Downregulation of KLF4 activates embryonic and fetal globin mRNA expression in human erythroid progenitor cells

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Abstract. The Krüppel-like factor (KLF) family dominates highly conserved three zinc finger DNA binding domains at the C-terminus and variable transactivation domains at the N-terminus. Humans possess 18 *KLF* genes that are differentially expressed in various tissues. Several KLFs recognize a specific CACCC DNA motif that is commonly found within hematopoietic-specific promoters. To investigate those KLFs that are involved in human hemoglobin (Hb) switching, the present study analyzed a previous microarray data set from fetal and adult erythroid cells and validated the mRNA expression levels of 18 *KLF*s by reverse transcription-quantitative PCR (RT-qPCR). *KLF* with a decreased expression level in the fetuses was selected for a functional study in human erythroid progenitor cells using lentiviral-based short hairpin RNA knockdown. The fetuses demonstrated a lower level of *KLF4* mRNA expression when compared with the adults. Downregulation of KLF4 in erythroid progenitor cells from healthy individuals and individuals with β^0-thalassemia/HbE evidenced the increasing embryonic and fetal globin mRNA expression with neither significant cytotoxicity nor gene expression alteration of the examined globin regulators, KLF1, B-cell lymphoma/leukemia 11A and lymphoma/leukemia-related factor. These findings demonstrate that the downregulation of KLF4 is associated with increased embryonic and fetal globin gene expression in human erythroid progenitor cells. Moreover, identifying putative compounds or molecular approaches that effectively downregulate KLF4 and further induce embryonic globin expression may provide an alternative therapeutic strategy for α-globin substitution in severe α-thalassemia.

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Introduction

Fetal hemoglobin ($\alpha_2\gamma_2$; also termed HbF) to adult Hb ($\alpha_2\beta_2$; also termed HbA) switching is usually completed within one year after birth and results in a normal Hb profile in adulthood, comprising HbF (<1%), HbA_2 ($\alpha_2\delta_2$; 2-3%) and HbA (≥95%) (1). This obscure scheme is controlled by many zinc finger transcription factors, such as Krüppel-like factor 1 (KLF1) (2), B-cell lymphoma/leukemia 11A (BCL11A) (3) and leukemia/lymphoma-related factor (LRF) (4). KLF1 is known as an erythroid-specific transcription factor that orchestrates the expression of hundreds of erythroid essential genes, including fetal and adult globin genes (2,5). KLF1 regulates fetal to adult Hb switching by direct activation of β-globin (*HBB*) gene expression and indirect repression of the γ-globin (*HBG*) gene by stimulating the expression of BCL11A and LRF, two major *HBG* gene repressors (6,7). Furthermore, the docking of KLF1 on the CACCC core box within *HBB*, *BCL11A* and *LRF* promoters was mentioned as an initial step of gene expression (6,7). Downregulation of KLF1 reduces BCL11A expression and further increases *HBG* gene and HbF production (2,7). In addition, KLF1 occupies the *LRF* promoter in human erythroid cells and activates *LRF* gene expression (6). KLF1 is categorized as a member of the KLF family. To date, 18 *KLF* genes have been described in human chromosomes (8,9). KLF members are characterized by three Cys_2His_2 zinc finger DNA binding domains at the C-terminus, and variable transactivating domains at the N-terminus. The binding domains of KLFs recognize specific DNA motifs containing GC-rich or CACCC boxes, whereas the transactivating domains harness transcriptional levels of the target genes upon which co-activators or co-repressors are recruited (10). Notably, the CACCC motif has been identified as a common regulatory element in erythroid gene promoters, particularly in globin gene promoters (6,11,12).

Furthermore, KLF1 amino acid sequences have the highest homology with KLF2 and KLF4 (13). KLF2 (or lung KLF) regulates embryonic globin gene expression and is essential for erythroid maturation within the yolk sac in transgenic mice (14,15). KLF4 (or gut KLF) binds to the CACCC motif within the *HBG* gene promoter and activates *HBG* gene expression in early erythroid progenitor cells from adults (16). Additionally, KLF4 is able to dock on the α-globin (*HBA*) gene promoter and stimulate the gene expression in the human chronic myeloid leukemia (K562) cell line (17). In zebrafish, *klf4* acts as an embryonic globin gene suppressor; however, the arrangement of globin genes in zebrafish differs from that of humans (18).

The present study proposed that additional KLF members may be crucial for human globin gene regulation in erythroid cells. To ascertain a potential for KLF in exploiting globin gene regulation, the mRNA expression of 18 *KLF*s in erythroid progenitor cells from fetal liver and adult peripheral blood samples were screened for their relative differences in terms of the globin gene expression status at specific developmental stages. *KLF* with the greatest decrease of mRNA expression in the fetus was chosen for a functional study using RNA interference in adult peripheral blood-derived erythroid progenitor cells. Effects of *KLF* knockdown during *in vitro* erythropoiesis were assessed. Reactivation of embryonic or fetal globin gene expression after the *KLF* knockdown was investigated. These changes in globin gene expression can be exploited for potential therapeutic implications for severe thalassemias in which a specific globin gene was insufficient.

Materials and methods

Samples. Existing cDNA samples from a previous microarray study (19) were used as templates for investigating *KLF* expression patterns in the present study. The cDNA samples were prepared from the RNA of hematopoietic progenitor cells from fetal liver samples (n=3) and adult peripheral blood samples (n=3). Whole blood samples were drawn from six adults who were aged ≥18 years, consisting of healthy volunteers (50 ml; n=3) and patients with β^0-thalassemia/HbE (25 ml; n=3) at Faculty of Allied Health Sciences, Thammasat University (Pathum Thani, Thailand) in June 2020 after obtaining written informed consent. Anticoagulant Citrate Dextrose Solution, Solution A (Terumo Bct, Inc.) was used as a preservative. Thalassemia and other hemoglobinopathies were ruled out in the healthy subjects. The most common deletional α-thalassemia mutations, including-$\alpha^{3.7}$, -$\alpha^{4.2}$, --^{SEA} and --^{THAI} were screened for exclusion, and *HBB* genes were genotyped in the subjects with β^0-thalassemia/HbE. At the time of sampling, the patients had not received any blood transfusions for ≥1 month before venipuncture. All hematological parameters and Hb typing were obtained from an automated blood cell analyzer (ADVIA 2120; Bayer AG) and the VARIANT II β-thalassemia Short Program (Bio-Rad Laboratories, Inc.), respectively. The hematological data of the subjects are presented in Table SI.

Erythroblast culture. Peripheral blood mononuclear cells (PBMCs) were immediately harvested from the blood samples using Lymphoprep (Axis-Shield). Hematopoietic stem cells or progenitor cells ($CD34^+$) were purified from the PBMCs using a CD34 MicroBead Kit (Miltenyi Biotec GmbH) with a magnetic activated cell sorting column according to the manufacturer's instructions. The isolated $CD34^+$ cells were placed in the 3-phase erythroblast culture system where the cells were able to grow, expand and differentiate into erythroid cells. Briefly, Iscove's modified Dulbecco's medium (Biochrom GmbH) supplemented with 20% v/v fetal bovine serum (FBS; EMD Millipore), 300 μg/ml holo-transferrin (ProSpec-Tany TechnoGene Ltd.) and 1% v/v penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) was prepared as a basal medium. In phase 1 (days 0-4), the basal medium was augmented with 10 ng/ml human interleukin-3 (IL-3; Miltenyi Biotec GmbH), 50 ng/ml human stem cell factor (SCF; Miltenyi Biotec GmbH) and 2 U/ml erythropoietin (EPO; Janssen-Cilag Ltd.). In phase 2 (days 4-8), the basal medium was fortified by 10 ng/ml SCF and 2 U/ml EPO. In phase 3 (days 8-14), the basal medium was only supplemented with 4 U/ml EPO. As the number of isolated $CD34^+$ cells varied among individuals, cell numbers were reserved at 1-2x10^6 cells/ml during the culture. The cells were incubated at 37˚C in a 5% CO_2 and 100% humidity atmosphere.

KLF4 knockdown. KLF4sh1 (cat. no. TRCN0000231078) and KLF4sh2 (cat. no. TRCN0000231079) short hairpin (sh)RNA sequences were obtained from the MISSION® shRNA library (Sigma-Aldrich; Merck KGaA; https://www.sigmaaldrich.com/catalog/genes/KLF4?lang=en&region=GB#shRNA%20Products) and cloned into the third-generation lentiviral vector, pLL3.7-puro, containing the mouse U6 promoter for driving shRNA expression and the puromycin resistance gene as a selectable marker. pLL3.7-puro was in-house modified from pLL3.7 (cat. no. 11795; Addgene, Inc.) by replacing the EGFP gene with the puromycin resistant gene. The 293T cells (cat. no. CRL-3216; American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% v/v FBS and 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.). When the cells reached ~80% confluency, lentiviruses expressing different shRNAs targeting *KLF4* mRNA were produced by co-transfecting the expression constructs with packaging plasmids, including 2.5 μg of pMD2.G (cat. no. 12259; Addgene, Inc.), 3.75 μg of pMDLg/pRRE (cat. no. 12251; Addgene, Inc.) and 3.75 μg of pRSV-Rev (cat. no. 12253; Addgene, Inc.) into the 293T cells using the X-tremeGENE™ HP Transfection Reagent (Roche Diagnostics GmbH). Supernatants were collected at 48 and 72 h after transfection and filtered through a 0.45-μm membrane. The filtrates were concentrated using a Lenti-X Concentrator (Clontech Laboratories, Inc.) and centrifuged at 4˚C, 1,500 x g for 1 h. Lentiviral titers were measured by transducing the viruses into 293T cells in the presence of 4.0 μg/ml polybrene (EMD Millipore) and were subsequently challenged by 2.0 μg/ml puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 48 h post-transduction. A non-targeting control shRNA sequence (cat. no. SHC016V; EMD Millipore) served as a negative control (shNTC).

Erythroid cells from day 4 of culture were transduced overnight with the lentiviruses at a multiplicity of infection of 20 in the phase 2 medium supplemented with 8.0 μg/ml

polybrene. Transduced cells were treated with 0.5 μg/ml puromycin at 48 h post-transduction and were consequently placed in the phase 3 medium at 48 h after puromycin selection.

Erythroid differentiation and morphology. Cultured cells ($5x10^4$ cells) were harvested on day 12 and resuspended in Dulbecco's phosphate-buffered saline (HyClone; Cytiva). Erythroid differentiation was surveilled using antibodies against two erythroid-specific surface markers, phycoerythrin-conjugated mouse monoclonal anti-human CD71 (cat. no. CY1G4; BioLegend, Inc.) and allophycocyanin-conjugated mouse monoclonal anti-human CD235a (cat. no. GA-R2; BD Biosciences). Flow cytometry was achieved on BD Accuri™ C6 Plus (BD Biosciences). Data were analyzed using FlowJo version 10.3.0 (FlowJo LLC). To investigate erythroid cell morphology, cultured cells ($5x10^4$ cells) were fixed with absolute methanol and subsequently stained with modified Giemsa stain (MilliporeSigma) according to the manufacturer's instructions. The stained cells were examined under a light microscope.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cultured cells ($1x10^6$ cells) on day 10 using the TRIzol® Reagent (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The extracted RNA samples (1 μg each) were treated with DNase I (Thermo Fisher Scientific, Inc.) to remove DNA contamination and were reverse transcribed into cDNAs using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RT-qPCR was performed in duplicate using gene-specific primers, as presented in Table SII and FastStart™ Essential DNA Green Master (Roche Diagnostics GmbH) according to the manufacturer's instructions. qPCR was performed and analyzed using a CFX96 Real-Time System (Bio-Rad Laboratories, Inc.) with pre-incubation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec and 72˚C for 10 sec. All target gene expression levels were normalized to β-actin (*ACTB*) mRNA expression levels. Relative gene expression data were calculated using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. Nuclear and cytoplasmic proteins were extracted from a pellet of at least $5x10^6$ cultured cells on day 10 using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The protein concentration was determined using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. A total of 10 μg protein was run on 10% SDS-polyacrylamide gel, transferred to a polyvinylidene fluoride membrane and subsequently blocked at room temperature for 1 h with 5% skimmed milk in PBS supplemented with 0.05% Tween-20. Immunoblotting was performed by incubating the membranes with specific antibodies against their target proteins (Table SIII) at room temperature for 1 h. The membrane was washed three times for 10 min each with blocking buffer. HRP-conjugated secondary antibodies were then added and incubated at room temperature for 1 h before signal development. Chemiluminescent detection was conducted with Amersham™ ECL™ Prime Western Blotting Detection Reagent (Cytiva) according to the manufacturer's instructions.

Hb typing. Hemolysates were prepared from a minimum of $1x10^6$ cultured cells on day 14 and subjected to high-performance liquid chromatography for Hb type analysis using the Bio-Rad VARIANT II Hemoglobin Testing System with β-Thalassemia Short Program (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocols.

Statistical analysis. Statistical analysis was performed using SPSS version 26.0.0.0 (IBM Corp.). Unpaired student's t-test and one-way ANOVA with Tukey's post hoc test were performed to identify significant differences. Data are presented as means ± SEM and $P<0.05$ was considered to indicate a statistically significant difference.

Results

KLF4 mRNA expression levels decrease in erythroid progenitor cells from fetal liver samples. Our previous study conducted microarray analysis using Affymetrix GeneChip® Human Gene 2.0 ST Arrays to differentiate *KLF* expression patterns in erythroid progenitor cells from fetal liver and adult peripheral blood samples (19). According to the microarray data, *KLF4* and *KLF9* emerged as the top candidate genes due to their greater downregulation (0.79-fold; P=0.06495) and upregulation (1.91-fold; P=0.00628), respectively, in fetuses when compared with adults (Table SIV). The mRNA expression levels of 18 human *KLF*s, along with the key HbF regulators, *BCL11A* and *LRF,* and eight globin genes were subsequently validated by RT-qPCR using the existing cDNA samples obtained from our previous study (19). The results demonstrated that *KLF4* mRNA expression was significantly downregulated (0.32±0.08-fold; P=0.02413; Fig. 1), which was similar to that observed from the microarray data (Table SIV), whereas the upregulation of *KLF9* mRNA expression was not detected by RT-qPCR. Instead, the mRNA expression of *KLF7* was significantly upregulated (1.68±0.07-fold; P=0.03000; Fig. 1). However, certain *KLF* expression patterns obtained from RT-qPCR, for example, undetectable levels of *KLF8*, *KLF12* and *KLF14* (Fig. 1) were inconsistent with the results from the microarray. The discrepancy of the association between results of the microarray and RT-qPCR could be due to different platform conditions. The mRNA expression levels of the two well-characterized HbF regulators, *BCL11A* and *LRF* were also analyzed for their function as essential *HBG* repressors (3,4). Although the results demonstrated that the two were relatively decreased in fetuses when compared with the adults, only the mRNA expression level of *BCL11A* was significantly downregulated in the fetuses (Fig. S1). These results indicated that *KLF4* mRNA expression might be developmentally regulated and associated with changes in fetal globin expression, concomitant with the *BCL11A* mRNA expression level.

KLF4 knockdown increases embryonic and fetal globin gene expression. To determine the effects of KLF4 on globin expression, the expression levels of eight human globin genes were examined using RT-qPCR following KLF4 knockdown

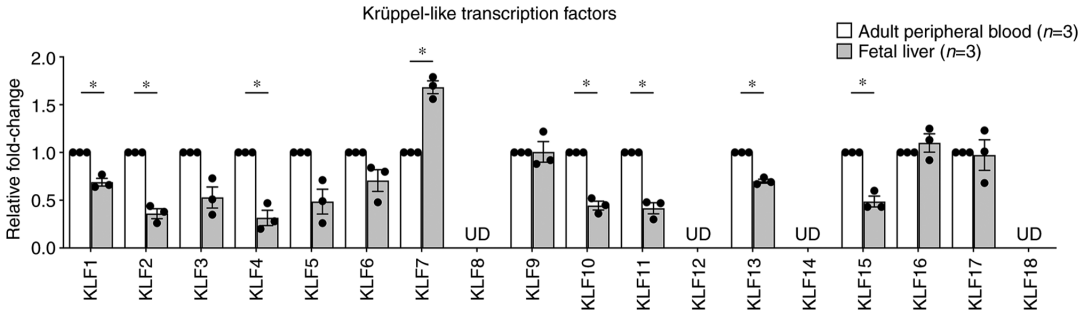

Figure 1. mRNA expression patterns of 18 *KLF*s in erythroid progenitor cells from FL and AB as examined by reverse transcription-quantitative PCR. Gene expression levels were normalized to β-actin. Data are presented as the means ± SEM of relative fold-change of FL (n=3) relative to AB (n=3). *P<0.05. *KLF*, Krüppel-like factor; FL, fetal liver; AB, adult peripheral blood; UD, undetectable.

in human erythroid progenitor cells obtained from healthy individuals and donors with compound heterozygous β^0-thalassemia/HbE, which exhibit high baseline HbF levels. KLF4 expression was more pronounced upon erythroid maturation in the primary erythroid culture system (Fig. S2). KLF4sh1 and KLF4sh2 reduced the KLF4 expression levels by >0.5-fold when compared with shNTC. Furthermore, downregulation of KLF4 did not significantly affect the three known HbF suppressors, KLF1, BCL11A and LRF (Fig. 2A and B). The results revealed that downregulation of KLF4 in hematopoietic progenitor cells from healthy individuals and donors with β^0-thalassemia/HbE evidently upregulated ζ-globin (*HBZ*), ε-globin (*HBE*) and moderately increased *HBG* mRNA expression when compared with shNTC in the two subject groups (Fig. 2C). This alteration was similar to that of globin mRNA expression patterns in hematopoietic progenitor cells from fetal liver samples (Fig. S1). In addition, marginally decreased expression levels of μ-globin (*HBM*), α-globin (*HBA*), θ-globin (*HBQ*), δ-globin (*HBD*) and *HBB* genes were observed when compared with the control (Fig. 2C). The increase of HbF (Δ%HbF) executed by experimental KLF4sh1 knockdown in healthy and β^0-thalassemia/HbE erythroid cells was 2.0±0.8 and 2.7±1.4%, respectively, above the control. The Δ%HbF observed by experimental KLF4sh2 knockdown in healthy and β^0-thalassemia/HbE erythroid cells was 1.5±1.0 and 5.0±1.3%, respectively, above the control (Fig. S3). These changes in HbF levels were consistent with the marginally increased *HBG* mRNA expression observed in Fig. 2C.

The effects of KLF4 knockdown on erythroid cell differentiation and maturation were assessed by evaluating the expression levels of two erythroid-specific surface markers, CD71 and glycophorin A (GPA), and by investigating erythroid morphology under a light microscope. Erythroid differentiation and gating proportions were analyzed based on the expression levels of CD71 and GPA, as follows: R1, $CD71^{high}/GPA^{low}$; R2, $CD71^{high}/GPA^{high}$; R3, $CD71^{medium}/GPA^{high}$; and R4, $CD71^{low}/GPA^{high}$ (Fig. 2D). In the culture system, β^0-thalassemia/HbE-derived erythroblasts demonstrated delayed erythroid differentiation when compared with the healthy erythroblasts (Fig. 2E and F). Downregulation of KLF4 in healthy erythroblasts using KLF4sh1 did not affect erythroid differentiation or maturation. However, KLF4sh2 knockdown in the healthy group insignificantly delayed erythroid differentiation as demonstrated by the reduction in the R4 portion when compared with the controls (Fig. 2F). Similar findings of the fairly delayed differentiation were also observed in the β^0-thalassemia/HbE-derived erythroblasts with KLF4sh2 knockdowns, in which the majority of the cell populations retained in R2 (Fig. 2F). Early-stage erythroid morphology was found in experimental KLF4sh2 knockdown in both healthy and β^0-thalassemia/HbE-derived erythroblasts when compared with the controls (Fig. 2E), confirming a slightly delayed erythroid maturation. Notably, modest promotion of cell differentiation was apparent in β^0-thalassemia/HbE-derived erythroblasts upon KLF4sh1 knockdown as evidenced by the increase of R4 when compared with the controls (Fig. 2F). Following KLF4sh1 knockdown, erythroid morphology accelerated erythroid maturation in β^0-thalassemia/HbE-derived erythroblasts when compared with the controls (Fig. 2E). However, this acceleration was not particularly noticeable in healthy subjects under the same circumstances.

Discussion

Hb switching is specific to human developmental stages, resulting in the production of different Hb molecules during ontogeny (1). However, the mechanisms underlying the switch remain unclear. KLFs are transcription factors classified by highly conserved three Cys_2His_2 zinc finger DNA binding domains at the C terminus (8-10). KLFs bind to a specific CACCC DNA motif, which are commonly found within human globin gene promoters (7,11,12). KLF1 is a member of the KLF family, regulating fetal to adult Hb switching by activating the expression of the major *HBG* repressor, BCL11A. Furthermore, KLF1 can dock on the CACCC boxes within the *HBB* gene promoter and can further activate *HBB* gene expression. Notably, the amino acid sequences of KLF1 are markedly similar to those of KLF2 and KLF4 (13). This similarity may indicate comparable functions between KLF1, KLF2 and KLF4 in relation to globin gene regulation.

In the present study, the mRNA expression pattern of 18 human *KLF*s in fetal and adult erythroblasts were investigated using RT-qPCR. The expression of *KLF4* was revealed to be the most significantly downregulated in erythroid progenitor cells from the fetal liver when compared with those from adults. The result was consistent with our previously reported microarray data (19). This indicates that *KLF4*

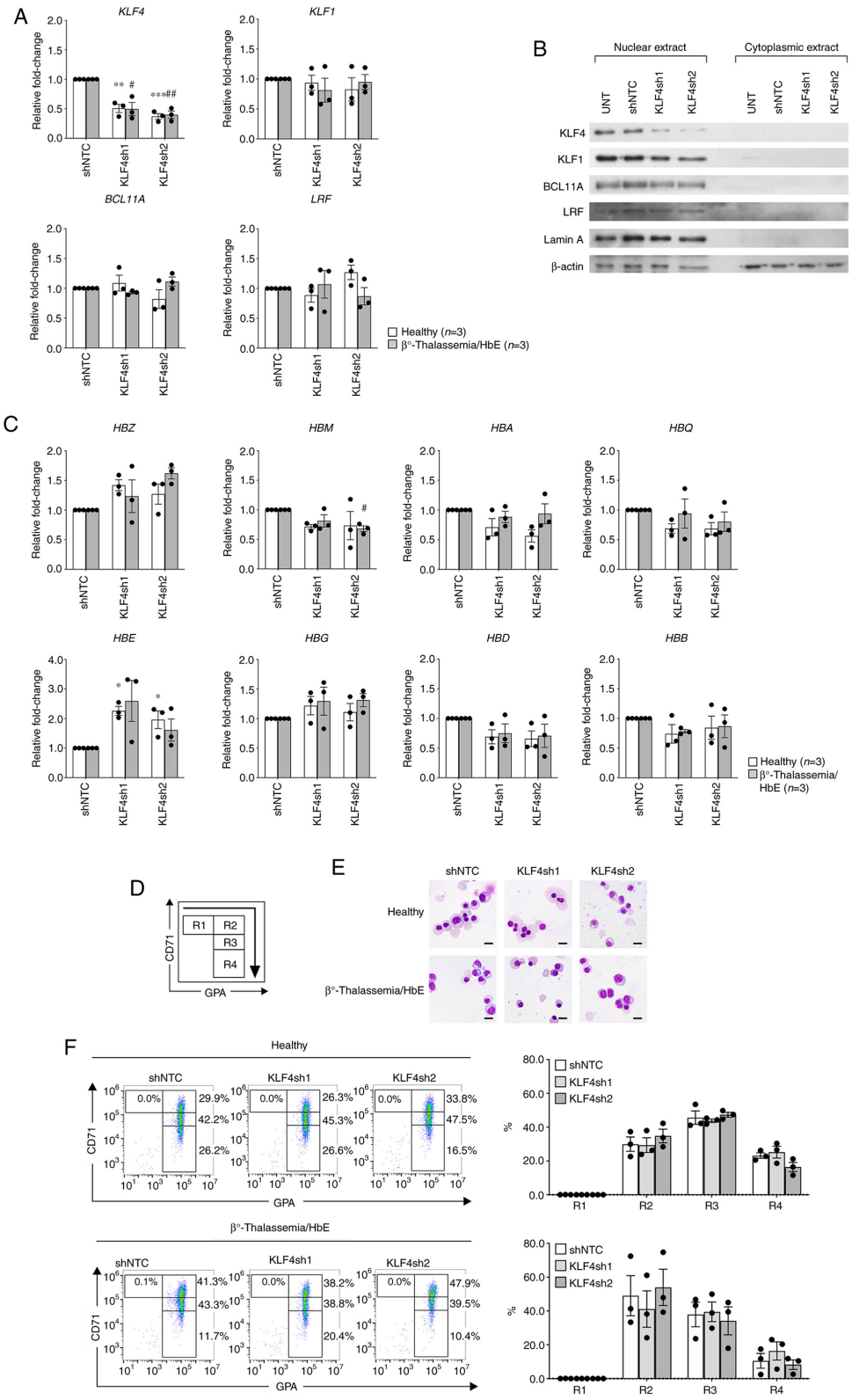

Figure 2. Effects of KLF4 knockdown in erythroid progenitor cells from healthy individuals and patients with β^0-thalassemia/HbE. (A) RT-qPCR demonstrating KLF4 knockdown efficiency and effects on *KLF1*, *BCL11A* and *LRF* mRNA expression levels. (B) Representative western blot analysis showing KLF4 knockdown efficiency and effects on KLF1, BCL11A and LRF protein expression levels. Lamin A and β-actin served as loading controls for nuclear and cytoplasmic protein origin, respectively. (C) RT-qPCR displaying relative fold-change of α-like globin (upper panel) and β-like globin (lower panel) mRNA expression levels. Relative fold-change represents the mRNA expression levels normalized to β-actin of shRNAs targeting KLF4 (KLF4sh1 and KLF4sh2) vs. shNTC. Data are presented as the means ± SEM of healthy individuals (n=3; *P<0.05, **P<0.005, ***P<0.0005) and patients with β^0-thalassemia/HbE (n=3; $^{\#}$P<0.05, $^{\#\#}$P<0.005). (D) Illustrative flow cytometric analysis on the basis of transferrin receptor, CD71 and GPA expression levels. Where R1=CD71^{high}/GPA^{low}; R2=CD71^{high}/GPA^{high}; R3=CD71^{medium}/GPA^{high}; and R4=CD71^{low}/GPA^{high}. (E) Representative cytospin preparations of modified Giemsa-stained cells on day 12 visualized under a light microscope (x1,000 magnification; scale bar, 10 μm). (F) Representative flow cytometric analysis of experimental KLF4 knockdown. The bar graph represents proportions of each cell population. KLF, Krüppel-like factor; HbE, hemoglobin E; RT-qPCR, reverse transcription-quantitative PCR; BCL11A, B-cell lymphoma/leukemia 11A; LRF, leukemia/lymphoma-related factor; shRNA, short hairpin RNA; shNTC, non-targeting control shRNA; GPA, glycophorin A; *HBZ*, ζ-globin; *HBM*, μ-globin; *HBA*, α-globin; *HBQ*, θ-globin; *HBE*, ε-globin; *HBG*, γ-globin; *HBD*, δ-globin; *HBB*, β-globin.

might be developmentally controlled. Furthermore, the lower mRNA expression level of *KLF4* was concomitant with the greater mRNA expression levels of embryonic and fetal globin, including *HBZ*, *HBE* and *HBG*. This result revealed the association between the modest expression of the *KLF4* mRNA level and the pronounced mRNA expression levels of embryonic and fetal globin in erythroid progenitor cells from the fetus. However, it was found that the mRNA levels of two major *HBG* gene repressors, *KLF1* and *BCL11A* were significantly downregulated in the fetus group when compared with the adult group. Therefore, the elevation of fetal globin mRNA expression in the present study might be due to the reduction of these two transcription factors.

To elaborate on the function of KLF4 on globin expression regulation at diverse HbF baseline levels, lentiviral shRNA-mediated *KLF4* knockdowns were performed in erythroid progenitor cells from healthy adults and patients with β^0-thalassemia/HbE. The results demonstrated that experimental *KLF4* knockdown could induce embryonic (*HBZ* and *HBE*) and fetal (*HBG*) globin mRNA expression in healthy subjects and those with β^0-thalassemia/HbE. Moreover, a marginal surge in the HbF level also corresponded with the modest elevated *HBG* mRNA expression. However, these findings were inconsistent with previous studies showing that KLF4 triggers *HBG* and *HBA* gene expression in human erythroid cells and the K562 cell line, respectively (16,17). This may be due to the disparate erythroid cell stage during experimental KLF4 knockdown and the dissimilar erythroid culture system. Notably, downregulation of KLF4 did not significantly affect the expression levels of well-known fetal globin gene repressors, KLF1, BCL11A and LRF, suggesting a possible independent globin regulation mechanism. Although KLF4 knockdown revealed insignificant effects on erythroid cell maturation, delayed erythroid differentiation was observed in subjects with β^0-thalassemia/HbE when compared with the healthy subjects. To date, the effort to induce embryonic *HBZ* expression in patients with α-thalassemia major (ATM) has been proposed as a novel therapy to improve the phenotype and survival rate of patients with ATM (21). Therefore, manipulations of KLF4 expression using chemical compounds or molecular approaches may also be considered as potential therapeutic strategies for *HBA* substitution via *HBZ* induction in those patients.

Thus, the present study revealed that the *KLF4* mRNA expression level is relatively downregulated in hematopoietic progenitor cells from the fetal liver. Additionally, experimental knockdown of KLF4 promoted embryonic and fetal globin mRNA expression in human hematopoietic progenitor cells. Despite a small sample size, this evidenced the inverse association between KLF4 and embryonic and fetal globin mRNA expression in human erythroid progenitor cells. Moreover, the development of drugs or molecular methods that reduce KLF4 and further increase embryonic globin gene expression for adult globin substitution may provide an alternative treatment for severe α-thalassemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PK conceptualized the idea, designed the research, performed experiments, analyzed data and wrote the manuscript. NJ performed erythroid culture and supplied resources. AT and SH provided microarray data, analyzed microarray data and supplied cDNA samples. PK and AT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study procedures were approved in accordance with compliance to the Declaration of Helsinki, The Belmont Report, Council for International Organizations of Medical Sciences guidelines and the International Conference on Harmonisation-Good Clinical Practice by the Ethical Review Sub-Committee Board for Human Research Involving Sciences (Thammasat University, Pathum Thani, Thailand; approval nos. 198/2561 and 031/2563). In our previous study (19), women who underwent pregnancy termination due to medical complication provided written informed consent to donate their fetus for research purposes. The protocols for the previous study were approved in accordance with the Declaration of Helsinki by the Committee on Human Rights related to research involving human subjects (Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand; Institutional Review Board no. MURA2013/363).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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