Hypoxia-inducible factor 1–mediated human GATA1 induction promotes erythroid differentiation under hypoxic conditions

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Abstract

Hypoxia-inducible factor promotes erythropoiesis through coordinated cell type–specific hypoxia responses. GATA1 is essential to normal erythropoiesis and plays a crucial role in erythroid differentiation. In this study, we show that hypoxia-induced GATA1 expression is mediated by HIF1 in erythroid cells. Under hypoxic conditions, significantly increased GATA1 mRNA and protein levels were detected in K562 cells and erythroid induction cultures of CD34+ haematopoietic stem/progenitor cells. Enforced HIF1α expression increased GATA1 expression, while HIF1α knockdown by RNA interference decreased GATA1 expression. In silico analysis revealed one potential hypoxia response element (HRE). The results from reporter gene and mutation analysis suggested that this element is necessary for hypoxic response. Chromatin immunoprecipitation (ChIP)-PCR showed that the putative HRE was recognized and bound by HIF1α in vivo. These results demonstrate that the up-regulation of GATA1 during hypoxia is directly mediated by HIF1α. The mRNA expression of some erythroid differentiation markers was increased under hypoxic conditions, but decreased with RNA interference of HIF1α or GATA1. Flow cytometry analysis also indicated that hypoxia, desferrioxamine or CoCl2 induced expression of erythroid surface markers CD71 and CD235a, while expression repression of HIF1α or GATA1 by RNA interference led to a decreased expression of CD235a. These results suggested that HIF1-mediated GATA1 up-regulation promotes erythropoiesis in order to satisfy the needs of an organism under hypoxic conditions.

Keywords: hypoxia • hypoxia-inducible factor 1 (HIF1) • hypoxia-response element (HRE) • GATA1 • erythropoiesis • erythroid differentiation

Introduction

The production of red blood cells is promoted by the hormone erythropoietin (EPO) in response to tissue hypoxia. It has been discovered that the hypoxic induction of erythropoietin serves as a paradigm of oxygen-dependent gene regulation and that hypoxia-inducible factor (HIF) serves as a key mediator of cellular adaptation to low oxygen. Erythropoietin is a glycoprotein hormone and its major action is the prevention of apoptosis in EPO-dependent colony-forming unit-erythroid cells and erythroblasts that have not begun haemoglobin synthesis [1]. Hypoxia-inducible factor is a heterodimeric transcription factor that is composed of a constitutively expressed HIFβ subunit and an oxygen-regulated HIFα subunit [2]. Both the stability and transcriptional activity of HIF are negatively regulated by oxygen-dependent hydroxylation of specific residues [3]. Recent experimental evidence suggests that HIF promotes erythropoiesis through coordinated cell type–specific hypoxia responses, which include increased EPO production in the kidney and liver, enhanced iron uptake and utilization, as well as changes in the bone marrow micro-environment that facilitate erythroid progenitor maturation and proliferation [1, 4].

Haematopoietic transcription factor GATA1 is the founding member of the GATA family of transcription factors. It is expressed in primitive and definitive erythroid cells, megakaryocytes, eosinophils, mast cells and the Sertoli cells of the testis [5]. GATA1 is essential for normal erythropoiesis [6, 7]. GATA1 is directly involved in cell survival. It activates transcription of the erythropoietin receptor (EpoR) [8], and EPO signalling is important for the survival of erythroid progenitors [9]. Bcl-xL that

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encodes an anti-apoptotic protein is another GATA1-regulated gene [10]. Furthermore, GATA1 has also been implicated in the regulation of Gv/S cell cycle progression [11] and the reprogramming of haematopoietic precursors [12]. GATA1 interacts with a variety of proteins and these interactions play important roles in haematopoesis. GATA1 induces the expression of many target genes, some of which are essential for the differentiation and maturation of erythroid cells.

The stimulation of red blood cell (RBC) production is one of the systemic adaptations to hypoxia, and caspase-mediated cleavage of GATA1 represents an important negative control mechanism in erythropoiesis. It is reported that erythropoiesis blockade following EPO deprivation was largely prevented by the expression of caspase-inhibitory proteins or caspase-resistant GATA1 in erythroid progenitors [13]. Previous study also revealed that the expression of GATA1 in the rat kidney fibroblast NRK-49F cell line was determined only under hypoxic conditions but not under normoxic conditions [14]. We therefore deduce that GATA1 is associated with cellular response to hypoxia. Here, we show that HIF1 induces the expression of human GATA1 under hypoxic conditions to promote erythropoiesis.

Materials and methods

Cell lines and cell culture

The human myelogenous leukaemia cell line K562 and the human breast adenocarcinoma cell line MCF-7 were, respectively, cultured in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium ( Gibco, Grand Island, NY, USA) with 10% foetal bovine serum (FBS) and penicillin/streptomycin. Cells maintained at 37°C in an incubator with 5% CO2. For hypoxic exposure, cells were placed in an incubator chamber that was tightly sealed and thoroughly flushed with 1% O2/5% CO2/balance nitrogen and incubated at 37°C. Where indicated, desferrioxamine (DFO) or cobalt chloride (CoCl2) (Sigma-Aldrich, Deisenhofen, Germany) was added to the medium at a final concentration of 100 µM.

Isolation and erythroid induction cultures of CD34+ haematopoietic stem/progenitor cells (HPCs)

Human umbilical cord blood (UCB) was obtained from normal full-term deliveries with informed consent and the relative research was approved by the Research Ethics Committee of the Military General Hospital of Beijing (China) and the Research Ethics Committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Mononuclear cell (MNC) fractions were isolated from UCB by percoll density gradient (d = 1.077; Amersham Pharmacia Biotech, Freiburg, Germany). CD34+ HPCs were enriched from MNCs through positive immunomagnetic selection (CD34 Multisort kit; Miltenyi Biotec, Bergisch-Gladbach, Germany). CD34+ cells were cultured in Iscove’s Modified Dulbecco’s Medium with 30% foetal bovine serum, 1% bovine serum albumin (BSA), 100 µM 2-ME, 2 ng/ml recombinant human interleukin-3 (IL-3), 100 ng/ml recombinant human stem cell factor (Stem Cell Technologies, Vancouver, British Columbia, Canada), 2 U/ml recombinant human EPO (R&D Systems Inc., Minneapolis, MN, USA), 60 mg/ml penicillin and 100 mg/ml streptomycin. Four days later, cells were cultured in normoxia or hypoxia for indicated time before harvested.

Plasmid constructs

The cDNA encoding HIF1α was amplified from the HA-HIF1α plasmid [15] (a gift from Dr. Yunjin Jung, Pusan National University, Korea) using the primers HIF1α-F (5’-ACGGTGACATGAGGCCCAGGC-3’) and HIF1α-R (5’-ACGGCCGCGTAACTGGATCACA-3’). This fragment (2480 bp) was inserted, in-frame, into the Kpn1/Apal sites of FLAG-tagged pcDNA6/V5-His B (Invitrogen, Carlsbad, CA, USA), yielding the construct pcDNA6/V5His/FLAG-HIF1α (pHIF1α).

The dominant-negative form of HIF1α DN, which completes with endogenous HIF1α for dimerization with HIF1β but forms an inactive transcription heterodimer, was amplified as described previously [16, 17]. The primers DN-F (5’-ACGGTGACATGAGGCCCAGGC-3’) and DN-R (5’-ACGGCCGCGTAACTGGATCACA-3’) were used for PCR amplification. The amplified fragment was double-digested with KpnI/ApaI and inserted to pcDNA6/V5HisB, yielding the construct pcDNA6/V5HisB/HIF1α-DN (pDN).

To specifically silence HIF1α, we constructed the plasmid psiSilencer 2.1-U6-HIF1α. The plasmid psiSilencer 2.1-U6neo (Ambion, Austin, TX, USA) was double-digested with BamH I and HindIII. The target sequences of HIF1α mRNA were amplified as described above using the primer pairs: (sense) 5’-ACGGTGACATGAGGCCCAGGC-3’; (anti-sense) 5’-ACGGCCGCGTAACTGGATCACA-3’. This plasmid psiSilencer 2.1-U6 vector was used to yield the plasmid psiSilencer 2.1-U6-HIF1α-RNAi (pshHIF1α).

To construct the recombinant plasmid plG3-GATA1, a 750-bp fragment amplified from human genomic DNA was double-digested using Sall and BamHI (MBI Fermentas, Burlington, ON, Canada) and inserted into the BamHI/Sall sites of the plG3-Promoter Vector (Promega, Madison, WI, USA). Mutations of the putative HRE sequence in the plG3-GATA1 plasmid were introduced through polymerase chain reaction (PCR)-based site-directed mutagenesis. The bases GAAAG replaced GCGTG at position 533 and GAAAC replaced GCGC GGCC at position 548 to construct plG3-GATA1-M12 with dual mutations. The following primers were used for amplification and mutation: pGATA1F, 5’-ACGGTGACATGAGGCCCAGGC GGGC GCGCAGGAAGTTGTCAAAACACACGCTGCACAGTGATCCACTGGTCTTTTCA-3’; pGATA1R, 5’-ACGGTGACATGAGGCCCAGGC GGGC GCGCAGGAAGTTGTCAAAACACACGCTGCACAGTGATCCACTGGTCTTTTCA-3’; M1F, 5’-ACGGTGACATGAGGCCCAGGC GGGC GCGCAGGAAGTTGTCAAAACACACGCTGCACAGTGATCCACTGGTCTTTTCA-3’; M1R, 5’-ACGGTGACATGAGGCCCAGGC GGGC GCGCAGGAAGTTGTCAAAACACACGCTGCACAGTGATCCACTGGTCTTTTCA-3’. This fragment was cloned in-frame with the putative HRE sequence into pcDNA6/V5HisB/HIF1α-DN (pDN).

Cell transfection

For transfection with plasmids, K562 cells were plated in 3.5-cm dishes at 70–80% confluency and cultured for several hours before transfected with 4 µg of plasmid. The transfection reagent Lipofectamine 2000 (Invitrogen) was used according to the manufacturer’s instructions.

For transfection with siRNAs, K562 cells were plated in 3.5-cm dishes at 70–80% confluency and cultured for several hours. The siRNAs (Dharmacon, Lafayette, CO, USA) were re-suspended and DharmaFECT
transfection reagents were used according to the manufacturer’s instructions. The siRNAs targeting HIF1A were siGENOME SMARTpool containing GGACACAGAAUUUGACUUUG, GAUGAGAACAGACUAGACAA, CGUGUUAUCUGGUCGCUUUG, and GAUGAAGAUAUIACCGAAG. The siRNAs targeting GATA1 were ON-TARGETplus SMARTpool containing GGACAGGCACUACCUUAUAG, AGCCGUGGCUACAGACA, GCUGGUGCCUUAGUGGUG and CCAAGAAGCCGGCAGAUGU.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from cell samples with TRIzol Reagent (Invitrogen) and quantified with NanoDrop 2000 Spectrophotometer (Thermo Scientific Inc., Bremen, Germany). The first strand of cDNA was synthesized by M-MLV reverse transcriptase (Invitrogen) according to the manufacturer’s instruction. The target mRNAs in cultured K562 cells and CD34+ HPCs were quantified by real-time PCR using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) within the iQ™ 5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each PCR reaction was performed in triplex tubes, and β-actin was used as an endogenous control to standardize the amount of the sample mRNA. The quantification data were analysed with the iQ® software (Bio-Rad). The following primers were used for real-time PCR: β-actin-f, 5’-CTGGACCAACACCTCTTACA-3’, β-actin-r, 5’-AGCACAGCCTGAGATGCAAC-3'; GATA1-f, 5’- CCTGCTTTGTGCACACATG-3’, GATA1-r, 5’-CTGCTCaACGATGCAAAG-3’, HIF1α-f, 5’-AGGTGGAATGTCCTGGGTG-3’, HIF1α-r, 5’-AAGGACAATTTGTTTGTG-3’, α-globin-f, 5’-GGTAACACTGAGCTCAAG-3’, α-globin-r, 5’-GCTCAAGAGCCGACAACTG-3’, β-globin-f, 5’-GCTACCTTGGACAAAGCGAGGTG-3’, β-globin-r, 5’-TGAGCAGCCGACATAAACG-3’, γ-globin-f, 5’-GGACCTTGCTCAGTCAGGT-3’, γ-globin-r, 5’-GGCAAGAGGTTGCTAAGG-3’, γ-globin-f, 5’-GGACCTTGCTCAGTGCAGCT-3’, γ-globin-r, 5’-GGCAAGAGGTTGCTGAGG-3’, CD71-f, 5’-CTTCCGAGATGCTCCATGG-3’, CD71-r, 5’-CACCGGAATATGTCTGGGTTG-3’, CD235a-f, 5’-GGCTGTGTTATGGGACAGCT-3’, CD235a-r, 5’-GAGGTTTTTACATCAGTGATGGCCTTT-3’.

Western blot assay

Cell samples were lysed with SDS Lysis Buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol). Total cell extracts were quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk solution for 2 hrs and incubated either with anti-HIF1α, anti-GATA1 (Abcam plc, Cambridge, UK), or anti-ACTB (ProteinTech Group Inc., Chicago, IL) monoclonal antibody overnight followed by peroxidase-conjugated affinipure goat anti-mouse or anti-rabbit IgG (H + L) (Zhongshan Goldenbridge, Beijing, China). After washed with TBS-T buffer, the membrane was treated with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and exposed to Kodak X-omat BT Film.

Dual-luciferase reporter assay

K562 cells or MCF-7 cells were plated in each well of 24-well plates at 50–60% confluence the day before co-transfection using the poly(ethylene) (PEI) method as described previously [19, 20] with a mixture of 1 μg pGL3 reporter plasmid and 20 ng pRL-TK reporter vector encoding Renilla luciferase, which was used to normalize for transfection efficiency. In other experiments, 0.6 μg of expression plasmids was co-transfected with 0.4 μg of pGL3-GATA1 and 0.1 μg pRL-TK in total as indicated in the figure legends. Six hours after transfection, cells were transfected to complete medium and incubated for 24 hrs under the specified condition. Cells were lysed with Passive Lysis Buffer, and the dual-luciferase activities were measured by a Modulus™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) using the dual-luciferase reporter assay system (Promega, Milano, Italy) according to the manufacturer’s instructions.

Chromatin immunoprecipitation-PCR (ChIP-PCR)

K562 cells in four 100-mm culture dishes containing 10 ml of growth media were cultured under normoxia (21% O2) or hypoxia (1% O2) for 24 hrs and then fixed in 1% formaldehyde (Sigma-Aldrich) at room temperature for 10 min. and quenched with glycine for 5 min. Cells were lysed and sonicated to get 200–1000 bp DNA fragments. Chromatin immunoprecipitation was performed using the EZ-ChIP™ Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer’s instructions with minor modifications. A ChIP-grade rabbit polyclonal antibody to HIF1α (Abcam plc) and a polyclonal antibody to p300 (Biovert, Atlanta, GA, USA) were used as the immunoprecipitating antibody and rabbit IgG (Santa Cruz Biotechnology, CA, USA) was used as the control. After reverse cross-linking and DNA purification, the INPUT and immunoprecipitated DNA samples were used as templates to amplify the target sequences by PCR, and the products were assayed by agarose gel electrophoresis. The primers used for ChIP-PCR are as follows: forward (5’- AGCAGGTTGATAAGGGTGTTG-3’) and reverse (5’- GTCATTGTTTCAGGCCAC-3’), with a 302 bp product covering the putative HREs of GATA1. We also used the primers for the positive control: forward (5’- CGCCCTGTCTCACACT-3’) and reverse (5’- GATAAGCCCTGTCCTTGAGCC-3’), with a 302 bp product covering the negative control: forward (5’- CTGTAAGACTACATGGAGG-3’) and reverse (5’- CCAAGATACACTACATCC-3’), with a 486 bp product covering no HREs.

Flow cytometry

Cells were harvested and washed in ice cold PBS. For indirect labelling, cells were fixed with formaldehyde and permeated with methanol; After rinsing with incubation buffer (PBS with BSA), cells were incubated with unconjugated primary antibody for 1 hr at room temperature and then incubated with fluorochrome-conjugated secondary antibody for 30 min. at room temperature. For direct labelling, cells were rinsed with ice cold PBS and then incubated with fluorochrome-conjugated antibody for 30 min. at 4°C in the dark. Finally, Cells were rinsed, suspended in PBS, and analysed using the Accuri C6 flow cytometer system (Accuri Cytometersm, Ann Arbor, MI). FITC Conjugated Anti-human CD235a (glycophorin A), Mouse IgG2b Isotype Control, PE Conjugated Anti-human CD71 (transferrin receptor), Mouse IgG1 kappa Isotype Control (eBioScience, San Diego, CA) and FITC Conjugated goat anti-rabbit IgG (Zhongshan Goldenbridge, Beijing, China) were used.

Statistics

The data were analysed with Student’s t-test (two-tailed). P values < 0.05 were considered significant.
Results

Hypoxia-induced expression of GATA1

To determine whether hypoxia induces expression of GATA1 under hypoxic conditions, K562 cells and CD34<sup>+</sup> HPCs were cultured in normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) before harvest. GATA1 mRNA was measured by real-time PCR. Significantly increased expression of GATA1 mRNA was detected in K562 cells treated with DFO or CoCl<sub>2</sub> or under hypoxic conditions (Fig. 1A). The expression of GATA1 mRNA also increased in CD34<sup>+</sup> erythroid cultures exposed to hypoxia (Fig. 1B). GATA1 protein expression was examined by Western blot assay. As expected, the protein levels of HIF1α and GATA1 obviously increased in K562 cells exposed to DFO or CoCl<sub>2</sub> or hypoxia (Fig. 1C). Increased GATA1 protein levels were also observed in CD34<sup>+</sup> erythroid cultures exposed to hypoxia (Fig. 1D). Furthermore, flow cytometry also showed a notable increase of GATA1 protein in K562 cells under hypoxic conditions (Fig. 1E). These results established that hypoxia could induce expression of GATA1.

Hypoxia-induced expression of GATA1 is regulated by HIF1

To determine whether HIF1 involves in the hypoxia-induced expression of GATA1, we constructed a HIF1α expression plasmid, pCDNA6/V5HisB/HIF1α (pHIF1α), and a plasmid expressing a HIF1α-specific interference RNA sequence, psilencer 2.1/U6-HIF1α-RNAi (pSiHIF1α). These plasmids or their corresponding empty vectors were, respectively, transfected into K562 cells. The mRNA levels of the HIF1α gene (HIF1A) and GATA1 were determined by real-time PCR, and their protein levels were determined by Western blotting. As expected, there was a dramatic rise in HIF1A mRNA and protein in K562 cells transfected...
with pHIF1α, and this HIF1α over-expression induced expression of GATA1 mRNA and protein (Fig. 2A and B). In contrast, there was a sharp decrease in HIF1α mRNA and protein in K562 cells transfected with siRNA targeting HIF1A (siHIF1α) or control siRNA (siCON). After 24 hrs of transfection, the transfection medium was replaced with complete medium and the cells were cultured under normoxia (the left two columns) or hypoxia (the right two columns) for an additional 24 hrs. (F) Western blotting assay of HIF1α and GATA1 protein levels in K562 cells transfected with siRNA targeting HIF1A or control siRNA. After 24 hrs of transfection, the transfection medium was replaced with complete medium and the cells were cultured under hypoxia for an additional 24 hrs. The numbers in brackets indicate the mean fluorescent intensities. (G) Real-time PCR analysis of HIF1α and GATA1 mRNA levels in K562 cells transfected with the plasmid pcDNA or pDN. After 6 hrs of transfection, the transfection medium was replaced with complete medium and the cells were cultured under normoxia (the left two columns) or hypoxia (the right two columns) for 36 hrs. pDN that consists of amino acids 28 through 390 of human HIF1α, contains the basic domain deletion that affects DNA binding, and the carboxyl-terminal truncation that affects transactivation. (H) Western blotting assay of GATA1 protein in K562 cells transfected with the plasmid pcDNA or pDN. After 6 hrs of transfection, the transfection medium was replaced with complete medium and the cells were cultured under normoxia or hypoxia for 36 hrs. *P < 0.01.
Identification of functional HRE in the human GATA1 gene

We searched the DNA sequence of the human GATA1 gene on NCBI and identified putative HRE core motifs present in the 3'-flanking region within 1 kbp DNA fragment of the human GATA1 gene. Two HRE core motifs RCGTG and another CACAG were located within a less than 100 bp region (Fig. 3A and B). This putative HRE was of extreme similarity to the consensus HRE described previously [21–23] (Fig. 3C). Such core motifs provide the potential for HIF1 recognition and HRE function.

To determine whether the putative HRE within the 3'-flanking region is actually involved in the hypoxic induction of GATA1, the DNA fragment containing this region was inserted into the 3'-clone site of a luciferase reporter gene vector (pGL3-promoter), yielding pGL3-GATA1. Specific mutations were individually introduced into the core motif sequences within this construct to generate different constructs. K562 cells and MCF-7 cells were transfected with these constructs and cultured under normoxic or hypoxic conditions. The wild-type pGL3-GATA1 construct showed a hypoxic induction of luciferase activity. However, this activation decreased when the consensus sequence RCGTG was replaced with RAAAG in the single mutant constructs pGATA1-M1 or pGATA1-M2, and dual mutations led to a greater loss of hypoxia-induced activity in both of K562 cells and MCF-7 cells (Figs 3D and S1). These results demonstrate that the putative HRE core motifs, located at 533 and 548 bp downstream from the human GATA1 gene, are responsible for the hypoxic induction of GATA1.

Validation of HIF1 binding to the GATA1 downstream region covering the putative HRE in vivo

We performed a ChIP assay to examine whether the GATA1 region covering the putative HREs was recognized and bound by HIF1 in vivo. DNA was isolated from K562 cells, fragmented by sonication and immunoprecipitated with an anti-HIF1 antibody. When these fragments were used as templates of PCR amplification using a primer pair located within the 3'-flanking region of the GATA1 gene, an obvious amplification fragment was detected in samples under hypoxic conditions (Fig. 4A and B). An obvious PCR product was also observed with the positive control (PC) primers, which amplify a region containing the HRE of PDK1, a previously verified target of HIF1. However, no obvious PCR product was detected when DNA fragments from cells cultured under normoxia were subjected to ChIP-PCR. The negative control (NC) fragment lacking of HRE motif was amplified from input DNA, but not from DNA subjected to ChIP, demonstrating that the positive ChIP results were not due to non-specific binding of HIF1 to a random sequence. The results from the IgG control excluded non-specific binding of the HIF1 antibody to other proteins. As HIF binding often recruits the co-activator p300, we also performed ChIP assay with anti-P300 antibody to examine if the HIF1 binding in the HRE region located in downstream of GATA1 gene resulted in recruitment of P300. The results showed that the DNA fragment bound by HIF1 under hypoxic conditions was also immunoprecipitated by anti-p300 antibody (Fig. 4C) which gave a positive answer. All of the results demonstrated that the GATA1 downstream region containing the putative HRE could be recognized and bound by HIF1 in K562 cells under hypoxic conditions.

Involvement of GATA1 in hypoxia-induced erythroid differentiation

As GATA1 is essential for red blood cells development, we used real-time PCR to examine association of the GATA1 expression with the expression of erythroid differentiation makers. It was
shown that, accompanying with hypoxia-induced expression of GATA1, the expressions of α-, γ- globin, CD71 and CD235a increased in K562 cells treated with DFO or CoCl2 under hypoxic conditions (Fig. 5A), and the expressions of α-, β-, γ- globin, CD71 and CD235a also increased in CD34+ erythroid cultures under hypoxic conditions (Fig. 5B). However, when K562 cells were transfected with siRNA targeting HIF1α or GATA1, the expressions of these erythroid differentiation markers significantly decreased (Fig. 5C). These results demonstrated that hypoxia-induced expression of GATA1 promoted the expressions of erythroid differentiation markers.

We also performed flow cytometry analysis to assay expressions of the erythroid surface markers CD71 and CD235a. As the curve shifted right, the expression of CD71 increased in K562 cells exposed to hypoxia for 32 hrs or 48 hrs (Fig. 6A), and significantly enhanced in K562 cells treated with DFO or CoCl2 (Fig. 6B). In addition, the expression of CD235a gradually enlarged under hypoxia (Fig. 6C), and also notably increased in DFO- or CoCl2-treated K562 cells (Fig. 6D). While CD71 (also known as transferrin receptor) has been verified as a target gene of not only HIF1 but also GATA1 [24–26], we additionally determined if the expression of CD235a was affected by siRNAs that targets HIF1α or GATA1. The expression of CD235a obviously decreased when the K562 cells with knocked down of HIF1α or GATA1 were cultured in normoxia (Fig. 6E). Under hypoxic conditions, a significantly reduced expression of CD235a in K562 cells with knockdown of GATA1 was detected, while a slightly decreased expression of CD235a in K562 cells with HIF1α knockdown (Fig. 6F). Furthermore, when a positive peak was marked according to the negative control, a notable decreased percentage of CD235a-positive cells was detected in the K562 cells with HIF1α knockdown (Fig. 7). These showed that knockdown of HIF1α or GATA1 leads to a decreased expression of CD235a.

These results demonstrated that hypoxia-induced expression of GATA1 increased the expression of erythroid differentiation makers, which suggested that HIF1-mediated human GATA1 gene induction participated in the hypoxia-induced erythropoiesis.

Discussion

In this study, we demonstrate that the expression of human erythroid-specific GATA1 is regulated by HIF1 and the upregulation of GATA1 expression contributes significantly to erythropoiesis in response to hypoxia.

Erythropoiesis is a necessity throughout the life for the limited lifespan of circulating erythroid cells. During erythropoiesis, haematopoietic stem cells (HSCs) first give rise to common myeloid progenitors (CMPs), and then differentiate into bipotential megakaryocytic and erythroid progenitors (MEPs) [27]. The earliest erythroid-committed progenitors are erythroid burst-forming units (BFU-Es), which further differentiate through erythroid colony-forming units (CFU-Es) and proerythroblasts into erythroblasts [4]. Erythroid progenitors have the potential to proliferate rapidly in response to anaemia and hypoxia stimuli, a process referred to as stress erythropoiesis. Hypoxia alters the response of progenitor cells to BMP4 and SCF, and they cooperatively regulate the expansion of stress BFU-E [28]. However, EPO is required for late stage differentiation. In particular, the number of CFU-Es increases markedly during stress erythropoiesis. The receptor for erythropoietin (EpoR), which is expressed abundantly in CFU-E stage progenitors [29], plays a crucial role in promoting the erythropoietic response [30].

Both EPO-EpoR signalling and GATA1 are required for normal erythroid cell development. They regulate the survival, proliferation, differentiation and maturation of erythroid cells [5, 31, 32]. Erythropoietin- and EpoR-deficient mice die during embryogenesis with severe anaemia because of a lack of post-progenitor maturation of erythroid cells. Loss of GATA1 results in fatal embryonic anaemia [33], and GATA1-deficient cells are arrested
at a proerythroblast stage [34]. Erythropoietin acts as the key upstream signal in stress erythropoiesis. In fact, during erythroid cell expansion increased plasma EPO concentration precedes an increase in G1-HRD-luc, which is under the control of the GATA1 haematopoietic regulatory domain [35]. It is reported that EPO stimulates phosphorylation and activation of GATA1 via the PI3-kinase/AKT signalling pathway. Moreover, there are reports showing that GATA1 is expressed prior to the EpoR but its expression is enhanced by EpoR-mediated signals [36, 37]. However, our data suggest that HIF1 directly induces GATA1 gene expression under hypoxic conditions, and this could contribute significantly to stress erythropoiesis. In our contention, EPO stimulates proliferation of erythroid progenitors to expand the progenitor population, and GATA1 promotes the differentiation and maturation of committed erythroid progenitor cells in response to hypoxia.

EPO/EpoR and GATA1 are essential for the survival of erythroid precursors, and their terminal differentiation into red blood cells. Loss of EPO signalling through gene targeting results in apoptosis of committed definitive erythroid precursors at the late CFU-E stage [38, 39], and EPO is required for optimal expression of bcl-xL in erythroid cells [40, 41]. GATA1-deficient erythroid cells undergo rapid apoptosis [34, 42] and embryonic red cell precursors in mouse embryos lacking GATA1 undergo maturation arrest [33]. GATA1 and EPO cooperate to induce bcl-xL expression, which in turn is critical for the survival of late proerythroblasts and early normoblasts [10]. HIF1-mediated induction of GATA1 promotes erythroid cell survival under hypoxic conditions.
GATA1 regulates virtually all erythroid-expressed genes including globins, heme biosynthetic enzymes, membrane proteins, and red blood cell transcription factors [43, 44]. GATA1 helps to establish and maintain the erythroid phenotype by activating these genes. GATA1 is involved in the regulation of G1/S cell cycle progression. Cell cycle control plays an important role in haematopoietic differentiation, since progenitors must be able to proliferate to proceed through haematopoietic development, but for terminal differentiation to occur cells must exit the cell cycle [45]. GATA1 inhibits the cell cycle progression and induces the G1 phase arrest during terminal differentiation. A variety of GATA1 target genes involve in cell cycle regulation or in proliferation and differentiation processes [11]. Therefore, HIF1-mediated induction of GATA1 contributes to the generation of mature erythroid cells under hypoxic conditions.

**Fig. 6** Flow cytometry analysis of the expression of CD71 and CD235a. (A) Induced expression of CD71 in K562 cells cultured under hypoxic conditions. (B) Induced expression of CD71 in K562 cells treated with 100 μM DFO or CoCl2. (C) Induced expression of CD235a in K562 cells cultured under hypoxic conditions. (D) Induced expression of CD235a in K562 cells treated with 100 μM DFO or CoCl2. (E) Expression of CD235a in K562 cells transfected with siRNA for 24 hrs and cultured under normoxic conditions for 36 hrs. (F) Expression of CD235a in K562 cells transfected with siRNA for 24 hrs and cultured under hypoxic conditions for 36 hrs. The numbers in brackets indicate the mean fluorescent intensities. NC: negative control, K562 cells without labelling; IC: isotype control, K562 cells labelled by isotype control IgG.
In summary, this study demonstrated that HIF1 induces human GATA1 gene expression to promote erythroid differentiation under hypoxia. This discovery should be an important complement to the mechanism of stress erythropoiesis.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Identification of the functional HRE in MCF-7 cells. MCF-7 cells were co-transfected with a construct carrying the wild-type or motif-mutant of the 3’ GATA1 sequence, and pRL-TK that provided an internal control. After transfection, the cells were cultured at 21% or 1% O2 for 24 hrs. The mean relative luciferase activity ratio is shown (±S.E.M., n = 3) relative to the activity in cells incubated at 21% O2. *P < 0.01.

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