interference that is used to silence specific proteins in the cell. The effects of RNA interference (RNAi) were first reported by Napoli and colleagues as a result of their efforts to increase the expression of chalcone synthetase (CHS), an enzyme responsible for plant color in petunia [18]. Today, in a wide range of diseases, therapeutic efforts are being made using siRNA [19–21].

Although many studies have been conducted on the β-globin locus, studies that focus on regulating the normal hemoglobin switch and the re-activation of HbF in erythroid progenitor cells are still of interest.

The main objective of this study was to examine the inhibition of the BCL11A in erythroid progenitors derived from $CD34^+$ cells by the RNA interference and its subsequent effects on the β and γ globin expression and fetal hemoglobin. In our work, due to the lack of globin-beta expression in K562 cells [22, 23], which was used in some previous studies, we used $CD34^+$ derived erythroid cells.

According to the studies that show the fetal hemoglobin switching process occurs around day 21 of the erythroid differentiation [24], we transfected BCL11A siRNA into differentiated erythroid cells on day 20. After transferring the siRNA into the differentiated erythroid cells, the expressions of β globin, γ globin, and BCL11A were measured at the mRNA level using qRT-PCR. Additionally, the level of BCL11A protein was measured by ELISA.

The results showed that the expression of the BCL11A protein was significantly reduced in cells treated with siRNA BCL11A compared with untreated groups. Reduction of BCL11A mRNA followed by twofold decrease of BCL11A protein and a relative increase in γ globin expression suggests that the fetal to adult hemoglobin switching process has been partially inhibited in $CD34^+$ derived erythroid cells, which ultimately leads to a 3.3-fold increase in fetal hemoglobin.

Up-regulation of fetal hemoglobin, due to the effect of reducing the severity of clinical symptoms in patients with thalassemia and SCD, can be used as a therapeutic goal in these patients. Although this study has been conducted

in vitro but may be a useful guide for in vivo studies and can be the basis for the design of the γ globulin-expressing cellular system for gene therapy purposes.

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Compliance with Ethical Standards

Conflict of interest All authors of this article declare that they have no conflict of interest.

Human and Animal Rights This article does not contain any studies with human participants performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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