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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 HIF1 *in vivo*. These results demonstrate that the up-regulation of *GATA1* during hypoxia is directly mediated by HIF1.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 $CoCl_2$ 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-x$_L$* 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 G_1/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 haematopoiesis. 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 detected 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 RMPI 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% CO_2. For hypoxic exposure, cells were placed in an incubator chamber that was tightly sealed and thoroughly flushed with 1% O_2/5% CO_2/balance nitrogen and incubated at 37°C. Where indicated, desferrioxamine (DFO) or cobalt chloride ($CoCl_2$) (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 (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 Phamacia Biotech, Freiburg, Germany). $CD34^+$ HPCs were enriched from MNCs through positive immunomagnetic selection (CD34 MultiSort kit; Miltenyi Biotec, Bergisch-Glad-bach, 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 nomoxia 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′-ACCGGTACCATGGAGGGCGCCGGCGGC-3′) and HIF1α-R (5′-ACGGGCCCGCGTTAACTTGATCCAAAGCTC-3′). This fragment (2480 bp) was inserted, in-frame, into the KpnI/ApaI sites of FLAG-tagged pcDNA6/V5-His B (Invitrogen, Carlsbad, CA, USA), yielding the construct pcDNA6V5HisB/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′-AACC**GGTACC**TCTCGGCGAAGTAAAGAATCTG-3′) and DN-R (5′-AAC**GGGCCC*GC***AAGTTTGTCAAAGAGGCTAC) were used for PCR amplification. The amplified fragment was double-digested with Kpn I/Apa I and inserted to pcDNA6/V5hisB, yielding the construct pcDNA6V5HisB/HIF1α-DN (pDN).

To specifically silence HIF1α, we constructed the plasmid pSilencer 2.1U6-HIF1α. The plasmid pSilencer 2.1-U6 neo (Ambion, Austin, TX, USA) was double-digested with BamHI and HindIII. The target sequences of HIF1α RNAi accorded with that described by Berchner-Pfannschmidt *et al.* [18]. To get the target sequences, we synthesized two oligonucleotides, 5′-AGCTTTTCCAAAAAACTAACTGGACACAGTGTGTTCTCTTGAAACACACT-GTGTCCAGTTAGCG-3′ (sense) and 5′-GATCCGCTAACTGGACACAGTGT-GTTTCAAGAGAACACACTGTGTCCAGTTAGTTTTTTGGAAA-3′ (anti-sense). The two oligonucleotides were annealed and inserted into the pSilencer 2.1-U6 vector to yield the plasmid pSilencer 2.1U6-HIF1α-RNAi (pSiHIF1α).

To construct the recombination plasmid pGL3-GATA1, a 750-bp fragment amplified from human genomic DNA was double-digested using SalI and BamHI (MBI Fermentas, Burlington, ON, Canada) and inserted into the BamHI/SalI sites of the pGL3-Promoter Vector (Promega, Madison, WI, USA). Mutations of the putative HRE sequence in the pGL3-GATA1 plasmid were introduced through polymerase chain reaction (PCR)-based site-directed mutagenesis. The bases GAAAG replaced GCGTG at position 533 to construct pGATA1-M1, AAAAG replaced ACGTG at position 548 to construct pGATA1-M2. We also constructed pGATA1-M12 with dual mutations. The following primers were used for amplification and mutation: pGATA1F, 5′-ATGGGATCCTGGCTCTCTGATGACAGTG-3′; pGATA1R, 5′-AAGGTCGAC GGGCTGAGCAAGATAAGAC-3′; M1F, 5′-TGGGCATGGGTGTGAAAGTGTGT-GCAT-3′; M1R, 5′-CGTAATGCACACACTTTCACACCCATG-3′; M2F, 5′-GTGTGTGTGCATTAAAAGTGGCATGTG-3′′; M2R, 5′-ATGGCACATGC-CACTTTTAATGCACAC-3′. All constructs were verified by sequencing.

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 GGACACAGAUUUAGACUUG, GAUGGAAGCACUAGACAAA, CGUGUUAUCUGUCGCUUUG, and GAUGAAAGAAUUACCGAAU. The siRNAs targeting *GATA1* were ON-TARGETplus SMARTpool containing GGACAGGCCACUACCUAUG, ACGCUGAGGCCUACAGACA, GCUGGUGGCUUUAUGGUGG and CCAAGAAGCGCCUGAUUGU.

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 iQ5 software (Bio-Rad). The following primers were used for real-time PCR: β-actin-F, 5′-CTGGCACCACACCTTCTACA-3′, β-actin-R, 5′-AGCACAGCCTGGATAGCAAC-3′; GATA1-F, 5′-CCTGCTTTGTTGCCAATG-3′, GATA1-R, 5′-CTGCTCCACTGTTACGGATAC-3′; HIF1α-F, 5′-AGGTGGATATGTCTGGGTTG-3′, HIF1α-R, 5′-AAGGACACATTCTGTTTGTTG-3′; α-globin-F, 5′-GGTCAACTTCAAGCTCCTAAGC-3′, α-globin-R, 5′-GCTCACAGAAGCCAGGAACTTG-3′; β-globin-F, 5′-GTCTACCCTTGGACCCAGAGGTTC-3′, β-globin-R, 5′-TGAGCCAGGCCATCACTAAAG-3′; γ-globin-F, 5′-GCAGCTTGTCACAGTGCAGTTC-3′, γ-globin-R, 5′-TGGCAAGAAGGTGCTGACTTC-3′; ε-globin-F, 5′-TGTGGAGCAAGATGAATGTG-3′, ε-globin-R, 5′-AGAGGGAGACGACAGGTTTC-3′; CD71-F, 5′-CTTTGGACATGCTCATCTGG-3′, CD71-R, 5′-GACCGAGATGGTGGAAACTG-3′; CD235a-F, 5′-GGCTGGTGTTATTGGAACGATC-3′, CD235a-R, 5′-GAGGTTTTACATCAGATGGGCTTT-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 (VIGOROUS, Beijing, China) within Synergy 4 (BioTek, Tucson, AZ, USA) and fractionated by electrophoresis on 10% SDS polyacrylamide gels. 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(ethyleneimine) (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 transferred 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% O_2) or hypoxia (1% O_2) 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 (Bioworld, 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′-AGCAGGTGATAAGGAGGTG-3′) and reverse (5′-CTCATGTTGTCAGCCACTC-3′), with a 302 bp product covering the putative HREs of GATA1. We also used the primers for the positive control: forward (5′-CGCCCTGTCCTTGAGCC-3′) and reverse (5′-CGGTATGGAGCGTCCCCT-3′), with a 100 bp product covering the HRE of *PDK1*, and the negative control: forward (5′-CTGATAAGACTACACTGGACG3′) and reverse (5′-CCCAAGATACTACACTACCATC-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 direst 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.

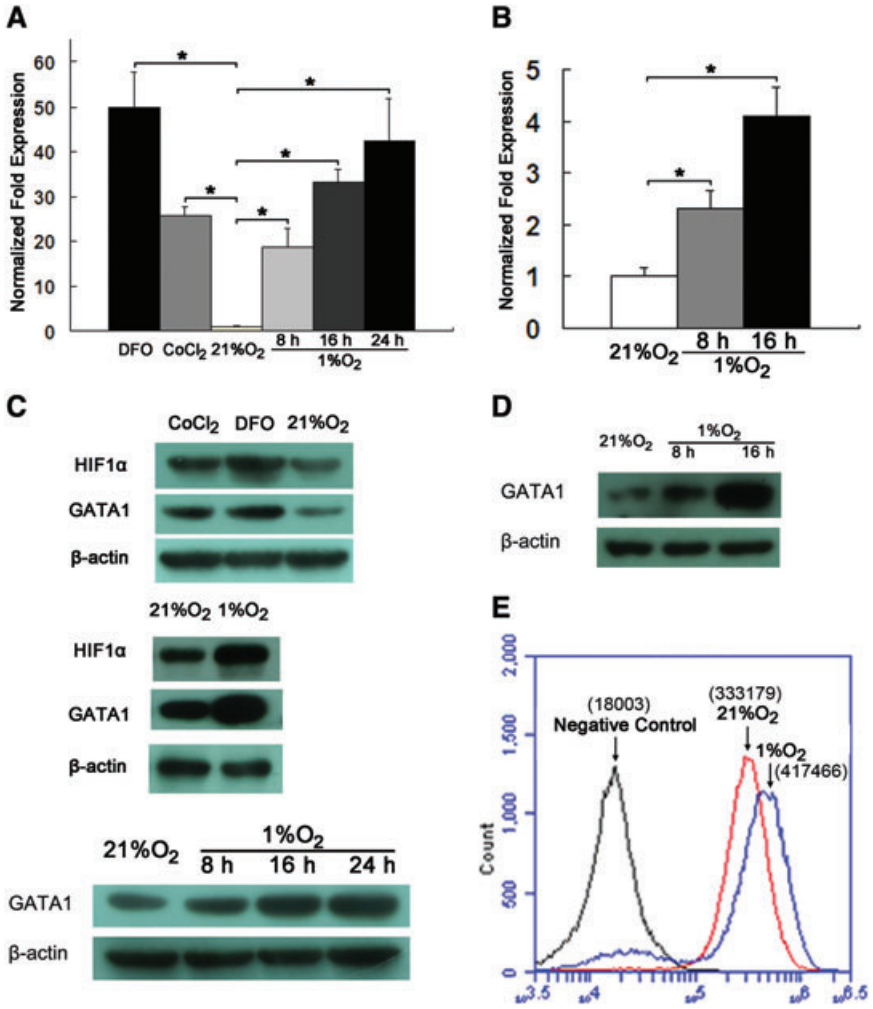

Fig. 1 Induction of *GATA1* gene expression under hypoxia. (**A**) Real-time PCR analysis of *GATA1* mRNA expression (mean ± S.E.M.) in K562 cells that were cultured with 100 μM DFO or $CoCl_2$, or under normoxia (21% O_2) or hypoxia (1% O_2). (**B**) Real-time PCR analysis of *GATA1* mRNA expression (mean ± S.E.M.) in $CD34^+$ erythroid cultures. $^*P < 0.01$. (**C**) Western blot assay of HIF1α, GATA1 and β-actin protein expression in K562 cells cultured in 100 μM DFO or $CoCl_2$, or under normoxia (21% O_2), or hypoxia (1% O_2) for 36 hrs or indicated time. (**D**) Western blot assay of GATA1 and β-actin protein expression in $CD34^+$ erythroid cultures. The UCB-derived $CD34^+$ cells were cultured in erythroid induction culture medium for 4 days and then were exposed to 21% O_2 (normoxia) for 16 hrs or 1% O_2 (hypoxia) for the indicated hours. (**E**) Flow cytometry analysis of GATA1 protein expression in K562 cells that were cultured under normoxia (21% O_2) or hypoxia (1% O_2) for 36 hrs. After being fixed and permeated, the cells were incubated with unconjugated primary antibody, and then with fluorochrome-conjugated secondary antibody. As a negative control, cells were incubated only with secondary antibody.

Results

Hypoxia-induced expression of GATA1

To determine whether hypoxia induces expression of *GATA1* under hypoxic conditions, K562 cells and $CD34^+$ HPCs were cultured in normoxia (21% O_2) or hypoxia (1% O_2) 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 $CoCl_2$ or under hypoxic conditions (Fig. 1A). The expression of *GATA1* mRNA also increased in $CD34^+$ 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 $CoCl_2$ or hypoxia (Fig. 1C). Increased GATA1 protein levels were also observed in $CD34^+$ 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, pcDNA6V5HisB/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

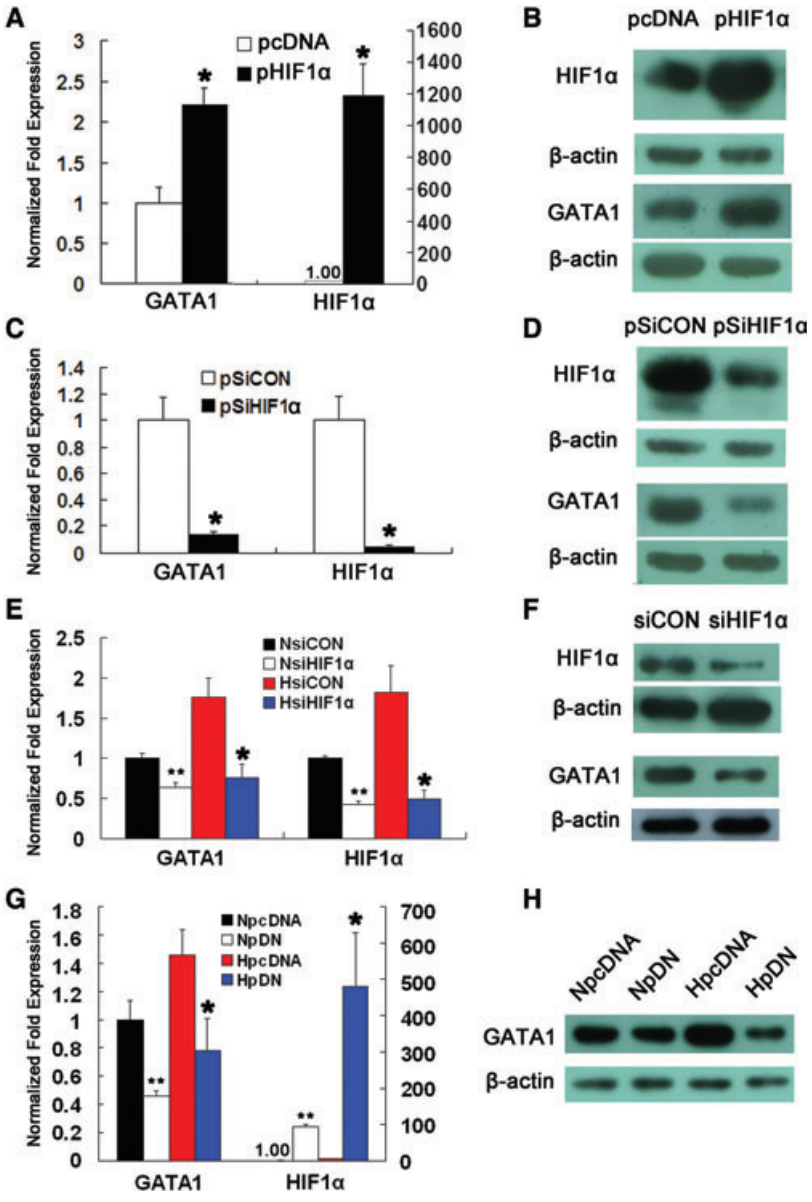

Fig. 2 Regulation of *GATA1* gene expression by HIF1. (**A**) Real-time PCR analysis of *HIF1A* and *GATA1* mRNA levels in K562 cells transfected with the HIF1α expression plasmid (pHIF1α) or empty plasmid (pcDNA) and subsequently incubated under hypoxia for 24 hrs. (**B**) Western blotting assay of HIF1α and GATA1 protein levels in K562 cells transfected with the HIF1α expression plasmid or corresponding empty vector and subsequently incubated under hypoxia for 48 hrs. (**C**) Real-time PCR analysis of *HIF1A* and *GATA1* mRNA levels in K562 cells that were transfected with the HIF1α interference vector (pSiHIF1α) or psilencer control (pSiCON) and subsequently incubated under hypoxia for 24 hrs. (**D**) Western blotting assay of HIF1α and GATA1 protein levels in K562 cells transfected with the HIF1α interference plasmid or the corresponding empty vector and subsequently incubated under hypoxia for 48 hrs. (**E**) Real-time PCR analysis of *HIF1A* and *GATA1* mRNA levels 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 *HIF1A* 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. $^*P < 0.01$.

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 *HIF1A* mRNA and protein in K562 cells transfected with pSiHIF1α, accompanied by a significant decrease in *GATA1* mRNA and protein levels (Fig. 2C and D). Furthermore, K562 cells were also transfected with siRNA that specifically targeted *HIF1A* (siHIF1α). Comparing with control siRNA (siCON), RNA interference-mediated knockdown of HIF1α resulted in a decreased expression of *GATA1* (Fig. 2E and F). Over-expression of HIF1α increased *GATA1* expression, while knockdown of HIF1α decreased *GATA1* expression. Moreover, transfection of K562 cells with the dominant-negative form of HIF1α (pDN) that competes with endogenous HIF1α for dimerization with HIF1β but forms an inactive transcription heterodimer, resulted in a significant decrease of GATA1 expression under hypoxic conditions (Fig. 2G and H). These results established that HIF1 regulates the expression of *GATA1* under hypoxia.

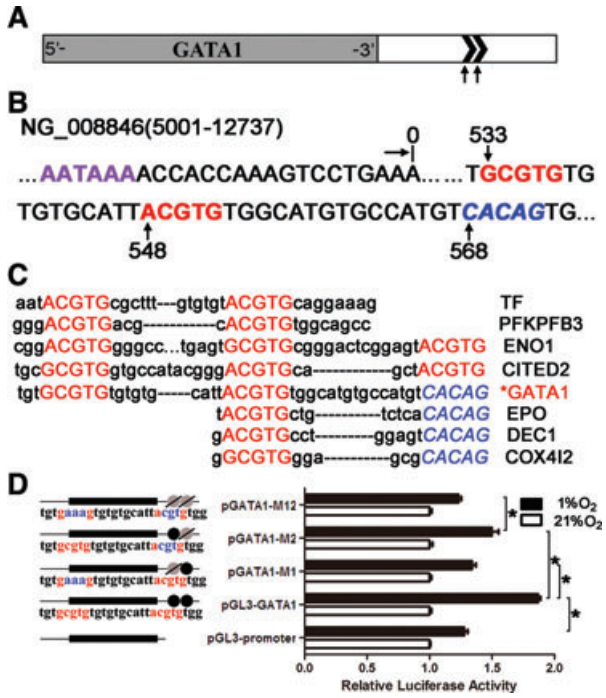

Fig. 3 Identification of the functional HRE in the human *GATA1* gene. (**A**) The location of the putative HRE in human *GATA1* gene. (**B**) Nucleotide sequences matching the consensus HRE core motif are present in the 3′-flanking region within 1 kbp DNA fragment of the human *GATA1* gene. The nucleotide sequences are numbered in relation to the transcription termination site. The putative HRE motif sequences are shown in red. The additional motif CACAG, which was identified in some HIF1 target genes but its function is not clear yet, is shown in blue. (**C**) Similarity of the putative HRE to the verified HRE in target genes of HIF1. (**D**) Identification of the functional HRE. K562 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% O_2 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% O_2. *$P < 0.01$.

Identification of functional HRE in the human *GATA1* gene

We searched the DNA sequence of the human *GATA1* gene on NCBI and identified putative HREs in the 3′-flanking sequence of the gene. Two HRE core motifs RCGTG and another CACAG are 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-HIF1α 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 nomoxia 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 HIF1α to a random sequence. The results from the IgG control excluded non-specific binding of the HIF1α antibody to other proteins. As HIF binding often recruits the co-activator p300, we also performed ChIP assay with anti-P300 antibody to exam 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

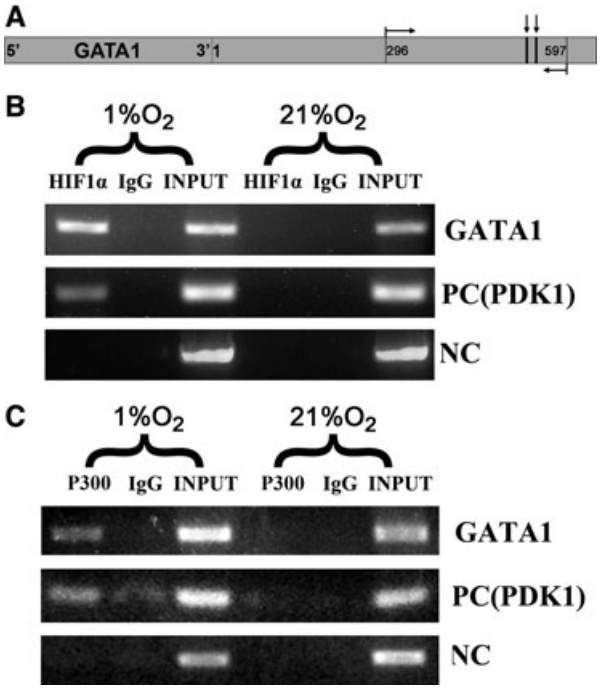

Fig. 4 ChIP-PCR analysis of HIF1's binding to the DNA region containing the putative HREs. (**A**) The region of PCR amplification. The two vertical arrows indicate the positions of the HRE core motifs in the 3′-flanking region of the *GATA1* gene. The two horizontal arrows indicate the locations of the two PCR primers. (**B**) PCR amplification of DNA fragments immunoprecipitated by anti-HIF1α. K562 cells were cultured under normoxia or hypoxia conditions for 24 hrs and subjected to ChIP assay as described in section Materials and methods. Rabbit polyclonal antibody to HIF1-α or IgG was used to precipitate sonicated chromatin. Sonicated cell lysate was used as an input control. NC: negative control; PC: positive control; *PDK1*: pyruvate dehydrogenase kinase 1 gene, which has been verified as a target gene of HIF1. (**C**) PCR amplification of immunoprecipitated DNA fragments by anti-p300 antibody.

shown that, accompanying with hypoxia-induced expression of *GATA1*, the expressions of α-, γ-, ε-globin, CD71 and CD235a increased in K562 cells treated with DFO or $CoCl_2$ or under hypoxic conditions (Fig. 5A), and the expressions of α-, β-, γ-, ε-globin, CD71 and CD235a also increased in $CD34^+$ erythroid cultures under hypoxic conditions (Fig. 5B). However, when K562 cells were transfected with siRNA targeting *HIF1A* or *GATA1*, the expressions of these erythroid differentiation makers 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 K652 cells exposed to hypoxia for 32 hrs or 48 hrs (Fig. 6A), and significantly enhanced in K562 cells treated with DFO or $CoCl_2$ (Fig. 6B). In addition, the expression of CD235a gradually enlarged under hypoxia (Fig. 6C), and also notably increased in DFO- or $CoCl_2$-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 *HIF1A* or *GATA1*. The expression of CD235a obviously decreased when the K562 cells with knocked down of *HIF1A* 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 *HIF1A* knockdown (Fig. 6F). Furthermore, when a positive peak was marked according to the negative control, a notably decreased percentage of CD235a-positive cells was detected in the K562 cells with *HIF1A* knockdown (Fig. 7). These showed that knockdown of *HIF1A* 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 erythoid 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

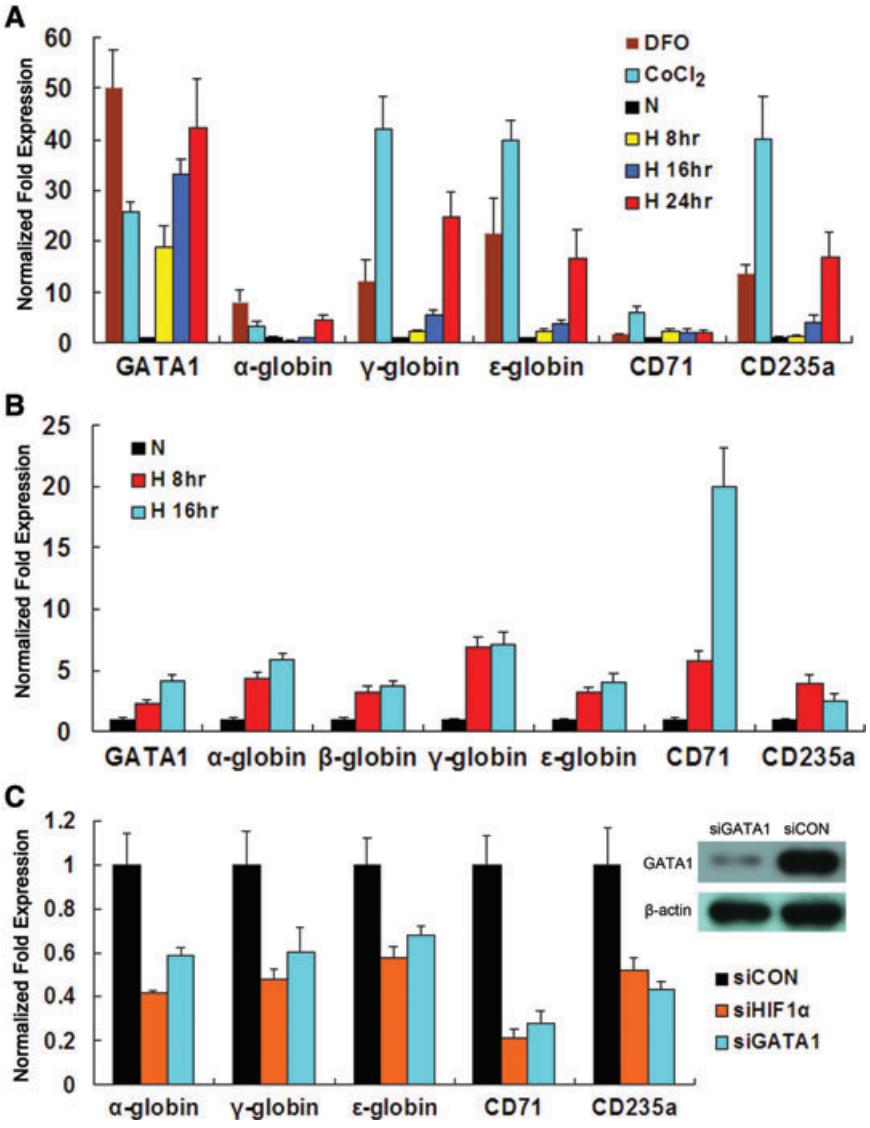

Fig. 5 Real-time PCR analysis of the expressions of α-, β-, γ-, ε-globin, CD71 and CD235a mRNAs. (**A**) K562 cells were cultured under normoxic or hypoxic conditions or treated with 100 μM DFO or $CoCl_2$ before harvest. (**B**) $CD34^+$ erythroid cultures were maintained under normoxic or hypoxic conditions before harvest. (**C**) K562 cells were transfected with siRNA targeting *HIF1A* (siHIF1α) or *GATA1* (siGATA1) or control siRNA (siCON). 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. $^*P < 0.01$. N: normoxia; H: hypoxia.

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-x_L 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-x_L 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.

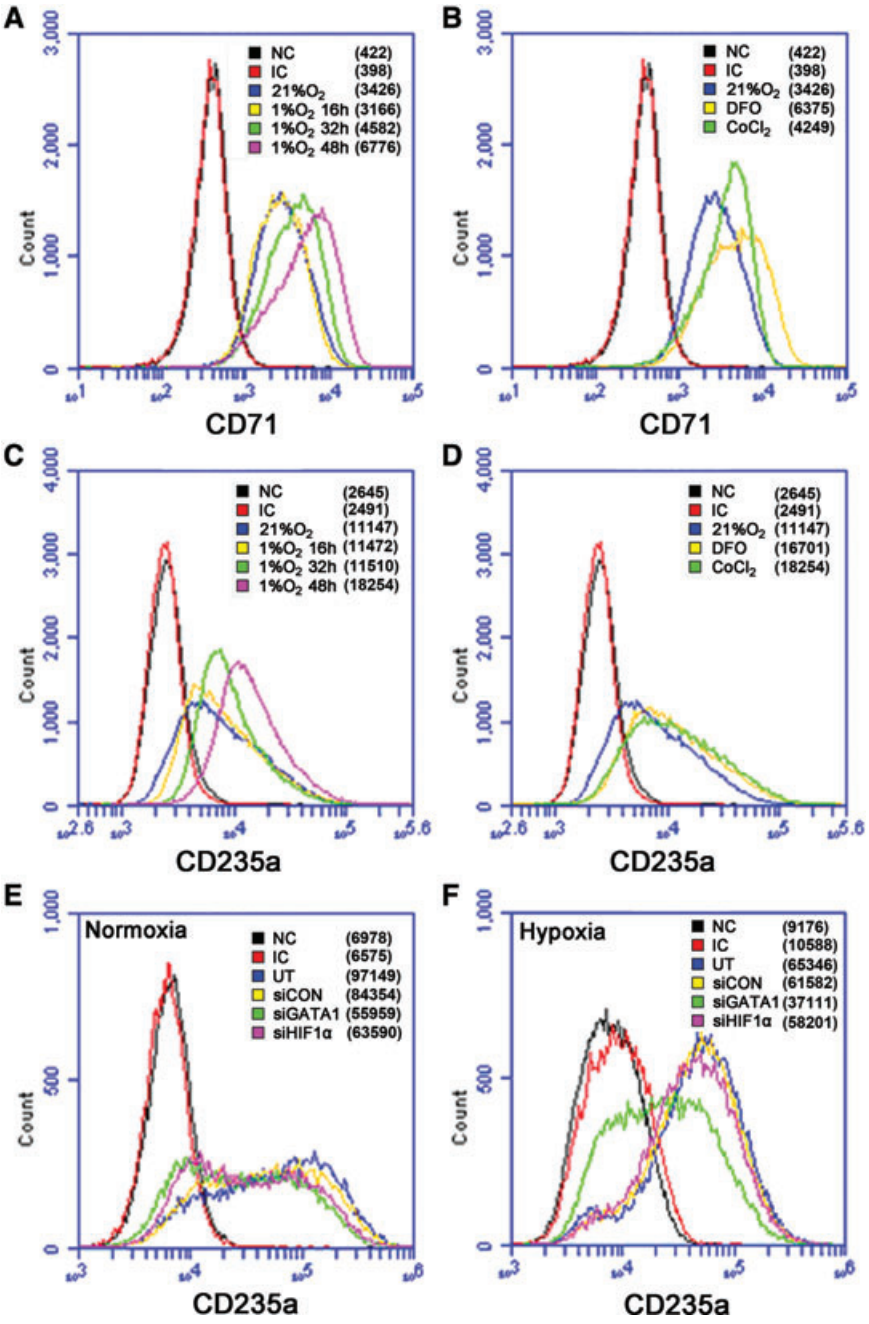

Fig. 6 Flow cytometry analysis of the expression of CD71 and CD235a. (**A**) Induced expression of CD71 in K562 cells cultured under hypoxic conditions. (**B**) Induced expression of CD71 in K562 cells treated with 100 μM DFO or $CoCl_2$. (**C**) Induced expression of CD235a in K562 cells cultured under hypoxic conditions. (**D**) Induced expression of CD235a in K562 cells treated with 100 μM DFO or $CoCl_2$. (**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.

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 involves in the regulation of G_1/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 G_1 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.

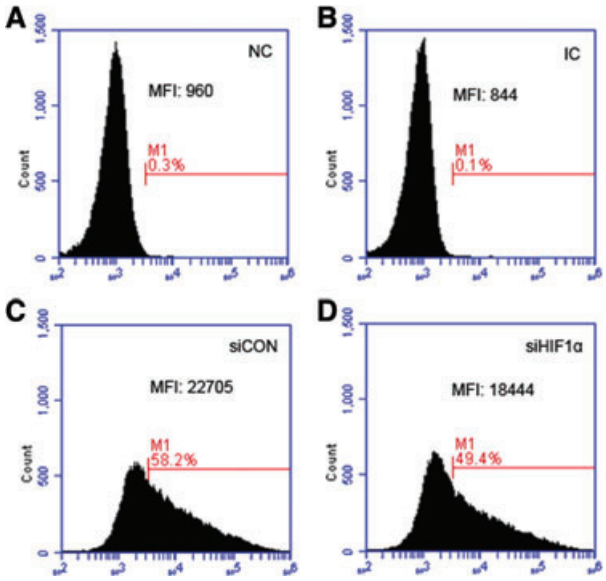

Fig. 7 Flow cytometry analysis of CD235a-positive cells. (**A**) The K562 cells were untreated and unlabelled as a negative control. (**B**) The K562 cells were untreated but labelled with IgG isotype control. (**C**) The K562 cells transfected with siRNA control for 24 hrs and cultured under hypoxic conditions for another 24 hrs, and then labelled with PE-anti-CD235a. (**D**) The K562 cells transfected with siRNA targeting *HIF1A* for 24 hrs and cultured under hypoxic conditions for another 24 hrs, and then labelled with PE-anti-CD235a. M1 mark indicates the CD235a-positive peak. MFI, mean fluorescent intensity.

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% O_2 for 24 hrs. The mean relative luciferase activity ratio is shown ($\pm$S.E.M., $n = 3$) relative to the activity in cells incubated at 21% O_2. $^*P < 0.01$.

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