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MiR-144 Regulates Hemoglobin Expression in Human Erythroid Cell Line[†]

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Abstract

The regulation of globin gene expression is significantly important to understand the pathogenesis of globin gene disorders. Recent findings have shown that microRNAs (miRNAs, miRs) play an important role in the regulation of globin gene expression. The miR-144 is an erythroid lineage-specific miRNA, in which its expression mediates *NRF2* gene silencing and inhibits fetal hemoglobin expression. However, roles of miR-144 to other globin genes expression especially in *α-globin cluster* remain unknown. This study, thus, examined the functional studies of miR-144 to globin gene expression in K562 human erythroid cell line. The results revealed that *α-globin* and *ζ-globin* gene expression were silenced by the overexpressed miR-144 and that correlated with the reduced expression of *KLF1*- the suspected target gene. By contrast, transfection with miR-144 inhibitor reversed the silencing effect of miR-144. On the other hand, miR-144 had no effect to *β-globin* gene expression. Our results sustain the findings of the previous studies that the overexpression of miR-144 correlates with the repressing of *NRF2* and ***γ-globin*** gene expression. Taken together, our results suggest that miR-144 plays a key role in globin gene expression by silencing ***γ-globin*** through *NRF2* target mRNA and repressing adult *α-globin* and embryonic *ζ-globin* gene expression possibly by targeting *KLF1* gene.

Keywords: MiR-144, γ-globin, *α-globin*, *ζ-globin*

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Introduction

In human, *α-globin* cluster comprises of *ζ*, *α1* and *α2* which developmentally express during primitive erythropoiesis in yolk sac (*ζ-globin*) and adult erythropoiesis in bone marrow (*α1-* and *α2-globin*) [1]. The *β-globin* cluster contains 5 genes that are differentially regulated; *ε-globin* expresses in yolk sac, $^{G}\gamma$- and $^{A}\gamma$-*globin* express in fetal liver, and *δ-* and *β-globin* express in bone marrow [2]. Studying the regulation of globin gene expression may give essential information on molecular mechanism underlying the pathogenesis of genetic hematological disease, such as sickle cell disease (SCD) and thalassemia [3,4].

Trans-acting elements including erythroid specific and general transcriptional regulators such as EKLF, GATA-1, and FOG-1 play an vital role in regulating the expression of globin genes [4,5]. Erythroid Kruppel-like factor (EKLF) or Kruppel-like factor 1 (*KLF1*) is a transcription factor regulating *γ-* and *β-globin* gene expression. *KLF1* controls globin gene switching by directly interacting with the CACCC boxes of *β*-globin promotor to activate *β-globin* expression and up-regulates *BCL11A*, a known repressor of γ-globin expression. [5-9]. Nuclear factor-like 2 (NRF2) is a transcription factor that regulates the expression of antioxidant proteins. It is bound with the γ-globin antioxidant response element, which can be inhibited by miR-144 mimic. The previous study showed that a miR-144/NRF2 had regulated the expression of γ-globin gene in SCD [10]. Although these advanced knowledges are well established in *β-globin* cluster, little is known about trans-acting elements regulating *α-globin* cluster.

MicroRNAs are endogenous small non-coding linear RNAs, approximately 20 nucleotides that regulate gene expression at the posttranscriptional level by targeting mRNAs for cleavage or translational repression [11]. Accumulating evidence indicates that miRNAs play a critical role in globin gene regulation. The expression of many miRNAs is correlated with the alteration of HbF synthesis. The miR-96 is associated with the silencing of *γ-globin* expression [12], whereas LIN28B is repressed by let-7 miRNA caused decreased expression of BCL11A as a mechanism of HbF induction [13]. Increased miR-15a and miR-16-1 expression in human erythroid cell is correlated with the increasing of fetal and embryonic Hb gene expression by downregulating MYB [14]. Similarly, miR-486-3p controls *γ-globin* expression by directly modulating *BCL11A* [15]. The miR-34a activates *γ-globin* expression in K562 cell line through STAT3 gene silencing [16]. The overexpressed miR-326 in K562 and $CD34^+$ hematopoietic progenitor cells regulates HbF synthesis by targeting EKLF mRNA [17]. Recently, the study in $CD34^+$ hematopoietic progenitor cells of sickle cell disease and KU812 cell line has demonstrated that miR-144 mediates *NRF2* gene silencing and inhibits fetal hemoglobin expression [10]. The first evidence revealed that the regulation of *α-globin* gene expression by miRNA was demonstrated in zebrafish model. This study also displayed that miR-144 was expressed at specific development stages and negatively regulated embryonic *α-globin* (*α-E1*) through Kruppel-like factor D (klfd) target gene [18]. However, roles of miR-144 to *α-globin* cluster of human erythropoiesis remains unknown.

In this study, the function of miR-144 in the regulation of globin gene expression through its target mRNA; *NRF2* and *KLF1* were investigated.

Materials and methods

Cell culture

K562 cells, a chronic myelogenous leukemia-derived cell line, were cultured in RPMI 1640 medium with 10 % heat-inactivated fetal bovine serum (FBS, GIBCO-Invitrogen, NY, USA), 2 mm^3 glutamine, and antibiotics under 5 % CO_2, 37 °C condition.

miR-144 mimic and anti-miR-144 inhibitor transfection

To study the effects of miR-144, gain and loss were performed. 2×10^5 K562 cells/well with 40 nm hsa-miR-144 miRNA mimic and negative mimic control (mirVana™ miRNA mimic; Applied Biosystem, Foster City, CA, USA) was used to increase miRNA expression. MiR-144 inhibitor with 400 nm and negative miRNA inhibitor (Anti-miR™ miRNA Inhibitor; Applied Biosystem) was used for decrease miRNA expression. MiRNA mimic and inhibitor were transfected to K562 cell using RNAiMAX

(Invitrogen). Cells were harvested at 72 h after transfection. The expression of miR-144, its target and globin genes were examined by qRT-PCR.

miRNA and target gene expression

Total RNAs were extracted from transfected K562 cell line using the Hybrid-R™ miRNA Isolation Kit (GeneAll, Seoul, South Korea) according to the manufacturer's instructions. The concentration of RNA was detected with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, DE, USA). Mature miRNAs expression specific for miR-144 was evaluated by TaqMan® Small RNA Assays (Applied Biosystems) and using RNU48 as a reference gene. Briefly, total RNA was reversely transcribed using the specific looped primer, then reverse transcription quantitative PCR was conducted using the Taqman miRNA assay protocol. Amplification of miRNA by qRT-PCR was measured by LightCycler® 480 PCR System (Roche Molecular System, CA, USA). The experiments were carried out in triplicate. The expression of miRNA normalized on reference gene was calculated using $2^{-\Delta\Delta Ct}$ (comparative Ct) method.

To determine the levels of gene expression including nuclear factor-erythroid 2-related factor 2 (*NRF2*), *KLF1, β-globin*, ***γ****-globin*, *α-globin* and ***ζ****-globin* were analyzed by qRT-PCR. Total RNAs extracted from reticulocyte portion and K562 cell line were subjected to reverse transcription to complementary DNA (cDNA) using a High-Capacity RNA-to-cDNA kit (Applied Biosystems) according to established protocols. The amplification of cDNAs by the qRT-PCR method was done using SYBR Premix EX Taq TM (Takara Bio, Shiga, Japan) with specific primers, *NRF2*: forward primer, 5'-GACGGTATG CAACAGGACATTGAG-'3, reverse primer, 5'- AACTTCTGTCAGTTTGGCTT CTGGA-3', GATA-1: forward primer 5'- CCCAAGAAGCGCCT GATTGT-3', reverse primer, 5'-GTGTAGCTTGTAGTAGAGGCCGC-3', *KLF1* or *EKLF*: forward primer 5'-TTGCGGCAAGAGCTACACC-3', reverse primer 5'- CAGGCGTATGGCTTCTCCC-3', *β-globin*: forward primer 5'- TAACAG CATCAGGAGTGGACAGA-3', reverse primer 5'-GCAAGGTGAACGTG GATGAAG-3', **γ**-globin: forward primer 5'-GGCAACCTGTCCTCTGCCTC-3', reverse primer 5' GAAATGGATTGCCAAAACGG-3', α-globin: forward primer 5'-GCCCTGGAGAGGATGTTC- 3', reverse primer 5'-AGGGTCACCAGCAGGCAGT 3', ζ-globin: forward primer 5'- GCCACCCGCAGACCAAGACC-3', reverse primer 5'-AGGCGTGCAGCTCGCTCAG-3'and GAPDH: forward primer, 5'- GAAGGTGAAGGTCGGAGTC-3', reverse primer, 5'- GAAGATGGTGATGGG ATTTC-3'. Amplification was performed with an initial denaturation at 95 °C for 30 s. followed by 40 cycles of denaturation at 95 °C for 3 s., annealing at 60 °C for 30 s, and melt curve stage. GAPDH served as the internal control. Normalized values ($2^{-\Delta\Delta Ct}$) were compared among samples and the experiments were carried out in triplicate.

Statistical analysis

Student's t-test was used to calculate statistical significance. A *p-value* less than 0.05 was considered statistically significant (SPSS version 13).

Results and discussion

NRF2 and *KLF1* gene silencing by miR-144 represses *γ-globin*, *α-globin* and *ζ-globin* gene expression in K562 cell line

To test the functional effect of miR-144 for regulating *NRF2* and *KLF1* target mRNA to control globin gene, the gain evidence of miR-144 was investigated. After K562 cells were transfected with miR-144 mimic, the expression of miR-144 increased compared to that of the untreated and negative control ($p < 0.01$) (**Figure 1**). It was observed that 8.4-fold and 2.8-fold decreased in *NRF2* and *KLF1* target mRNA levels after miR-144 overexpression ($p < 0.05$), respectively (**Figure 1**). The upregulation of miR-144 was associated with *γ-globin, α-globin* and *ζ-globin* gene silencing by 3.3-fold, 2.7-fold, and 2.5-fold, respectively but had not effect to *β-globin* gene expression (**Figure 2**).

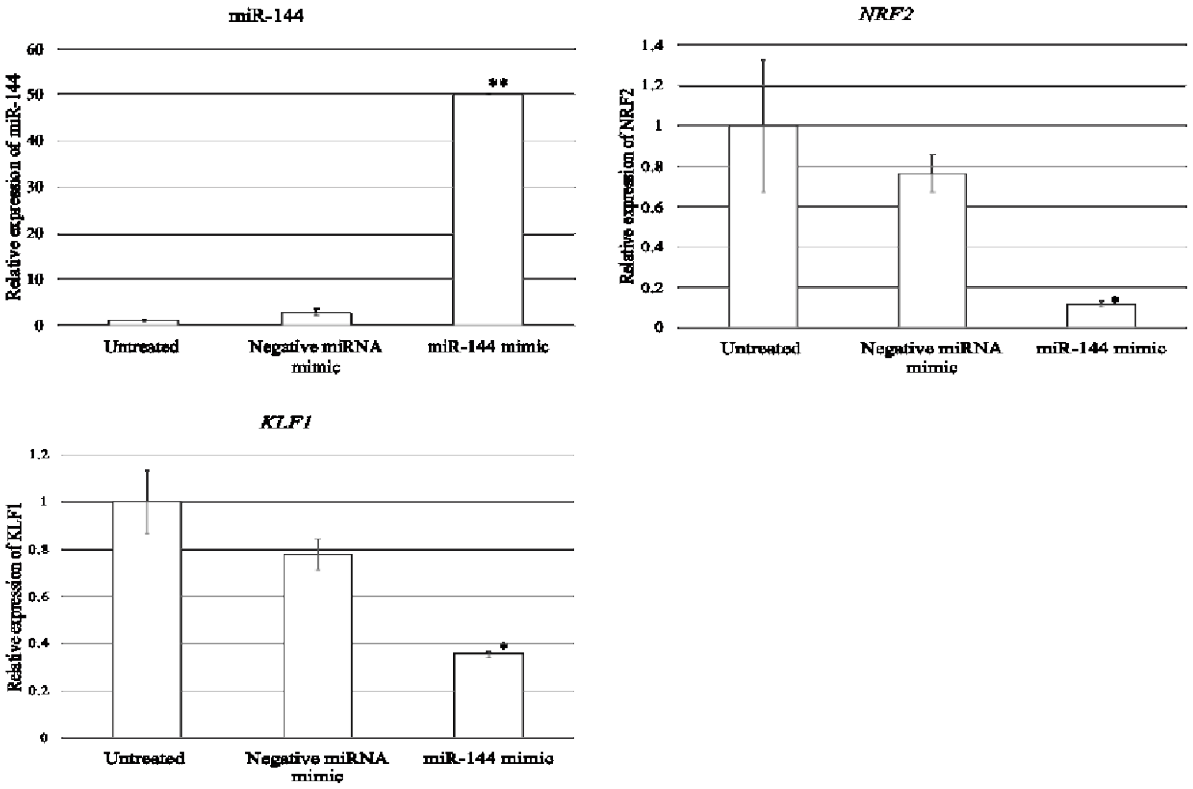

Figure 1 Overexpression of miR-144 regulated mRNA target in erythroblastic cell line. K562 cells were treated with hsa-miR-144 miRNA mimic and negative control. Expression of miR-144 and *NRF2, KLF1* target gene expression was examined by qRT-PCR. Data are means of triplicate determinations.; *$p < 0.05$, **$p < 0.01$.

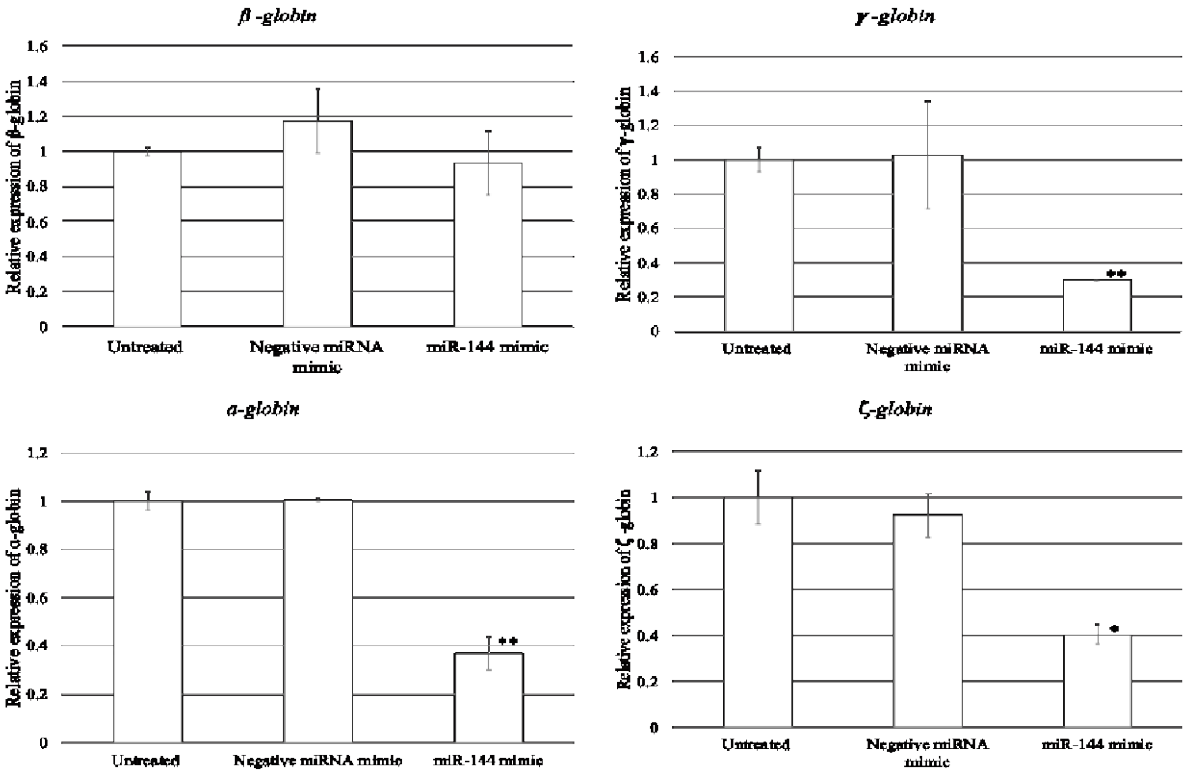

Figure 2 Overexpression of miR-144 regulated mRNA target involving in hemoglobin synthesis. K562 cells were treated with hsa-miR-144 miRNA mimic and negative control. Expression of *β-globin*, ***γ-globin***, *a-globin* and ***ζ-globin*** gene expression was examined by qRT-PCR. Data are means of triplicate determinations.; *$p < 0.05$, **$p < 0.01$.

The transfection of miR-144 inhibitor increases *NRF2 and KLF1 gene expression and promoted γ-globin, α-globin and ζ-globin gene expression in K562 cell line*

Conversely, inhibition of miR-144 expression by anti-miR-144 inhibitor produced 3.7-fold and 1.3-fold increase in *NRF2* and *KLF1* target mRNA levels ($p < 0.05$) compared that of untreated cell (**Figure 3**). Moreover, anti-miR-144 inhibitor promotes an increase in ***γ-globin, a-globin*** and ***ζ-globin*** gene transcription by 1.7-fold, 2.1-fold and 6.0-fold, respectively (figure 4). Anti-miR-144 inhibitor had no effect on *β-globin* gene expression (**Figure 4a**).

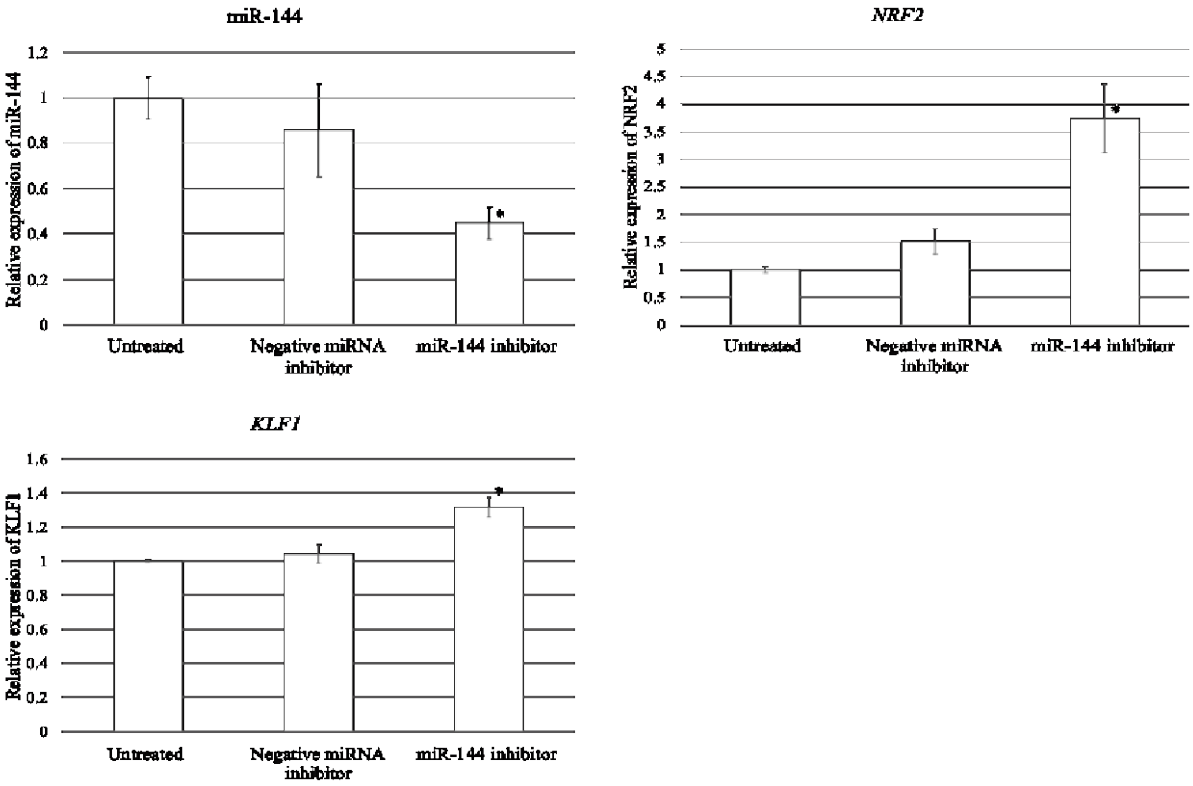

Figure 3 Loss of miR-144 expression in erythroblastic cell line. K562 cells were treated with hsa-miR-144 miRNA inhibitor and negative control. Expression levels of miR-144 and *NRF2*, *KLF1* target gene were examined by qRT-PCR. Data are means of triplicate determinations; * $p < 0.05$, ** $p < 0.01$.

This study identified that miR-144 was involved in ***γ-globin, α-globin*** and ***ζ-globin*** gene expression. The functional study in K562 cell line confirmed the ability of miR-144 in the regulation of ***γ-globin*** gene expression through *NRF2*. Likewise, in the loss of function, miR-144 inhibitor produced a fold change of *NRF2* gene and increased ***γ-globin***. The result of this study is similar to that of Li, B *et al*. who investigated in normal and sickle erythroid progenitor cells. They observed the regulation of miR-144 to *NRF2* and ***γ-globin*** at both mRNA and protein levels. They demonstrated a 30 - 50 % decrease in NRF2 mRNA levels after miR-144 overexpression. Conversely, inhibition of miR-144 by inhibitor alone produced a 1.4-fold increase in NRF2 gene expression. Chromatin immunoprecipitation confirmed the binding of NRF2 to the γ-globin antioxidant response element, which was inhibited by miR-144 mimic treatment [10]. These data suggest that miR-144 may directly regulate NRF2 expression. However, chromatin immunoprecipitation or luciferase reporter assays are required for confirming the binding of miR-144 and NRF2 gene. The function of miR-144 has no effects on *β-globin* gene expression. However, in the gain of miR-144 function, the reduction of *NRF2* gene and ***γ-globin*** in K562 cell line seems to be higher than in erythroid progenitor cells. As a result, types of cell transfection, productivity, or efficiency of miRNA mimic generation, cannot be excluded. Besides *NRF2*, previous report had demonstrated that BCL11A was a repressor of γ-globin expression during hemoglobin switching [7,8,19].

The finding of this study is the first report on the effect of miR-144 inactivation or overexpression to adult *α-globin* and embryonic ***ζ-globin*** gene expression through *KLF1*- the suspected target mRNA. Evidently, chromatin immunoprecipitation or luciferase reporter assays are required for confirming the binding of *KLF1* to *α-globin*, ***ζ-globin and miR-144 genes***. The overexpression of miR-144 results in

decreased *α-globin* gene expression and that correlates with *KLF1* silencing. This result was consistent with *KLF1* mutation in α^0-thalassemia carriers. Red blood cell indices of these patients presented the significantly lower mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) [20]. Previous study showed that miR-144 regulated *klfd* signaling in embryonic *α-globin* regulation, but not *β-globin* expression in zebrafish embryo. The klfd selectively binds to the CACCC boxes in the promoters of both *α-globin* and miR-144 genes to activate their transcriptions and thus lead to a negative feedback circuit to regulate the expression of embryonic *α-globin* gene [18]. Zebrafish *klfd* conserved elements are similar to human and mouse *KLF1* [21]. This transcription factor expresses within both embryonic and definitive erythropoiesis. The embryonic *KLF1-/-* mouse showed lower *ζ-globin* gene expression than wild type [22].

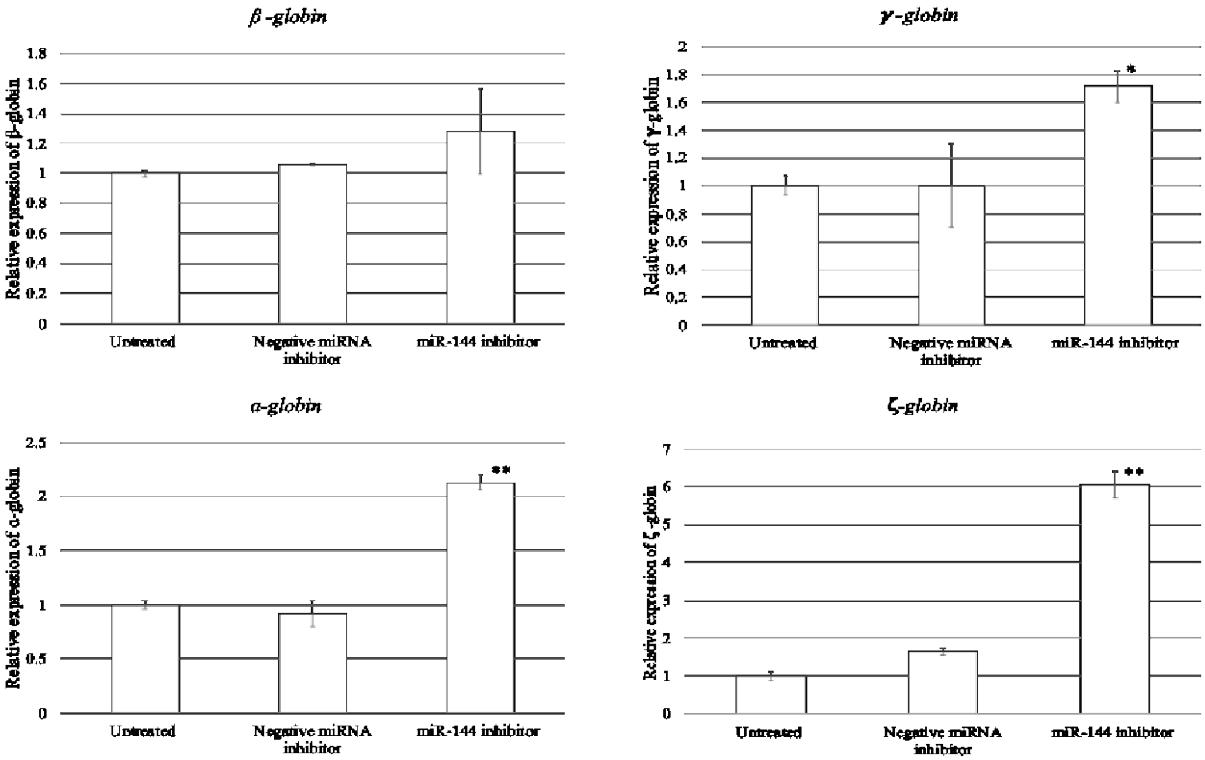

Figure 4 Loss of miR-144 regulated mRNA target involving in hemoglobin synthesis. K562 cells were treated with hsa-miR-144 miRNA mimic and negative control. Expression levels of *β-globin*, *γ-globin*, *α-globin* and *ζ-globin* gene were examined by qRT-PCR. Data are means of triplicate determinations.; *p < 0.05, **p < 0.01.

Conclusions

The regulation of globin gene synthesis is considerably essential to understand the principle controlling globin gene expression and the pathogenesis of hemoglobinopathy disease. The present study has demonstrated the functional studies of miR-144 in human erythroid cell line. The findings illustrate the role of miR-144 in the regulation of hemoglobin expression, especially *γ-globin, α-globin,* and *ζ-globin* gene expression through target mRNAs, *NRF2* and *KLF1*, respectively. These new insights may

potentially provide new therapeutic opportunities for treating thalassemia by developing miRNA as a therapeutic agent to reduce excess α-globin or induce Hb F production in the future.

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