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Knockdown of transcription factor forkhead box O3 (FOXO3) suppresses erythroid differentiation in human cells and zebrafish



Hai Wang^{a, d, 1}, Yanming Li^{a, c, 1}, Sifeng Wang^{b, c, 1}, Qian Zhang^a, Jiawen Zheng^{a, c},
Yadong Yang^a, Heyuan Qi^{a, c}, Hongzhu Qu^a, Zhaojun Zhang^a, Feng Liu^{b, **},
Xiangdong Fang^{a, *}

^a CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China

^b State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

^c University of Chinese Academy of Sciences, Beijing 100049, China

^d China National Committee for Terms in Sciences and Technologies, Beijing 100717, China

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ABSTRACT

Our previous study on the dynamic transcriptomes activated during human erythropoiesis suggested that transcription factor forkhead box O3 (FOXO3) possibly plays a role in erythroid differentiation. Functional studies in human cell line TF-1 indicated that FOXO3 knockdown repressed erythropoietin (EPO)-induced erythroid differentiation by activating promoter region of B-cell translocation gene 1 (BTG1), thereby regulating its expression. In zebrafish, injection of *foxo3b*-specific morpholinos (*foxo3b* MO) resulted in reduced globin (*hbα1* and *hbβ2*) and *gata1* gene expression. Transcriptome analyses of erythroid lineage cells isolated from the control and *foxo3b* morphants revealed the dynamic regulation of *foxo3b*. Further study suggested that *BTG1* is partially responsible for FOXO3 regulation in erythroid differentiation of TF-1 cells but is inconsequential in zebrafish. Taken together, we found that FOXO3 plays an important role in erythroid differentiation in both human TF-1 cells and zebrafish, but the mechanism underlying this regulation still remains unclear.

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1. Introduction

Erythroid differentiation generates sufficient counts of red blood cells necessary to fulfill daily need of vertebrates. The regulatory mechanism of normal and abnormal erythroid differentiation has been studied for years to better understand the critical functions of erythrocytes. Transcription factors, which bind to specific DNA sequences to regulate the transcription, were found to play important roles in erythroid differentiation. The two most-widely studied erythroid transcription factors are GATA1 [1–3] and KLF1 [2,4,5], owing to their indispensable role for the survival and terminal differentiation of erythrocytes; however, increasing number of transcription factors have been found to play

a role in erythroid differentiation. Forkhead box O3 (FOXO3) belongs to the O subfamily of forkhead transcription factors and was recently found to play a role in mice erythroid differentiation [6,7].

Human erythroid leukemia cell line TF-1 is often used to study inducible erythroid differentiation in response to erythropoietin (EPO) [8]. Zebrafish (*Danio rerio*) serve as another powerful model to investigate erythroid differentiation because of their small size and their ability to generate large numbers of embryos that enables large-scale analysis, also, their transparency facilitates the visualization of erythroid cell migration [9]. In addition, the highly conserved hematopoietic genes among vertebrates make studies using zebrafish feasible.

Our previous report on the dynamic transcriptomes utilized during human erythropoiesis identified FOXO3 as one of central nodes in the progression from human embryonic stem cells (HESCs) to HESC-derived erythroblasts (ESERs) [10], and led us to speculate that FOXO3 may play a role in erythroid differentiation. Thus, we initiated a study on the function of FOXO3 in erythroid differentiation in TF-1 cells and zebrafish.

* Corresponding author. No.1–104 Beichen West Road, Chaoyang, Beijing 100101, China. Fax: +86 10 8409 7720.

** Corresponding author. 1 Beichen West Road, Chaoyang District, Beijing 100101, China. Fax: +86 10 6480 7307.

E-mail addresses: liuf@ioz.ac.cn (F. Liu), fangxd@big.ac.cn (X. Fang).

¹ Contribute equally to this work.

2. Materials and methods

2.1. Cell culture

TF-1 was obtained from ATCC (CRL-2003; Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1% penicillin–streptomycin (P/S; Invitrogen, Waltham, MA), and 3.0 ng/mL of granulocyte-macrophage colony stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ, USA) for long term growth. Prior to the induction of differentiation, cells were kept in GM-CSF-free medium for 16 h. Subsequently, EPO (3 U/mL) (Procrit, Amgen, Thousand Oaks, CA, USA) was added. Human embryonic kidney cell line HEK 293T was maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) with 10% FBS and 1% P/S in a 37 °C incubator with 5% CO₂.

2.2. siRNA-mediated knockdowns

Three siRNAs specific to human *FOXO3* (5'-GGGCGACAGCAACAGCTCT-3' [11]; 5'-GCACAACCTGTCTACTGCAT-3' [12]; 5'-GGAGCTTGAATGTGACAT-3' [12]) were synthesized and cloned into pRNAT-U6.1/Neo (GenScript, San Diego, CA, USA) to generate pRNAT-FOXO3-sh1, pRNAT-FOXO3-sh2, and pRNAT-FOXO3-sh3. In addition, *BTG1*-specific siRNA (5'-GCTCTCTGTACATTTGCTAGC-3' [13]) was synthesized and cloned into the same vector to yield pRNAT-BTG1-sh. TF-1 cells were then transfected with pRNAT-U6.1/Neo or siRNA-containing derivatives (e.g. pRNAT-FOXO3-sh1) by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA, USA) at 180 V, 975 μ F to generate control (TF-1 CTRL) and knockdown cell lines (e.g. TF-1 FOXO3-sh1).

2.3. Dual-luciferase reporter assay

Primers were designed in Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) to amplify the promoter region and FOXO3 binding site upstream of *BTG1*. The promoter with or without the binding region was cloned into luciferase vector pGL4.10 (kindly provided by Dr. George Stamatoyannopoulos, University of Washington, Seattle, WA, USA) as shown in Fig. 4B. FOXO3 binding site mutagenesis primers were designed in QuickChange Primer Design (<http://www.genomics.agilent.com/primerDesignProgram.jsp>). The QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) was for site-directed mutagenesis. In addition, *FOXO3* cDNA was amplified and cloned into pcDNA3.0 (kindly provided by Dr. Yungui Yang, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China) to generate the overexpression plasmid pcDNA-FOXO3. HEK 293T cells were co-transfected with altered pGL4.10 (Fig. 4B), pcDNA-FOXO3 and a luciferase control vector (pRL-TK, also provided by Dr. Stamatoyannopoulos) with Lipofectamine 2000 Transfection Reagent (Life Technologies). Cells were harvested 48 h after transfection and subjected to dual-luciferase reporter assays as previously described [14]. Each experiment was performed in triplicate. Primer sequences can be found in Supplemental Table 1.

2.4. Quantitative real-time PCR (qRT-PCR) and western blot

Total RNA was extracted from cells using TRIzol Reagent (Life Technologies) and reverse transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). qRT-PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad) using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Primers were designed using Primer 5 and PrimerBank (<http://pga.mgh.harvard.edu/primerbank/index.html>). Transcript levels were normalized to that of glyceraldehyde-3-

phosphate dehydrogenase (*GAPDH*). All qRT-PCR primers are listed in Supplemental Table 2.

For western blot analysis, total protein was extracted from cells using RIPA lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate], and quantified using the BCA™ Protein Assay Kit (Thermo Scientific). Anti-GAPDH (ab75834) and anti-FOXO3 (ab12162) primary antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-rabbit and anti-mouse IgG secondary antibodies were purchased from ZSGB-BIO (Beijing, China).

2.5. Zebrafish

Zebrafish strains, including AB and *gata1:GFP* [15], were raised and maintained at 28.5 °C. Embryos were obtained by natural mating of adult male and female fish and staged according to standard methods. This study was approved by the Ethical Review Committee of the Institute of Zoology, Chinese Academy of Sciences. Standard and *foxo3b*-specific morpholinos [16] (MO) (5'-TGGCTCCAGGGTTGTCTCTGCCATC-3') were purchased from Gene Tools (Philomath, OR, USA). Capped *btg1* mRNA was synthesized from linearized pCS2⁺ constructs using the mMessage mMachine SP6 kit (Ambion, Austin, TX, USA). Zebrafish embryos were injected with *foxo3b* MO (3.2 ng/embryo) and/or *btg1* mRNA (400 pg/embryo) at the one-cell stage. The fixation of zebrafish embryo and whole-mount in situ hybridization (WISH) was performed with probes directed to *gata1*, *hbae1*, *hbbe1.1*, and *hbbe2* mRNA (α_{e1-} , β_{e1-} , and β_{e2} -globin, respectively) according to previously published methods [17]. O-Dianisidine staining was performed as previously described [18].

2.6. Transcriptome sequencing and bioinformatics analyses

Flow cytometry (BD FACSAria II) was used to isolate Gata1⁺ cells from control and *foxo3b* morphants at 24 and 48 h post-fertilization (hpf). Total RNA was isolated by TRIzol and subjected to RNA sequencing by HiSeq 2000 (Illumina, San Diego, CA, USA). Sequencing reads were processed and aligned with the UCSC zebrafish reference genome (build Zv9/danRer7, Jul. 2010) using TopHat (version 1.3.3), which incorporates the Bowtie v0.12.7 algorithm to perform alignments [19]. TopHat-aligned files were then entered into Cufflinks (version 1.2.1) software for further analyses [20]. To calculate gene expression intensity, read counts were normalized to the number of fragments per kilobase of transcript per million mapped reads (FPKM) according to the gene length and total mapped reads [19]. Confidence intervals for estimates of FPKM were calculated using the Bayesian inference method [21]. Differential expression tests at the level of transcripts, primary transcripts, and genes were then processed and characterized according to the criterion of fold-change >2 and p-value <0.05 using Cuffdiff. Average linkage hierarchical clustering of gene expression intensity was performed using Pearson distance to measure the distance between genes and clones. Computation and visualization were achieved using the heatmap plus package in R (www.r-project.org/). DAVID tools (<http://david.abcc.ncifcrf.gov/>) were used to identify enriched biological themes and functional related gene groups [22–24]. GO enrichment results were accepted at a threshold of ≥ 3 gene counts with a p-value < 0.05.

The data have been deposited in the Gene Expression Omnibus database (accession numbers: GSE66860).

2.7. Statistical analysis

Group data were expressed as mean \pm standard error (SEM). Statistical significance was evaluated using unpaired, two-tailed *t*-

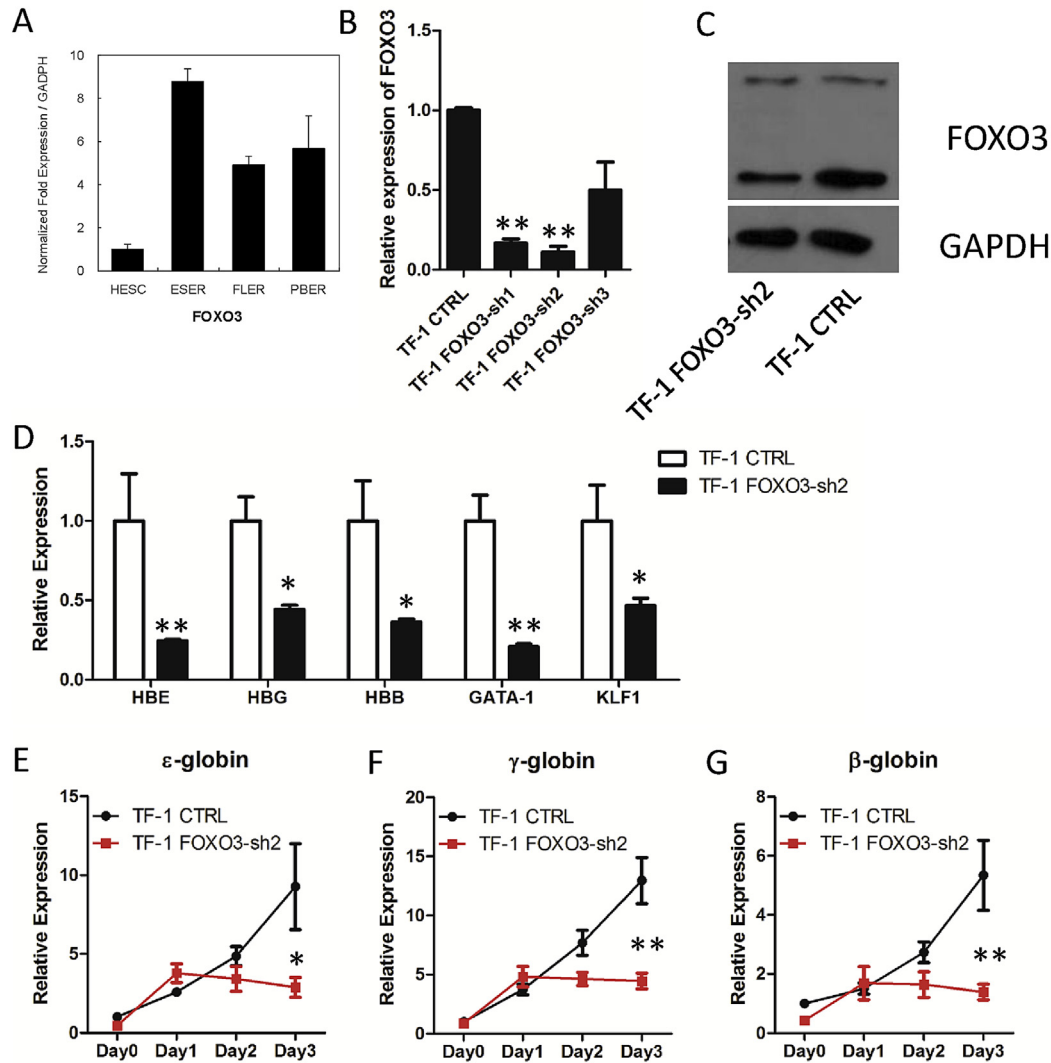


Fig. 1. FOXO3 plays a role in the induction of TF-1 erythroid differentiation. Relative FOXO3 expression at (A) different erythroid differentiation and development stages and (B) in control and TF-1 FOXO3-sh cells as determined by qRT-PCR. (C) Representative western blots showing FOXO3 expression in control and TF-1 FOXO3-sh2 cells. (D) Relative mRNA expression of globin genes, *GATA1* and *KLF1*, in the control and TF-1 FOXO3-sh2. The relative expressions of (E) ϵ -globin, (F) γ -globin, and (G) β -globin during EPO-induced erythroid differentiation in control and TF-1 FOXO3-sh2 cells. All relative mRNA expression was normalized to *GAPDH*. Asterisks indicate statistically significant differences according to Student's *t*-tests, **p* < 0.05, ***p* < 0.01.

tests in GraphPad Prism® version 5.0 (GraphPad Software, La Jolla, CA, USA), and *p*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. FOXO3 expression increase during erythroid differentiation

Our previous study on the dynamic transcriptomes that regulate erythropoiesis included four cell types: undifferentiated human embryonic stem cells (HESC), embryonic stem cells-derived erythroid cells (ESER), fetal liver-derived erythroid cells (FLER) and adult mobilized peripheral blood CD34⁺ cells-derived erythroid cells (PBER). The transcription factor FOXO3 has drawn our attention as one of central nodes in the differentiation from HESCs to ESERs [10], and exhibited a sharp increase in expression pattern during erythroid differentiation and development was confirmed using qRT-PCR (Fig. 1A). In addition, FOXO3 expression is similar to globin genes *HBE1* and *HBZ*, which encode ϵ - and ξ -

globin, respectively (Table 1). Thus, we speculated that FOXO3 possibly plays a role in erythroid differentiation.

3.2. Reduced levels of FOXO3 suppress erythroid differentiation in human

TF-1 cells were transfected with three FOXO3-specific siRNAs to generate knockdown cell lines. TF-1 FOXO3-sh2 was the most successful according to qRT-PCR analysis (Fig. 1B). The reduced expression of FOXO3 was confirmed by western blot (Fig. 1C). Examination of the expression of globin genes (*HBE*, *HBG*, and *HBB*) and critical erythroid transcription factor genes (*GATA1* and *KLF1*) revealed that FOXO3 knockdown repressed the expression of those genes (Fig. 1D). To further study the relationship between FOXO3 and erythroid differentiation in TF-1 cells, we induced both control and TF-1 FOXO3-sh2 cells undergoing erythroid differentiation with EPO. Analysis of ϵ -, γ -, and β -globin gene expression during differentiation by qRT-PCR indicated that both control and TF-1 FOXO3-sh2 cells exhibited an increased expression of globin

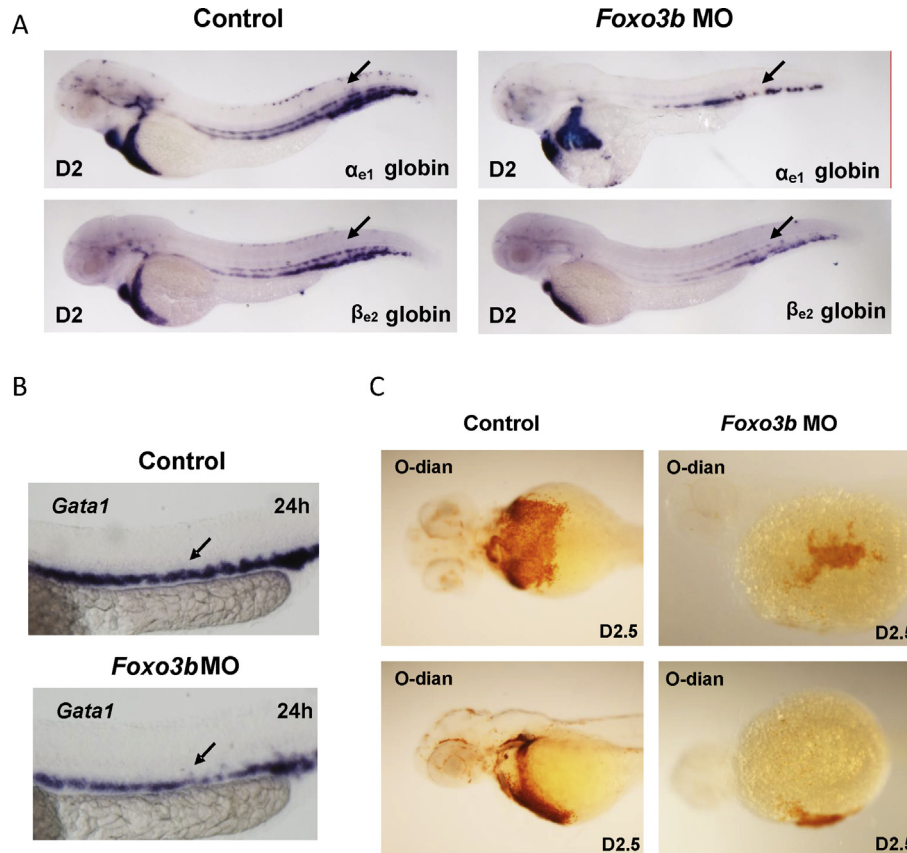


Fig. 2. Primitive erythroid differentiation is abnormal in *foxo3b* MO. (A) Representative whole-mount in situ hybridization (WISH) results showing α_{e1} globin (top) and β_{e2} globin (bottom) expression in control and *foxo3b* MO at 48 h post-fertilization (hpf). (B) Representative WISH showing *gata1* expression at 24 hpf in control and *foxo3b* MO. (C) O-Dianisidine staining showing hemoglobin expression at day 2.5 post-fertilization in control and *foxo3b* MO.

genes that associated with EPO induction (Fig. 1E–G). However, this increase appeared significantly delayed in TF-1 FOXO3-sh2 cells than vector-only controls (Fig. 1E–G).

3.3. Primitive erythroid differentiation is abnormal in *foxo3b* morphant

FOXO3 is a highly conserved in vertebrates, with a 68% sequence homology mapped between human FOXO3 and zebrafish *foxo3b*. To study the function of *foxo3b* in zebrafish, we injected standard and *foxo3b*-specific MO into zebrafish embryos. Expressions of the hemoglobin alpha embryonic-1 (α_{e1}) and hemoglobin beta embryonic-2 gene (β_{e2}) genes were detected by WISH at 2 day post-fertilization (2 dpf). Notably, both α_{e1} and β_{e1} globin gene expression were significantly decreased in *foxo3b* MO (Fig. 2A). *Gata1* is a critical transcription factor in zebrafish erythroid differentiation and its expression was detected at 24 hpf. In comparison to the control, *gata1* expression in *foxo3b* MO was significantly suppressed (Fig. 2B). At 2.5 dpf the matured erythrocytes were also observed decreasing by O-dianisidine staining (Fig. 2C).

3.4. Transcriptome dynamics in *foxo3b* MO

To get a further view of *foxo3b* MO, *Gata1*⁺ cells isolated from control and *foxo3b* MO at 24 and 48 hpf were subjected to RNA-seq and related analyses. These studies revealed that a substantial variation occurred from 24 to 48 hpf in the transcriptome, while the variation between control and *foxo3b* MO was much smaller (Fig. 3A). At 24 hpf, 194 genes were differentially expressed

between control and *foxo3b* MO, and 193 genes at 48 hpf (Fig. 3B, Supplemental File 1). At 24 hpf, 130 genes were up-regulated, while 64 genes were down-regulated out of 194 genes with differential expression (Fig. 3B, Supplemental File 1). Moreover, 79 genes were up-regulated and 114 genes were down-regulated at 48 hpf (Fig. 3B, Supplemental File 1). To get a further insight in the transcriptome mechanics, we analyzed the enriched functions of differently expressed genes. It is worth mentioning that mainly GO terms associated with the down-regulated genes in *foxo3b* MO at 24 hpf included “hemoglobin complex”, “oxygen transport”, “cytosolic part”, and “heme binding”, amongst others (Fig. 3C); while the functions of significantly down-regulated genes at 48 hpf covered a much broader range (Fig. 3D).

3.5. *BTG1* is a target gene of FOXO3 in human cells

To further study the mechanism of FOXO3-dependent gene regulation, transcriptome data from human HESC, ESER, FLER, and PBER cells were analyzed to construct a functional network central on FOXO3 by Ingenuity Pathways Analysis (IPA) (Supplemental Fig. 1). *BTG1* and B-cell CLL/lymphoma 2-like 1 (*BCL2L1*) were thought to be involved in the FOXO3-dependent regulation of erythroid differentiation due to the correlated expression pattern of FOXO3 with erythroid differentiation and development [10]. Sequence analysis was performed at the *BTG1* and *BCL2L1* promoter region to find FOXO3 binding motif 5'-TTGTTTAC-3'. However, we only found a FOXO3 binding site 159 bp upstream of the *BTG1* transcriptional start site, which is highly conserved in human and mouse (Supplemental Fig. 2).

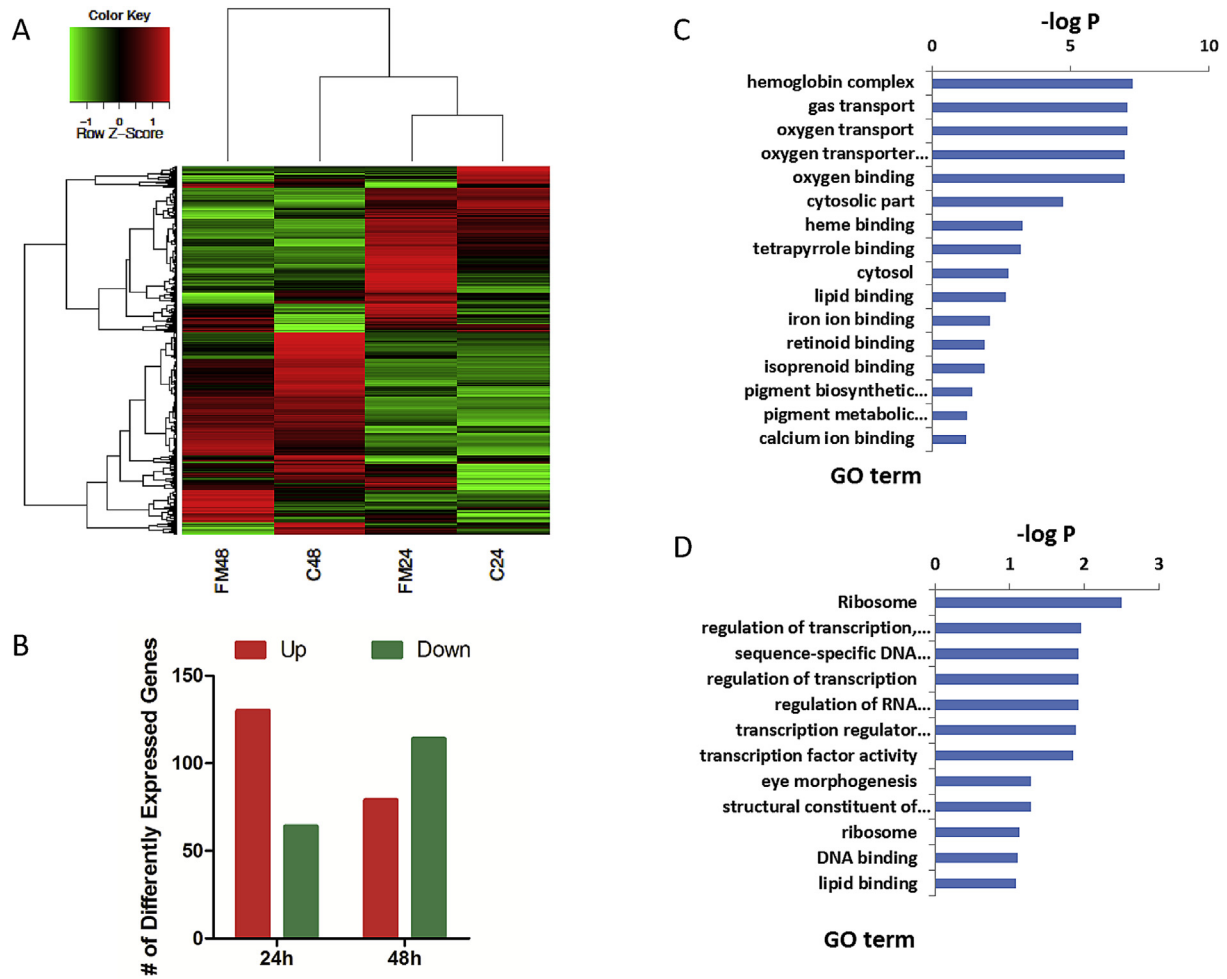


Fig. 3. Transcriptome dynamics in *foxo3b* MO. (A) Hierarchical clustering of genes expressed in control and *foxo3b* MO *Gata1*⁺ cells at 24 hpf and 48 hpf. Rows and columns represent individual genes and cell types, respectively. Expression intensities are displayed from green (low expression) to red (high expression). Lines on the top and left represent the similarity between cell types and genes, respectively. Genes and cell types with the most similar expression profiles were clustered together with the shortest branches and represented in the dendrogram to illustrate their relationship. (B) Numbers of differently expressed genes between control and *foxo3b* MO samples at 24 and 48 hpf. The top enriched functions of down-regulated genes at (C) 24 hpf and (D) 48 hpf listed according to the negative logarithm of p-value and corresponding GO term.

A dual-luciferase reporter system was applied to estimate whether FOXO3 activated the *BTG1* promoter directly. For this, plasmids were constructed as described in materials and methods (Fig. 4B). As shown in Fig. 4B and C, the *BTG1* promoter fragment significantly increased relative luciferase activity, indicating that the proper promoter region was cloned into pGL4.10. Further investigation revealed that additional FOXO3 binding site did not enhance the luciferase activity beyond that of the *BTG1* promoter (Fig. 4C, the third column). Neither did *FOXO3* expression vector (Fig. 4C, the fourth column). However, *BTG1* promoter with binding site and *FOXO3* expression vector together increased the relative luciferase activity significantly (Fig. 4C, the fifth column), which was reversed to *BTG1* promoter-only levels by mutation of the FOXO3 binding site (Fig. 4C, the last column). In addition, examination of *BTG1* expression in control and TF-1 *FOXO3*-sh2 cells by qRT-PCR revealed that it was depressed in response to *FOXO3* knockdown in TF-1 cells (Fig. 4A). Taken together, these data indicate that FOXO3 regulated *BTG1* expression by directly regulating the promoter region of *BTG1*.

3.6. *BTG1*-knockdown impaired inducible erythroid differentiation in TF-1 cells

The function of *BTG1* in erythropoiesis is still unclear. To study its function, we constructed TF-1 *BTG1* knockdown, henceforth referred to as TF-1 *BTG1*-sh. The effect of *BTG1* knockdown on gene expression was examined by qRT-PCR (Fig. 4D). Further, we induced control and TF-1 *BTG1*-sh cells towards erythroid differentiation to estimate the influence of *BTG1*. Analysis of gene expression by qRT-PCR revealed that *BTG1* knockdown attenuated the expression of the ϵ -, γ -, and β -globin genes during erythroid differentiation, similar to that observed in *FOXO3* knockdown TF-1 cells (Fig. 4E–G).

3.7. *Btg1* overexpression attenuated erythroid gene expression in zebrafish

Given the regulation of *FOXO3* on *BTG1* and its function on erythroid differentiation in TF-1 cells, we further assessed the regulation and function of *btg1* in zebrafish. However, the *btg1*

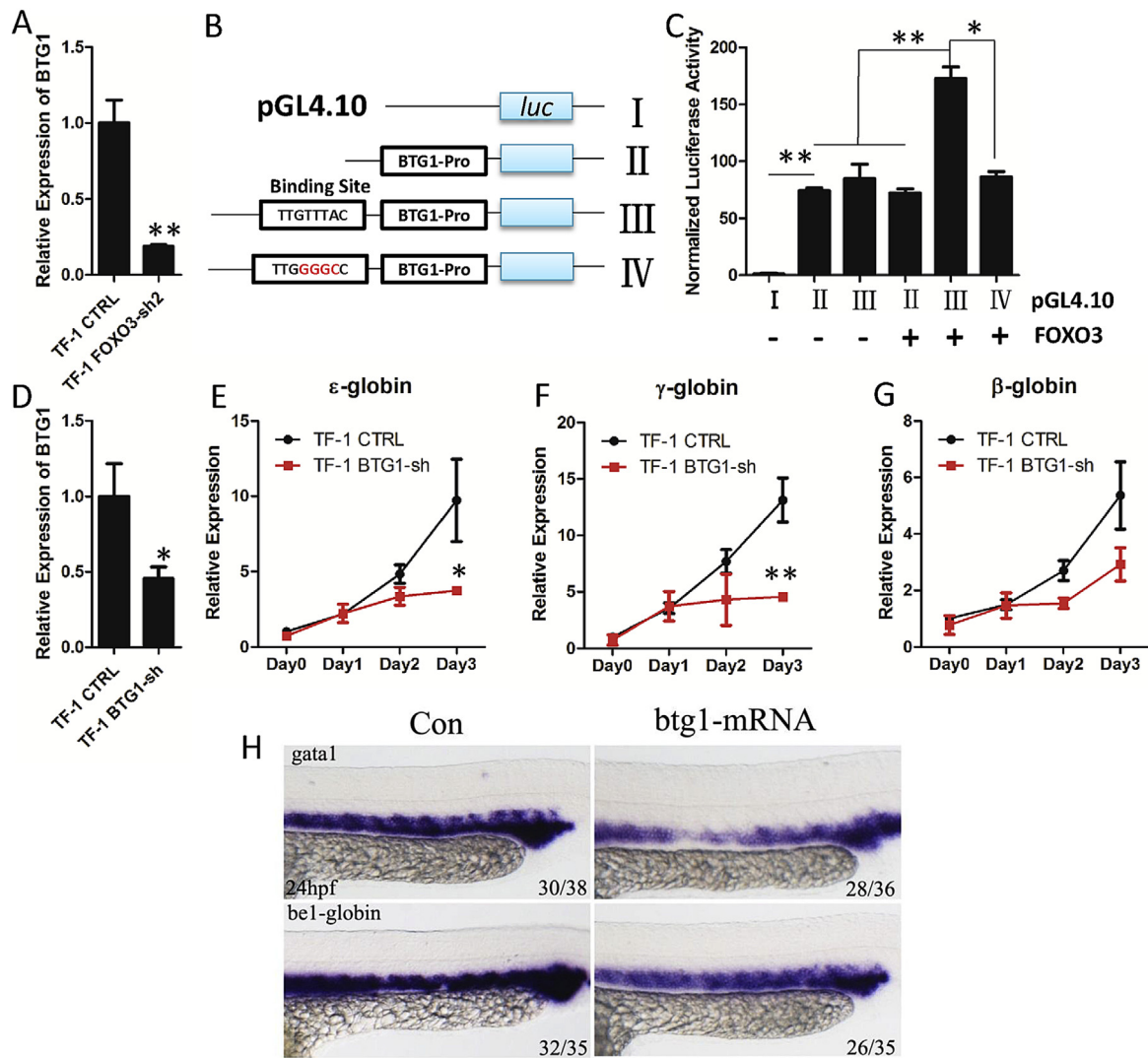


Fig. 4. Function of BTG1 in erythroid differentiation in TF-1 cells and zebrafish. (A) Relative *BTG1* expression in control and TF-1 *FOXO3*-sh2 cells using qRT-PCR. (B) Construction strategy of pGL4.10 for the dual-luciferase reporter assay. (C) HEK 293T cells were transfected with different plasmid combinations as shown. Relative luciferase activity was evaluated 48 h after transfection. (D) Relative *BTG1* expression in control and TF-1 *BTG1*-sh cells. The relative expression of (E) ϵ -globin, (F) γ -globin, and (G) β -globin genes during EPO-induced erythroid differentiation in control and TF-1 *BTG1*-sh cells. (H) Representative WISH results showing the *gata1* (up panel) and β_{e1} globin (bottom panel) expression in control (left panel) and *foxo3b* MO (right panel) at 24 hpf. All expression values were normalized to *GAPDH*. Asterisks indicate statistically significant differences according to a Student's *t* tests, **p* < 0.05, ***p* < 0.01.

Table 1
Expression (RPKM) of *FOXO3*, *HBE1*, and *HBZ* in HESC, ESER, FLER, and PBER cells, respectively.

	HESC	ESER	FLER	PBER
FOXO3	4.064	44.111	5.541	5.062
HBE1	1.423	49456.2	168.962	1.305
HBZ	0.851	11897.3	348.944	2.956

expression was not down-regulated significantly by *foxo3b* knock-down according to our zebrafish transcriptome data (Supplemental File 1). *Btg1* exhibited a small but detectable increase at 24 hpf (FPKM value increase from 36.4609 to 59.447); whereas, *btg1* expression showed a modest decrease in *foxo3b* MO when compared to the control at 48 hpf (45.3949–43.7774). Since the transcriptome data only represents expression at mRNA level, we thus speculated that *btg1* might be subjected to *foxo3b*-mediated regulation beyond the mRNA level. For this, *btg1*

mRNA was injected into zebrafish to study its function. The expressions of *gata1* (Fig. 4H, up panel) and hemoglobin beta embryonic-1 (β_{e1}) (Fig. 4H, bottom level) were detected by WISH at 24 hpf and the results revealed that this overexpression attenuated both *gata1* and β_{e1} gene expression in zebrafish at 24 hpf.

4. Discussion

In this study, we studied the function of *FOXO3*, a central node in the differentiation of HESC and ESER, in both human TF-1 cells and zebrafish. Reduced levels of *FOXO3* in both TF-1 cells and zebrafish resulted in impaired EPO-induced erythroid differentiation and primitive erythroid differentiation, respectively. Further RNA-seq analysis of *Gata1*⁺ cells sorted from the control and *foxo3b* MO at 24 hpf and 48 hpf revealed that erythroid-related functions were the main physiological impairments in *foxo3b* MO at 24 hpf. Our data presented here and generated in our previous reports imply that *BTG1* may be responsible for *FOXO3* function in erythroid

differentiation. In TF-1 cells, *FOXO3* knockdown decreased the expression of *BTG1* by directly activating its promoter region. Further, *BTG1* knockdown in TF-1 cells impaired EPO-induced erythroid differentiation. However, *foxo3b* MO exhibited an increase of *btg1* expression than the control embryo at 24 hpf and a modest decrease at 48 hpf. Altogether, these data suggest that *FOXO3* plays a role in erythroid differentiation in both human cells and zebrafish; however, the underlying mechanism still remains unclear.

In a murine erythroid cell line, the inappropriate activation of *FoxO3a* accelerates the differentiation of erythroid progenitors to erythrocytes [6]; whereas, a significant decrease in the rate of erythroid cell maturation was found in *Foxo3*-deficient mice [7]. In the present study, we found impaired EPO-induced erythroid differentiation as a result of *FOXO3* knockdown in human cell line TF-1. We also found that primitive erythroid differentiation was impaired in *foxo3b* MO. Taken together, it is apparent that *FOXO3* plays a role in erythroid differentiation across species.

The mechanism of *FOXO3* regulation in erythroid differentiation remains unclear. One explanation is that *FOXO3* regulates oxidative stress, which influences the mTOR pathway activation to subsequently influence erythroblast maturation [7,25]. In addition, *FoxO3a* was found to control erythroid differentiation possibly by regulating the expression of *Btg1* and its subsequent regulation of protein arginine methyl transferase (PRMT1) activity [6]. Here, we show that *FOXO3* can regulate *BTG1* expression through activating its promoter region in human cells. Further investigations confirmed that *BTG1* plays a role in induced TF-1 erythroid differentiation; however, a reduced level of *Foxo3b* did not decrease the expression of *btg1* in zebrafish at 24 hpf, as seen in murine [6] and TF-1 cells. Moreover, data acquired in zebrafish indicates that aberrant increase of *btg1* resulted in the down-regulation of *gata1* and β_{e1} , which is inconsistent with observations in murine [6] and TF-1 cells. We speculated that *foxo3b* regulation mechanism was simple and effective in zebrafish. The mechanisms were complicated in mice and human cells to form sophisticated regulation to face stresses and mutations. Involvement of down-stream effectors like *BTG1* makes regulation of *FOXO3* elaborate, accurate and widely.

Altogether, we found *FOXO3* regulates erythroid differentiation in both human cells and zebrafish. However, insight into the regulatory mechanism mediating these effects requires further study.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2015.03.128>.

Transparency document

Transparency document related to this article can be found online at <http://10.1016/j.bbrc.2015.03.128>.

References

- [1] T. Moriguchi, M. Yamamoto, A regulatory network governing Gata1 and Gata2 gene transcription orchestrates erythroid lineage differentiation, *Int. J. Hematol.* 100 (2014) 417–424.
- [2] J. Palis, Primitive and definitive erythropoiesis in mammals, *Front. Physiol.* 5 (2014) 3.
- [3] K.R. Katsumura, A.W. DeVilbiss, N.J. Pope, K.D. Johnson, E.H. Bresnick, Transcriptional mechanisms underlying hemoglobin synthesis, *Cold Spring Harbor Perspect. Med.* 3 (2013) a015412.
- [4] Y.Y. Yien, J.J. Bieker, EKLF/KLF1, a tissue-restricted integrator of transcriptional control, chromatin remodeling, and lineage determination, *Mol. Cell. Biol.* 33 (2013) 4–13.
- [5] M.R. Tallack, A.C. Perkins, Three fingers on the switch: Kruppel-like factor 1 regulation of gamma-globin to beta-globin gene switching, *Curr. Opin. Hematol.* 20 (2013) 193–200.
- [6] W.J. Bakker, M. Blazquez-Domingo, A. Kolbus, J. Besooyen, P. Steinlein, H. Beug, P.J. Coffey, B. Lowenberg, M. von Lindern, T.B. van Dijk, FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1, *J. Cell Biol.* 164 (2004) 175–184.
- [7] D. Marinkovic, X. Zhang, S. Yalcin, J.P. Luciano, C. Brugnara, T. Huber, S. Ghaffari, Foxo3 is required for the regulation of oxidative stress in erythropoiesis, *J. Clin. Invest.* 117 (2007) 2133–2144.
- [8] S. Chretien, P. Varlet, F. Verdier, S. Gobert, J.P. Cartron, S. Gisselbrecht, P. Mayeux, C. Lacombe, Erythropoietin-induced erythroid differentiation of the human erythroleukemia cell line TF-1 correlates with impaired STAT5 activation, *EMBO J.* 15 (1996) 4174–4181.
- [9] K. Kulkeaw, D. Sugiyama, Zebrafish erythropoiesis and the utility of fish as models of anemia, *Stem Cell Res. Ther.* 3 (2012) 55.
- [10] Y. Yang, H. Wang, K.H. Chang, H. Qu, Z. Zhang, Q. Xiong, H. Qi, P. Cui, Q. Lin, X. Ruan, Y. Li, C. Shu, Q. Li, E.K. Wakeland, J. Yan, S. Hu, X. Fang, Transcriptome dynamics during human erythroid differentiation and development, *Genomics* 102 (2013) 431–441.
- [11] R.R. Gomis, C. Alarcon, W. He, Q. Wang, J. Seoane, A. Lash, J. Massague, A FoxO-Smad synexpression group in human keratinocytes, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 12747–12752.
- [12] Y. Zhang, Y. Xing, L. Zhang, Y. Mei, K. Yamamoto, T.W. Mak, H. You, Regulation of cell cycle progression by forkhead transcription factor FOXO3 through its binding partner DNA replication factor Cdt1, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 5717–5722.
- [13] J.C. van Galen, R.P. Kuiper, L. van Emst, M. Levers, E. Tijchon, B. Scheijen, E. Waanders, S.V. van Reijmersdal, C. Gilissen, A.G. van Kessel, P.M. Hoogerbrugge, F.N. van Leeuwen, BTG1 regulates glucocorticoid receptor autoinduction in acute lymphoblastic leukemia, *Blood* 115 (2010) 4810–4819.
- [14] Q. Xiong, Z. Zhang, K.H. Chang, H. Qu, H. Wang, H. Qi, Y. Li, X. Ruan, Y. Yang, R. Sandstrom, P.J. Sabo, Q. Li, G. Stamatoyannopoulos, J.A. Stamatoyannopoulos, X. Fang, Comprehensive characterization of erythroid-specific enhancers in the genomic regions of human Kruppel-like factors, *BMC Genomics* 14 (2013) 587.
- [15] Q. Long, A. Meng, H. Wang, J.R. Jessen, M.J. Farrell, S. Lin, GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene, *Development* 124 (1997) 4105–4111.
- [16] X.W. Xie, J.X. Liu, B. Hu, W. Xiao, Zebrafish foxo3b negatively regulates canonical Wnt signaling to affect early embryogenesis, *PLoS one* 6 (2011) e24469.
- [17] C. Thisse, B. Thisse, High-resolution in situ hybridization to whole-mount zebrafish embryos, *Nat. Protoc.* 3 (2008) 59–69.
- [18] H.W. Detrich 3rd, M.W. Kieran, F.Y. Chan, L.M. Barone, K. Yee, J.A. Rundstadler, S. Pratt, D. Ransom, L.I. Zon, Intraembryonic hematopoietic cell migration during vertebrate development, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 10713–10717.
- [19] C. Trapnell, B.A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M.J. van Baren, S.L. Salzberg, B.J. Wold, L. Pachter, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, *Nat. Biotechnol.* 28 (2010) 511–515.
- [20] A. Roberts, C. Trapnell, J. Donaghey, J.L. Rinn, L. Pachter, Improving RNA-Seq expression estimates by correcting for fragment bias, *Genome Biol.* 12 (2011) R22.
- [21] H. Jiang, W.H. Wong, Statistical inferences for isoform expression in RNA-Seq, *Bioinformatics* 25 (2009) 1026–1032.
- [22] G. Dennis Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, R.A. Lempicki, DAVID: database for annotation, visualization, and integrated discovery, *Genome Biol.* 4 (2003) P3.

- [23] W. Huang da, B.T. Sherman, R.A. Lempicki, Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists, *Nucleic Acids Res.* 37 (2009) 1–13.
- [24] D.W. Huang, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, *Nat. Protoc.* 4 (2009) 44–57.
- [25] X. Zhang, G. Camprecios, P. Rimmele, R. Liang, S. Yalcin, S.K. Mungamuri, J. Barminko, V. D'Escamard, M.H. Baron, C. Brugnara, D. Papatsenko, S. Rivella, S. Ghaffari, FOXO3–mTOR metabolic cooperation in the regulation of erythroid cell maturation and homeostasis, *Am. J. Hematol.* 89 (2014) 954–963.