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

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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 (hbab1 and hbce2) 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) serves 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.
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’-GGGCGACACGAACGACGCCTTCTGTAAG-3’ [11]; 5’-GCCAACCTGTCACTGCAATCCTG-3’ [12]; 5’-GGGACCTTGAAGTACGTCAAGTAC-3’ [13]) 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’-CGCTTCTGTCATTTGCTAGC-3’) 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, 100 V, and 795 μF to control electroporation of TF-1 cells 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 Regent (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 ReverTra™ 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] (5’-TGCTTCAAGGTTGCTTCGTGGCATC-3’) were purchased from Gene Tools (Philomath, OR, USA). Capped bg1 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 bg1 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, hhb1, and hhb2 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, hhb1, and hhb2 mRNA (400 pg/embryo).

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 previously published methods [21]. Differential expression tests at the level of transcripts, primary transcripts, and genes were then performed 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.

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

2.7. Statistical analysis

Group data were expressed as mean ± standard error (SEM). Statistical significance was evaluated using unpaired, two-tailed t-
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 ζ-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 expression during differentiation by qRT-PCR indicated that both control and TF-1 FOXO3-sh2 cells exhibited an increased expression of globin
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 (αe1) and hemoglobin beta embryonic-2 gene (βe2) genes were detected by WISH at 2 day post-fertilization (2 dpf). Notably, both αe1 and βe1 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).
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 c-, 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 bg1 in zebrafish. However, the bg1
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 e 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 (βe1) (Fig. 4H, 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

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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
differential. 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 erythrophagocytes [6]; whereas, a significant decrease in the rate of erythroid cell maturation was found in Foxa3-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 b3γ, 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.

References

