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Regulatory Roles of KLF3 in Hematopoiesis of K562 Leukemia Cells

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Abstract

Objective: *KLF3* (Krüppel-like factor 3) is involved in the differentiation and development of a wide range of cell lineages. However, the regulatory roles of *KLF3* in hematopoiesis of *K562* leukemia cells remain largely unknown.

Methods: The public gene expression databases that represent the heterogeneity of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) disease were downloaded from GEO datasets in NCBI. *KLF3*-deficient *K562* stable cells were established and microarray datasets were analyzed. Gene Ontology (GO) analysis was used to identify the affected hematopoiesis-associated genes in *KLF3*-deficient *K562* cells. Ingenuity Pathways Analysis (IPA) was used to identify the affected hematopoiesis-associated functions, interaction networks, pathways and the upstream regulators involving differentially expressed genes (DEGs). *KLF3*-deficient *K562* cells were respectively induced towards erythrocytes and megakaryocytes with hemin and PMA, and phenotypic analyses were performed. We also utilized qPCR technique to analyze *KLF3* expression during erythroid and megakaryocyte differentiation of *K562* cells.

Results: We report that *KLF3* is aberrantly expressed at low levels in primary acute leukemia blast cells from patients that are associated with the accumulation of immature myeloid or lymphoid phenotypes of leukemia cells. The bioinformatics analyses reveal that *KLF3* is closely involved in hematopoiesis-associated functions in *K562* leukemia cells, and potentially regulates hematopoiesis of different blood cell lineages, including erythrocytes and megakaryocytes. *KLF3* is probably associated with the pathology of hematological diseases. Finally, the functional analysis demonstrated that *KLF3* deficiency accelerates *K562* leukemia cells towards erythroid and megakaryocyte differentiation, and is indispensable for the early stage of hematopoiesis in *K562* leukemia cells. We also proposed the potential mechanisms that *KLF3* regulates hematopoiesis in *K562* cells.

Conclusion: Our results first reveal that *KLF3* regulates erythroid and megakaryocyte differentiation of *K562* leukemia cells, and *KLF3* deficiency is indispensible for the early stage of erythroid and megakaryocyte differentiation in *K562* leukemia cells.

Keywords: *KLF3*; Hematopoiesis; Megakaryopoiesis; Erythropoiesis; *K562*; IPA; Leukemia; Microarray

Introduction

Krüppel-like factors (KLFs) are a subfamily of zinc-finger proteins that contain three highly conserved Cys_2His_2 zinc fingers at the carboxyl terminus. By regulating the expression of abundant genes containing GC-rich or CACCC sequences in their promoters, KLFs participate in several biological processes, including hematopoiesis, adipogenesis, and tumorigenesis. Hematological defects were observed in several Klf knockout mice, such as Klf1, Klf2, *KLF3*, Klf6, and Klf13 [1-6] demonstrating the significant role of Klfs in hematopoiesis. The role of Klf members in leukocyte development and hematopoiesis-associated diseases has also been reported [7]. Moreover, due to similarities in their structures and functions, cross-regulation of Klf members was observed during hematopoiesis in several hematopoietic blood cell lineages, such as erythrocytes and B lymphocytes [8-11].

KLF3 was first cloned from erythroid tissue, and was identified in a screening for factors with DNA-binding homology to the erythroid specific transcription factor, KLF1 [12]. *KLF3* is involved in various differentiation events such as erythropoiesis [6], lymphopoiesis [8,13], adipogenesis [14,15], skeletal muscle differentiation [16] and cardiovascular development [17], by predominantly functioning as a repressor, or exerting its activator functions. Moreover, *KLF3* also has regulatory roles in lipid metabolism and obesity control [18,19]. These observations suggest that *KLF3* may regulate the differentiation and development of diverse cell types.

KLF3 is expressed in various cell types, however, it is highly expressed in erythroid cells [20,21], where its expression is specifically regulated by an alternative and erythroid-specific *KLF3* promoter under the control of Klf1 *in vivo* [22,23]. *KLF3* knockout mice exhibit mild compensated anemia and defects in erythroid maturation in fetal liver [6]. Moreover, *KLF3* dysfunction is also associated with impaired B lymphocyte development, and *KLF3* expression is the driving force toward MZ B cell maturation [8,13]. *KLF3* is also involved in the differentiation or activation of monocytes/macrophages and in leukocyte-related diseases [7]. These observations demonstrate the role of *KLF3* in regulating hematopoiesis of different blood cell lineages. However, it is largely unknown whether megakaryopoiesis or erythropoiesis is regulated by *KLF3* in *K562* leukemia cells.

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Leukemia refers to a group of hematological malignancies that affect the bone marrow and blood. AML is characterized by the accumulation of early myeloid blood cells that fail to mature and differentiate. ALL is another acute form of leukemia characterized by the overproduction of cancerous, immature lymphoblasts. Here, the expression of *KLF3* in AML and ALL harboring defects in differentiation and development of blood cell lineages has been evaluated, and the results demonstrated that *KLF3* was aberrantly expressed in primary leukemia blasts from AML and ALL patients, suggesting that *KLF3* plays a pivotal role in leukemia cells.

The *K562* cell line was originally established from the pleural effusion of a 53-year-old female with myelogenous leukemia in terminal blast crisis, and it is recognized as a multi-potential hematopoietic precursor that differentiates into erythrocytes and megakaryocytes under different conditions [24-26]. Here, *K562* cells serve as a model to assess the regulatory roles of *KLF3* on erythroid differentiation and megakaryocyte differentiation of leukemia cells. Our genome-wide gene expression analysis of *KLF3*-deficient *K562* cells demonstrates that *KLF3* is specifically associated with hematopoiesis-associated functions. We first reported that *KLF3* regulates hematopoiesis of *K562* leukemia cells, including erythropoiesis and megakaryopoiesis, and *KLF3* deficiency is indispensible for the early stage of erythroid differentiation and megakaryocyte differentiation in *K562* leukemia cells, indicating that *KLF3* may play significant role during initial progression of leukemia or anemia.

Materials and Methods

Validation of *KLF3* expression in leukemia blast cells

KLF3 expression in leukemia patient blast cells was analyzed according to the public gene expression database that represents the heterogeneity of individual leukemia disease. The gene expression datasets for leukemia diseases were downloaded from GEO datasets in NCBI. *KLF3* expression levels in 285 diagnosed AML patients (GSE1159) and 26 diagnosed ALL patients (GSE33615) were evaluated in samples of peripheral blood or bone marrow from patients and healthy donors. The statistical significance of differences in gene expression between leukemia patients and healthy donors were analyzed using the Student's *t*-test. A value of $P<0.05$ indicated statistical significance.

K562 Cell culture

K562 cells were purchased from ATCC and grown in culture flasks containing RPMI 1640 medium supplied with 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (0.1 mg/ml). Cells were maintained at 37°C in a 5% CO_2 incubator.

FACS analysis

K562 cell erythroid differentiation was induced with 200 μM hemin at an initial density of 2×10^5 cells/ml, and megakaryocyte differentiation was induced using 20 nM PMA at an initial density of 3×10^5 cells/ml. *K562* cells were harvested at indicated times and washed twice on ice with 1×PBS supplemented with 2% FBS and 2 mM EDTA. To analyze the effect of *KLF3* deficiency on erythroid differentiation induced with hemin, cells were incubated with PE-conjugated anti-CD235a (BD Pharmingen) and APC-conjugated anti-CD71 (Milteny Biotec) antibodies for 10 min on ice. To analyze the effect of *KLF3* deficiency on megakaryocyte differentiation induced with PMA, cells were incubated with a FITC-conjugated anti-CD61 (Milteny Biotec) antibody for 10 min on ice. Flow cytometry analysis was performed on a FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Establishment of *KLF3*-deficient *K562* stable cells

K562 cells were seeded into 6-well plates at a density of 1.0×10^6 cells/well and transiently transfected with 1 μg of *KLF3* shRNA plasmid (sc-44963-SH) or control shRNA plasmid (sc-108060) using lipofectamine LTX and Plus reagents (Invitrogen) following manufacturer's instructions. Puromycin (3 μg/ml) was added to the culture medium after 48 h to enrich the positive clones. *KLF3* deficiency in *K562* cells was evaluated by qPCR analysis.

Quantitative Real-time PCR

Total RNA was isolated using Trizol reagents (Invitrogen) followed by the removal of residual genomic DNA using the TURBO DNA-free™ Kit (Ambion). DNA-free total RNA (1 μg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. The generated cDNA library was diluted 4 fold, and 1 μl of diluted cDNA was used as a template in a 20 μl qPCR reaction system. Real-time PCR was performed in triplicate using SYBR Green PCR Master Mixes (2×) (Fermentas) on a CFX96™ Real-Time PCR Detection System (Bio-Rad). Transcript expression levels were normalized to 18s rRNA. The purity of qPCR products was further confirmed by analyzing the melting curves, and running a 1.5% agarose gel. The qPCR primers are listed in supplementary Table 1.

Microarray analysis

Established *KLF3* knockdown and shRNA control *K562* stable cells were collected in RNA-later reagents (Sigma) and submitted to the UT Southwestern Microarray Core Facility for microarray analysis. The expression profile of each RNA sample was determined in duplicate. Genes with a p-value of < 0.01 between control and experimental cells were selected for further bioinformatics analysis. DEGs were identified based on the screening criteria of > 2-fold change in expression ($P<0.01$).

IPA analysis

Gene interaction networks and signaling pathways were generated using IPA software (http://www.ingenuity.com/) from Ingenuity® Systems. This software was used to analyze data from a variety of experimental platforms and to provide accurate biological insights into interactions between genes, proteins, chemicals, pathways, cellular phenotypes, and disease processes. DEGs were submitted to IPA for biological function, canonical pathway, and interaction network analyses as previously described [27]. To determine the significance of enrichment in a particular function, IPA calculates the significance value based on the measure of involvement of the gene in the input data set with its respective molecular functions and signaling pathways. The significance of networks was calculated using Fisher's exact test, and p-values were executed using negative logarithmic transformation as previously described [28]. A value of $P<0.05$ indicated statistical significance.

Databases

The datasets supporting the results of this article are available under accession number GSE54437, in which two mRNA-chip libraries of *K562* control and *KLF3* knockdown cells were generated in parallel and the expression profiles of each sample was determined in duplicates. Two gene expression datasets for AML (GSE1159) and ALL (GSE33615) diseases were respectively downloaded from GEO datasets in NCBI. The expression profiles of blasts or mononuclear cells from these two leukemic patient samples were presented by microarray.

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Results

KLF3 expression is aberrantly decreased in AML and ALL patient blasts

KLF3 was detected in diverse blood cell lineages including erythrocytes [9,12,21,22]. B lymphocytes [8,13] and macrophages [29] and was reported to regulate differentiation and development of different blood cell lineages. In this study, we investigated the specific functions of *KLF3* in leukemia cells. We first evaluated *KLF3* expression in human primary patient leukemia blast cells that were impaired in blood cell differentiation. AML and ALL are both hematological malignancies that are characterized by the overproduction of immature blood cell lineages. Considering the heterogeneity of AML and ALL patient blasts, we selected representative datasets of 285 AML patients and 26 ALL patients to analyze the gene expression of *KLF3* [30,31]. The *KLF3* expression was aberrantly decreased in AML patient leukemia blasts compared to normal bone marrow from healthy donors (Figure 1a). Meanwhile, its expression was also dramatically reduced in ALL patient leukemia blasts compared to control $CD4^+$ T cells from healthy donors (Figure 1b). These results indicate that the aberrant decrease in *KLF3* expression is associated with the accumulation of immature myeloid or lymphoid phenotypes of leukemia cells, suggesting a particular role of *KLF3* in hematopoiesis of leukemia cells.

Bioinformatics analysis shows that *KLF3* is involved in hematopoiesis in *K562* leukemia cells

Next, we investigated whether *KLF3* plays a regulatory role in the hematopoiesis of leukemia cells. Here, we established stable *KLF3*-deficient *K562* leukemia cells and performed genome-wide expression profiling of these cells using microarray analysis. We confirmed *KLF3* deficiency by qPCR analysis (Supplementary Figure 1a). Two mRNA-chip libraries of *K562* control and *KLF3* knockdown cells were generated in parallel and the expression profiles of each sample were determined in duplicate. The microarray results showed 47 322 probes on the human-6-V3 chips and all four arrays produced highly reproducible and consistent gene expression data (Supplementary Figure 1b). A total of 14 118 genes ($P<0.01$) were present in each group and the median expression levels from the duplicates were used in the statistical analyses. We identified 101 DEGs (> 2-fold change, $P<0.01$) that were affected by *KLF3* deficiency in *K562* cells, of which 57 genes were upregulated and 44 were downregulated (Figure 2a, Supplementary Table 2). To assess the quality of the microarray data, the expression of 15 selected genes was further validated by qPCR analysis. A good correlation was observed (Supplementary Figure 1c), indicating that we obtained an acceptable dataset of transcripts from the evaluated cells. A number of hematological genes were affected by *KLF3* deficiency in *K562* cells (Figures 2a and b). Several DEGs, including *ALAS2, SPTA1, TF, HBE, HBD,* and *HBA*, which are well-known hematopoiesis-associated genes, were derepressed in *KLF3*-deficient *K562* cells and were considered as potential targets of *KLF3* in *K562* leukemia cells (Supplementary Figure 1c and Supplementary Table 1). For example, SPTA1 encodes an actin crosslinking protein, Spectrin, whose tetramer structure may increase plasma membrane elasticity and deformability of erythrocytes

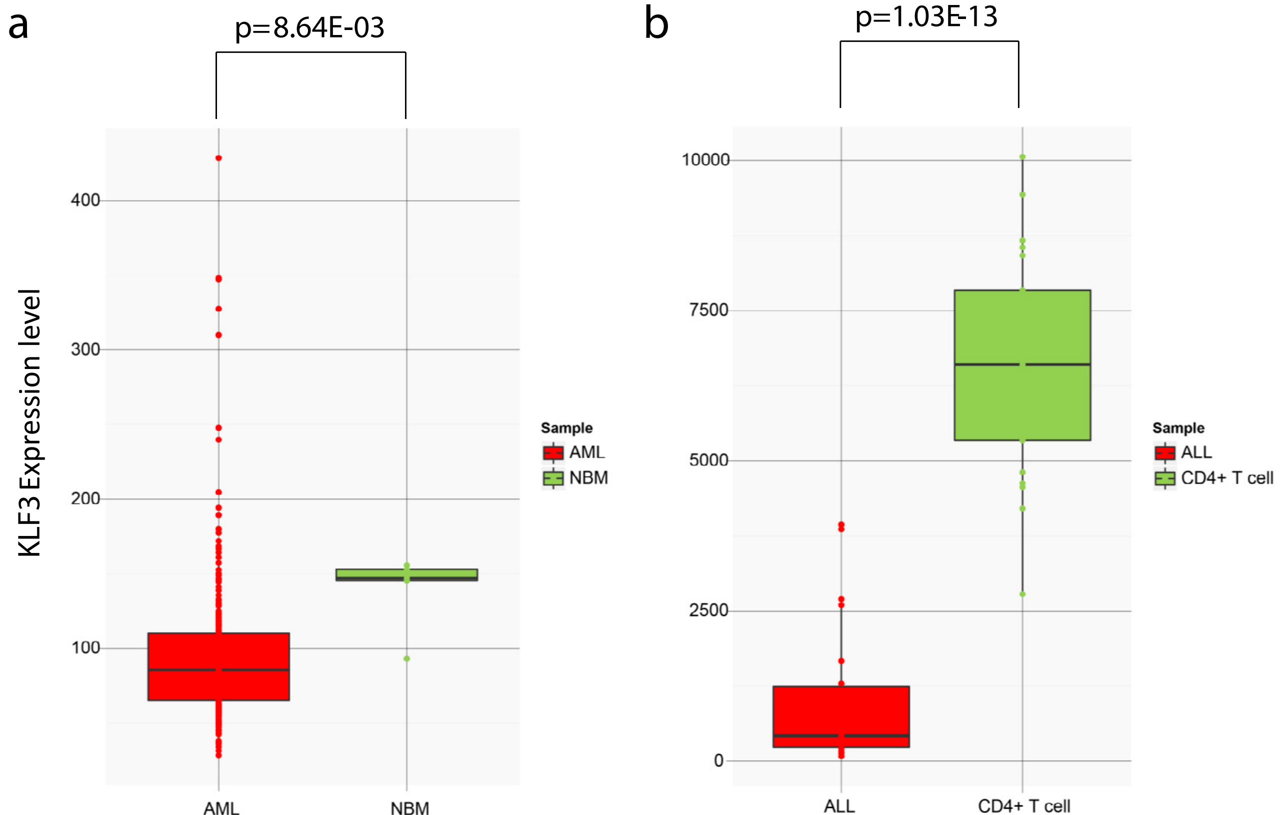

Figure 1: *KLF3* expression in blast cells from AML and ALL patients. (a,b) *KLF3* expression was respectively evaluated in blasts/mononuclear cells from AML (a) and ALL (b) patients. The gene expression of *KLF3* from different datasets (AML, GSE1159; ALL, GSE33615) was analyzed. The statistical significance of differences between *KLF3* expression in patients and healthy donors were analyzed using the Student's t-test. AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, Normal bone marrow: NBM.

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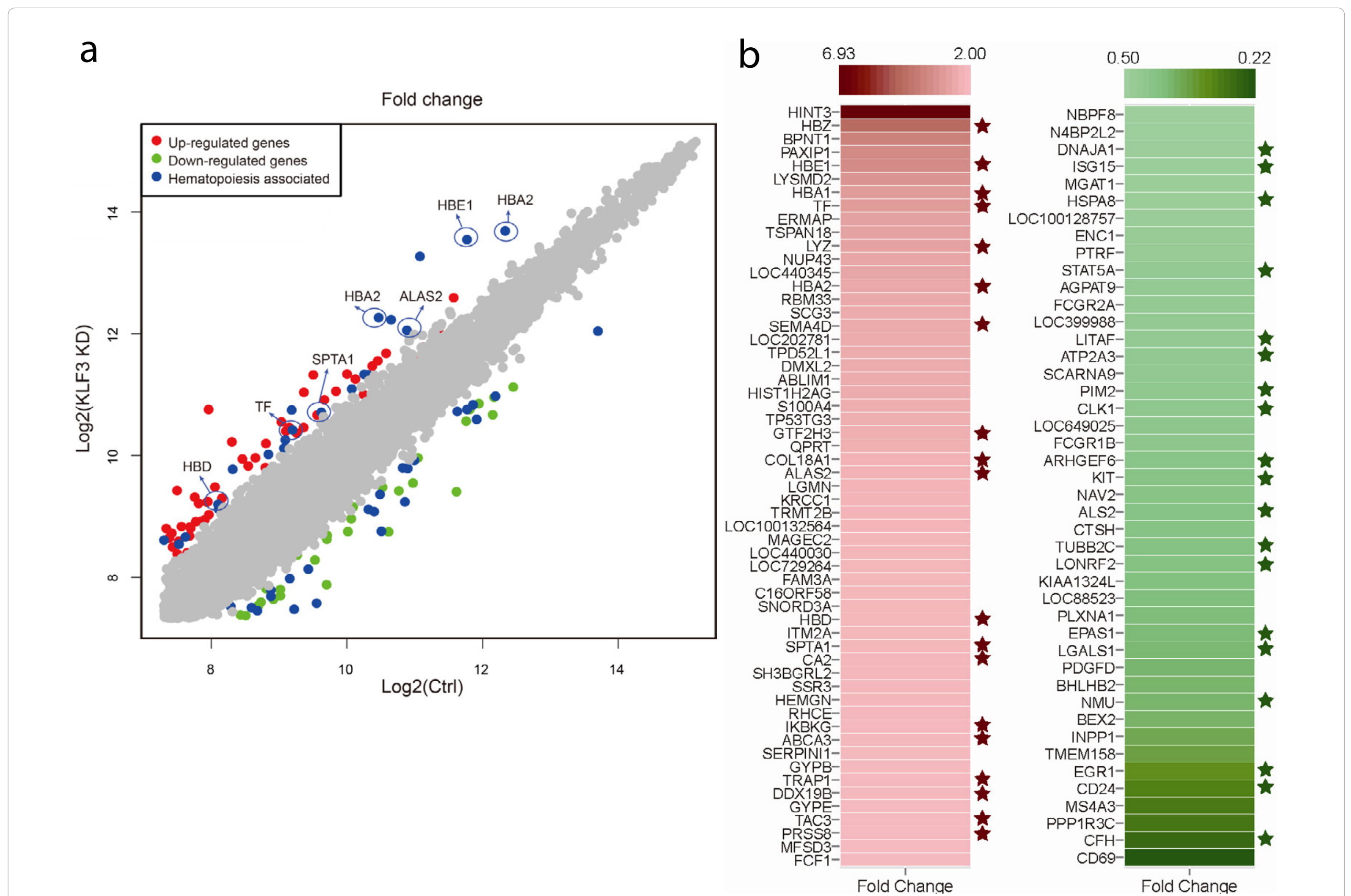

Figure 2: Hematopoiesis-associated genes were affected by *KLF3* deficiency in *K562* leukemia cells. (a) Scatter plot of DEGs compared with unchanged genes. Red and green dots are the up-regulated and down-regulated genes (> 2-fold change, P < 0.01), respectively. Blue dots are differentially expressed hematopoiesis-associated genes in *KLF3*-deficient *K562* cells identified by GO analysis. (b) Heatmap analysis of the affected genes in *KLF3*-deficient *K562* cells. The asterisks indicate the perturbed hematopoiesis-associated genes in *KLF3*-deficient *K562* cells. Red: upregulated genes, Green: down-regulated genes.

and megakaryocytes [32]. Mutations in SPTA1 resulted in a variety of hereditary erythrocyte disorders including hereditary elliptocytosis [33]. Destruction of the spectrin-based membrane skeleton affected mouse megakaryocyte membrane systems and formation of proplatelets and platelets [32]. Here, we present the genome-wide expression profile of *KLF3*-deficient *K562* leukemia cells, which is critical to studying the biology of *KLF3* in leukemia cells.

To further determine the hematopoiesis-associated functions of *KLF3* in *K562* leukemia cells, all DEGs were first subjected to IPA. These DEGs were highly enriched in the functions of "hematopoiesis", "hematological system development", and "hematological diseases" (Supplementary Table 3). Specifically, for example, these DEGs were particularly involved in differentiation (P=0.0035) and development (P=0.0016) of red blood cells, quantity maintenance (P=0.0026) and cell spreading of blood platelets (P=0.0024) (Figure 3). We speculated that these functions could be associated with erythroid and megakaryocyte differentiation regulated by *KLF3*. In addition to the various functions that *KLF3* fulfills in normal hematopoiesis, *KLF3* was also observed in various hematological disorders including anemia (P<0.0001), hematological neoplasia (P=0.0009) and leukemia (P=0.0048) (Figure 3), of which *KLF3* was confirmed to be abnormally expressed in AML and ALL leukemia patient blasts (Figure 1). Moreover, the IPA analysis also showed that the molecular interacting network regarding 'hematological diseases, hematological system development and function, and tissue morphology' in *K562* leukemia cells was the most significantly affected by *KLF3* deficiency (Supplementary Table 4). Taken together, the present results demonstrate that *KLF3* potentially exerts multiple roles in regulating hematopoiesis-associated functions and diseases in *K562* leukemic cells.

KLF3 deficiency promotes erythroid- and megakaryocyte differentiation of *K562* leukemia cells

The bioinformatics analyses showed that *KLF3* is likely able to regulate erythroid and megakaryocyte differentiation of *K562* leukemia cells. We therefore assessed the functions of *KLF3* in erythropoiesis and megakaryopoiesis using the *K562* cell model. To investigate the effect of *KLF3* on erythropoiesis of *K562* cells, we evaluated the expression of the erythroid-specific surface markers CD235a and CD71 by flow cytometry after hemin induction, using *KLF3*-deficient *K562* cells. Hemin-induced *K562* cells erythroid differentiation was assessed by the presence of a dark red cell pellet (data not shown). FACS analysis revealed a relatively higher percentage of CD235a$^+$ and CD71$^+$ positive cells in hemin-induced *KLF3*-deficient *K562* cells compared to shRNA control cells (Figures 4a and 4b), demonstrating that *KLF3* knockdown accelerates erythroid differentiation of *K562* cells under hemin induction. This finding is consistent with a previous study that showed increased

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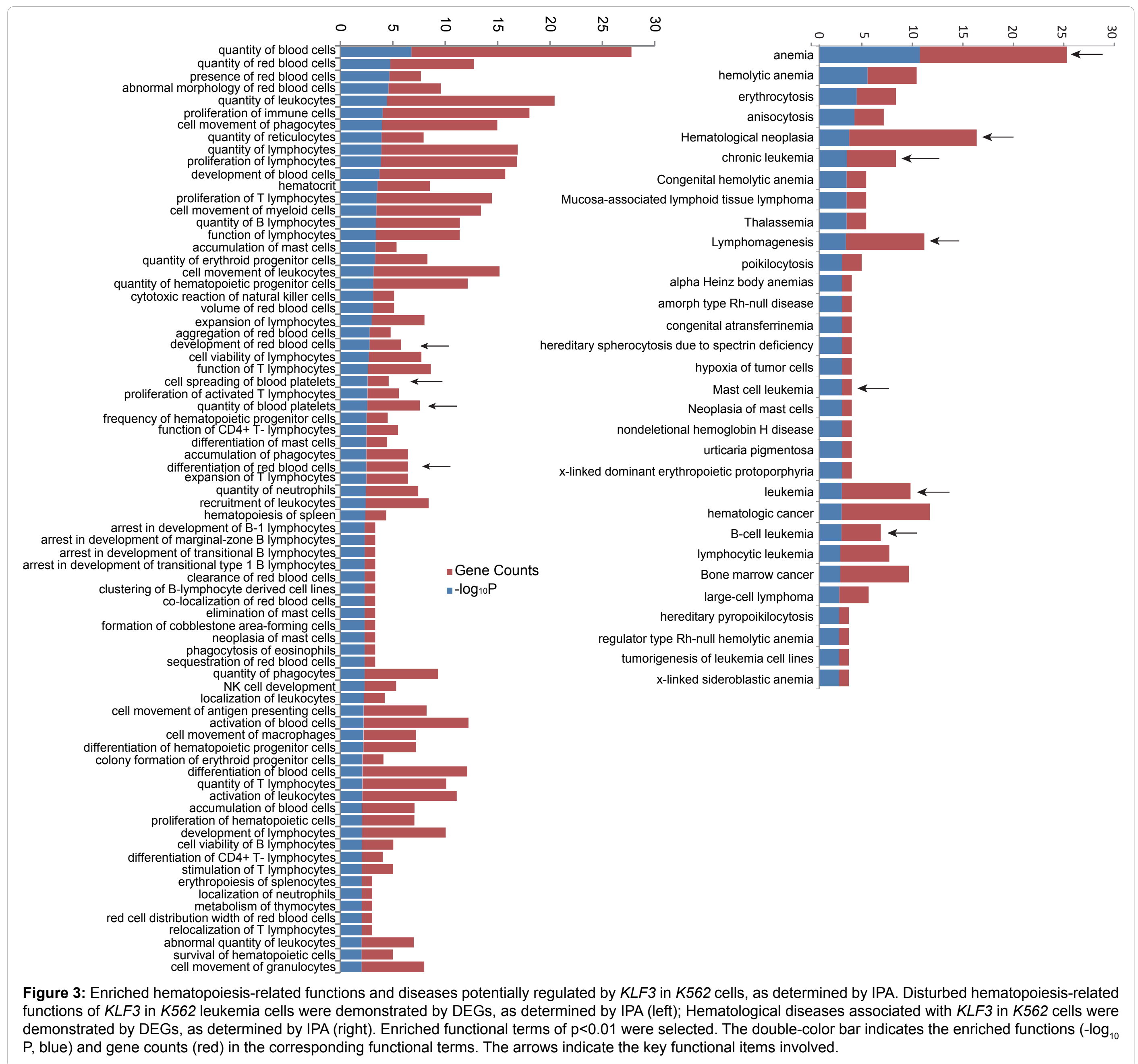

Figure 3: Enriched hematopoiesis-related functions and diseases potentially regulated by *KLF3* in *K562* cells, as determined by IPA. Disturbed hematopoiesis-related functions of *KLF3* in *K562* leukemia cells were demonstrated by DEGs, as determined by IPA (left); Hematological diseases associated with *KLF3* in *K562* cells were demonstrated by DEGs, as determined by IPA (right). Enriched functional terms of p<0.01 were selected. The double-color bar indicates the enriched functions ($-\log_{10}$ P, blue) and gene counts (red) in the corresponding functional terms. The arrows indicate the key functional items involved.

early erythroid populations, mainly including proerythroblasts and basophilic erythroblasts in *KLF3* deficient mice [6]. This further confirms the role of *KLF3* in compensatory stress erythropoiesis. Platelets are produced from terminally differentiated megakaryocytes, with morphological changes regulated by cytoskeletons [34]. *KLF3*-deficient mice also displayed significantly reduced circulating platelets in peripheral blood [6]. Here we also speculate that *KLF3* has a regulatory role in megakaryocyte differentiation of *K562* leukemia cells. Compared to shRNA control cells, PMA induction significantly increased the cell size of *KLF3*-deficient *K562* cells, of which a fraction exhibited dramatic morphological changes and developed pseudopodia (Figure 4c). This phenotypic change occurs as a result of rearrangement of the cytoskeleton during megakaryocyte differentiation of *K562* cells [35,36]. CD61 is a specific megakaryocyte surface marker. An increase in its expression indicates differentiation of *K562* cells toward the megakaryocyte lineage under PMA induction. CD61 expression dramatically increased when *K562* cells were induced with PMA (Figure 4d), indicating that most *K562* cells differentiated into megakaryocytes. Most importantly, *KLF3*-deficient *K562* cells were more prone to differentiate into megakaryocytes compared with control cells (Figure 4d and 4e), suggesting that *KLF3* deficiency promotes megakaryocyte differentiation of *K562* cells under PMA induction. Taken together, *KLF3* deficiency accelerates hematopoiesis of *K562* leukemia cells as far as erythropoiesis and megakaryopoiesis are concerned.

KLF3 deficiency is indispensible for the early stage of hematopoiesis in *K562* leukemia cells

We demonstrate that *KLF3* deficiency promoted hematopoiesis of

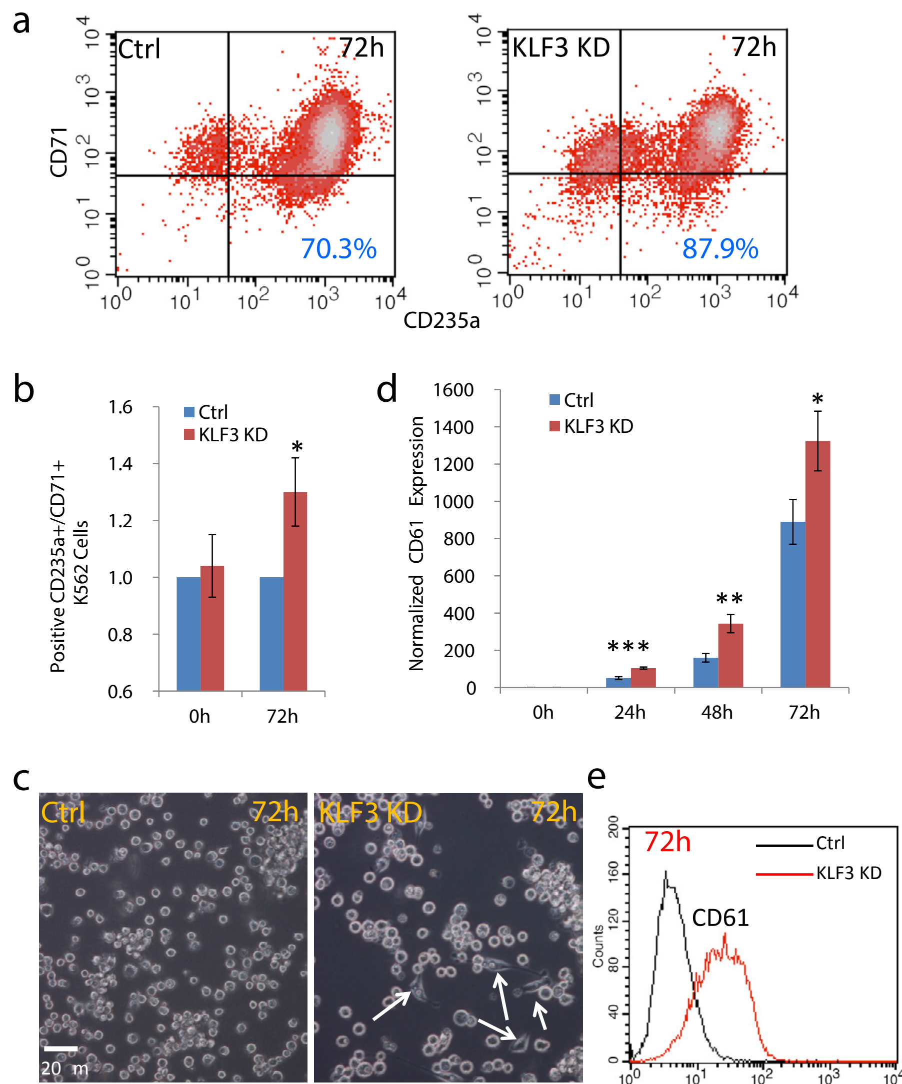

Figure 4: *KLF3* deficiency promotes erythroid differentiation and megakaryocyte differentiation of *K562* leukemia cells. (a) Representative FACS analysis of *KLF3* deficient *K562* cells and control shRNA transfected cells following hemin induction for 72 h. (b) Summary of FACS data showing the CD71⁺/CD235a⁺ population between *KLF3*-deficient and shRNA control *K562* cells after hemin induction at 72 h. The CD71⁺/CD235a⁺ population in *KLF3*-deficient cells was normalized to that of shRNA control cells. (c) PMA induced morphological changes of *KLF3*-deficient *K562* cells. PMA induced *K562* cells were analyzed under a microscope at 72 h. The white arrows indicate the cells with dramatic morphological changes toward megakaryocyte differentiation. Magnification, 20×. (d) *KLF3* deficiency triggers significantly increased expression of the megakaryocyte surface marker CD61 compared to shRNA control cells. Aliquots were isolated at the indicated time points and analyzed by qPCR. The CD61 expression was normalized to that of shRNA control cells at the indicated time points. The statistical significance of differences between *KLF3*-deficient and shRNA control cells was analyzed using the Student's t-test. ***: P<0.001, **: P<0.01, *: P<0.05; n=3. (e) Representative FACS analysis of *KLF3*- deficient *K562* cells and shRNA-transfected control cells following PMA induction for 72 h.

K562 cells, particularly erythropoiesis and megakaryopoiesis. Hence, we intend to examine whether *KLF3* expression decreases during hematopoiesis of *K562* cells under different induction conditions. It was reported that targeted disruption of *KLF3* demonstrates its particular role in adipogenesis, and *KLF3* expression decreases during adipocyte differentiation [14]. *K562* cells express high levels of embryonic and fetal globin genes and suppress the adult β-globin gene [37]. The expression of embryonic and fetal genes HBE and HBG drastically increased in the presence of hemin, the transcriptional regulator of globin genes in *K562* cells [38]. HBE and HBG expression was dramatically increased in the presence of 50 mM hemin, and the hemin-induced *K562* cell pellet became dark red (data not shown), indicating that *K562* cells were successfully induced to undergo erythropoiesis. *KLF3* expression decreased during the first 24 h of induction and was maintained at stable levels, but slightly increased at 72 h (Figure 5a), accompanying erythroid differentiation of *K562* cells. Similar to that observed during erythroid differentiation, *KLF3* expression also decreased within the first 24 h during megakaryocyte differentiation of *K562* cells and gradually increased thereafter (Figure 5b). We observed a decrease in *KLF3* expression during the early stages of erythroid- and megakaryocyte differentiation, which is consistent with the finding that *KLF3* deficiency promotes hematopoiesis of *K562* cells. This finding indicates that decreased *KLF3* expression is indispensable during the early stages of erythroid and megakaryocyte differentiation of *K562* cells. Our findings support the previous observations that *KLF3* deficiency leads to more erythroid precursors and fewer mature red blood cells

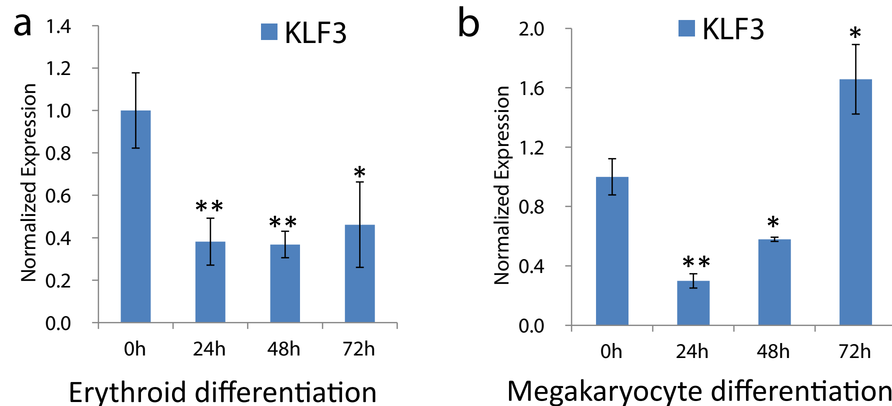

Figure 5: *KLF3* expression changes during erythroid differentiation and megakaryocyte differentiation of *K562* cells
(a) The expression profile of *KLF3* during erythroid differentiation of *K562* cells. Aliquots were isolated at the indicated time points for qPCR analysis. The gene expression was compared to that of the control sample without hemin induction. The statistical significance of differences between hemin-induced and uninduced cells was analyzed using the Student's t-test, a value of p<0.05 indicated statistical significance. (b) The expression profile of *KLF3* during megakaryocyte differentiation of *K562* cells. *K562* cells were treated with 20 nM PMA to initiate megakaryocyte differentiation. The gene expression was compared to that of the control sample without PMA induction. The statistical significance of differences between PMA-induced and uninduced cells was analyzed using the Student's t-test, a value of P<0.05 indicated statistical significance.

in mouse and zebrafish model [6,39]. Regarding megakaryopoiesis, we propose that the reduction of platelets quantity in peripheral blood is probably due to the abnormally accelerated megarkaryocyte precursors in *KLF3* deficient mice that block the maturation of platelets [6]. Our findings demonstrate that *KLF3* may play significant role during initial progression of leukemia or anemia. Moreover, since *KLF3* mainly serves as a repressor, the increase in *KLF3* expression during the later stages of differentiation is probably required to temper the expression of certain target genes or to involve KLF networks that are essential for late stage erythropoiesis or megakaryopoiesis.

Discussion

In this study, we demonstrate that *KLF3* can regulate hematopoiesis in *K562* leukemia cells, including erythropoiesis and megakaryopoiesis. Actually, the coordinate regulation of hematopoiesis by KLF members in blood lineage cells is extremely complicated. Take erythropoiesis as example, Klf1 knockout mice exhibited decreased *KLF3* expression and a concurrent overexpression of fetal globin chains, indicating that the excess of fetal globin might have resulted from the inability of residual *KLF3* to repress fetal globin transcription [12,40]. The observation that *KLF3* repressed several Klf1-driven target genes in erythroid cells also raises the possibility that these two factors coordinate to fine-tune gene expression during erythropoiesis [6]. Moreover, *KLF3* represses the expression of Klf8 [9] and *KLF3*/Klf8 double knockout causes embryonic lethality, although individual Klf8 or *KLF3* knockout mice exhibit a normal or mild erythroid phenotype, reflecting the genetic interaction between *KLF3* and Klf8 in fetal liver erythroid cells [9]. These observations suggest that the mechanism of *KLF3* regulation of erythropoiesis is probably extremely complicated, and may involve its genetic interaction with other KLF members. Our group has already characterized a novel combination with *KLF3* and three other KLF members that presumably regulate erythropoiesis of human hematopoietic stem cells (HSCs) via comprehensive analysis of the expression patterns of 17 KLF family members during differentiation and development of human cord blood HSCs (unpublished data). As far as megakaryopoiesis is concerned, here we first find that megakaryopoiesis can be regulated by *KLF3*, although *K562* leukemia cell model is currently utilized. In the near future, the detailed mechanism that *KLF3* regulate megakaryopoiesis will be further discovered with a combination of stem cell megakaryocyte differentiation and development system and NOD/SCID mouse transplantation model.

Our current bioinformatics results could provide some information regarding mechanism that *KLF3* regulates hematopoiesis in *K562* leukemia cells. GATA1 is a transcription factor that is widely expressed in different hematopoietic cells including erythroid, mast, and megakaryocyte cells [41]. GATA1 mutants were rendered nonfunctional in driving terminal erythroid and megakaryocytic differentiation, even when expressed at normal levels [42]. Through IPA analysis, we identified GATA1 as the most significant upstream regulator of DEGs in *KLF3*-deficient *K562* leukemia cells (P=2.18E-07) (Supplementary Table 5). Next, we searched for transcription factors that were physically associated with regulatory elements in the genomic regions of *KLF3*, in *K562* cells, by analyzing ChIP-Seq data of transcription factors in the ENCODE database. We found that GATA1 moderately bound to the promoter region of *KLF3* in *K562* cells. The hematopoietic transcription factor, TAL1 was also associated with the same genomic regions of *KLF3* (Supplementary Figure 2). In primary murine erythroid cells, Gata1 and Tal1 were reported to coordinately co-occupy the genomic region of *KLF3* to which Klf1 strongly binds [23,43]. Thus, our observation supports the finding that GATA1 and TAL1 coordinately regulate hematopoiesis [44,45], implying that GATA1 can also form an activation complex with TAL1 to regulate the hematopoietic functions of *KLF3* in *K562* cells. Moreover, IPA analysis also showed that another hematopoietic transcription factor NF-E2 might be the upstream regulator of DEGs in *KLF3*-deficient *K562* leukemia cells (P=2.79E-04). GATA1, TAL1, and NF-E2 are co-expressed in erythroid, megakaryocytic, and mast cells [41,46,47] and the co-expression of these three transcription factors has been implicated in the maturation of these cells [48]. Moreover, GATA1 and NF-E2 were both observed to regulate erythroid and megakaryocyte differentiation [49]. In megakaryopoiesis, NF-E2 knockout mice demonstrated a higher percentage of megakaryocytes and impaired platelet production in bone marrow. Additionally, the mice developed thrombocytopenia, which is suggestive of the role NF-E2 in the later stages of megakaryocyte maturation [48]. GATA1-deficient

megakaryocytic cells also demonstrated impaired platelet formation [50]. Our qPCR analysis showed that the expression of GATA1 and NF-E2 was unchanged in *KLF3*-deficient *K562* cells (Supplementary Figure 3), confirming that these two transcription factors are the upstream regulators of *KLF3* in *K562* cells. Taken together, our bioinformatics analysis suggests that GATA1, TAL1, and NF-E2 could also involve the hematopoiesis regulated by *KLF3* in *K562* leukemia cells, depending on the cell context and type of blood cell lineage differentiation.

KLF3-knockout mice exhibited defects in growth, a myeloproliferative disorder, and abnormalities in hematopoiesis, such as compensated anemia [5,6]. We demonstrate here that *KLF3* may be implicated in the regulation of genes involved in hematopoiesis-associated diseases in *K562* leukemia cells including anemia and leukemia. In erythropoiesis, *KLF3*-deficient mice had a higher percentage of erythroid progenitors and impaired erythroid maturation [6], which mimics myelogenous leukemia phenotype, and is consistent with our current findings that *KLF3* deficiency accelerates erythropoiesis of *K562* cells. Our data also showed that *KLF3* is abnormally expressed in leukemia patient blast cells (Figure 1, Supplementary Figure 2). It was reported that overcoming the maturation block in acute promyelocytic leukemia (APL) patients using all-trans retinoic acid therapy restored *KLF3* expression [51], indicating an association of low *KLF3* expression with an immature myeloid phenotype in leukemic cells. We also analyzed the affected signaling pathways in *KLF3*-deficient *K562* cells by IPA (Supplementary Table 6). The AML signaling pathway was significantly dysregulated in *KLF3*-deficient *K562* cells, and genes including STAT5A, KIT, and PIM2 were down-regulated in this pathway (Supplementary Figure 4). Our current findings propose that *KLF3* could be possible therapeutic target in AML.

In conclusion, our current results reveal that *KLF3* regulates erythroid and megakaryocyte differentiation of *K562* leukemia cells, and *KLF3* deficiency is indispensible for the early stage of erythroid and megakaryocyte differentiation in *K562* leukemia cells, which suggests that *KLF3* deficiency could accelerate the expansion of erythroid and megakaryocyte precursors, blocking the maturation of these blood cell lineages at later developmental stage. Since *K562* cells can't actually be developed into mature cells, the comprehensive investigation is proposed to be performed with stem cell erythroid and megakaryocyte development system or mouse model.

Conflict of Interests

The authors declare no competing interests.

Author Contributions

Z.Q. and D.N. performed the experiments, interpreted the results, and wrote the paper; X.Q performed the cDNA array experiments, analyzed the data, and prepared figures; L.Z., L.Y, and Z.J. performed research; L.Q. revised the manuscript and provided critical suggestions; Z.Z. and F.X. designed the experimental strategies, revised the manuscript, and approved the final version.

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