Effects of miR-210-3p on the erythroid differentiation of K562 cells under hypoxia

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Abstract. GATA binding protein 1 (GATA-1) is one of the most important hematopoietic transcription factors in the production of blood cells, such as platelets, eosinophils, mast cells and erythrocytes. GATA-1 regulates the participation of microRNA (miRNAs/miRs) in erythroid differentiation under normoxia. However, GATA-1 expression and the regulation of miR-210-3p in the context of erythroid differentiation under hypoxia remain unknown. The present study examined the expression levels of GATA-1 and miR-210-3p in the model of erythroid differentiation in K562 cells under hypoxia, and determined the effects of GATA-1, miR-210-3p and SMAD2 on erythroid differentiation through lentivirus transfection experiments. The present study detected increased GATA-1 expression under hypoxia. Moreover, miR-210-3p was identified as a positive regulator of erythroid differentiation, which was upregulated both during erythroid differentiation and in GATA-1 overexpression experiments under hypoxia. Importantly, in the K562 cell model of erythroid differentiation under hypoxia, miR-210-3p was upregulated in a GATA-1-dependent manner. Using a double luciferase reporter assay, miR-210-3p was identified as a downstream target of GATA-1-mediated regulation of erythropoiesis. Gain- or loss-of-function analysis of miR-210-3p identified its importance in erythroid differentiation. Furthermore, it was found that SMAD2 may be a downstream target gene for miR-210-3p. Bioinformatics predictions suggested that SMAD2 mediated miR-210-3p-induced regulation of erythroid differentiation. Collectively, the present study provides novel insights into the miRNA regulation of erythroid differentiation.

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Abbreviations: miRNAs/miRs, microRNAs; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; GATA-1, GATA binding protein 1

Key words: miR-210-3p, erythroid differentiation, GATA-1, hypoxia, SMAD2

Introduction

Erythropoiesis is a multistep process that produces erythroid cells, and it is regulated by transcription factors and oxygen concentration (1). The body oxygen level is one of the most critical factors affecting erythropoiesis. For example, low oxygen levels trigger erythropoiesis via erythropoietin release (2). Moreover, the oxygen level in the bone marrow microenvironment mediates the interaction between erythroid progenitors and stromal cells, further promoting erythroid differentiation (2). Changes in the oxygen level also alter the expression levels of globin genes, such as γ-globin (3-5).

GATA binding protein 1 (GATA-1), a member of the GATA transcription factor family, is expressed in hematopoietic progenitor cells, megakaryocytes, eosinophil granulocyte cells, testicular mast cells and testicular cells (6). GATA-1 is essential for normal erythroid cell proliferation and development (7,8). Under normoxia, GATA-1 modulates erythroid cells by inducing the expression of various erythroid differentiation- and maturation-related genes, such as zinc finger protein, FOG family member 1 and erythroid Krüppel-like factor (9,10). Accumulating evidence has indicated that microRNA (miRNA/miR) is a downstream target of GATA-1. For instance, GATA-1 activates miR-451a and completes a regulatory circuit that modulates erythroid maturation (11). However, the mechanism underlying GATA-1 regulation of erythroid differentiation under hypoxia remains unknown.

miRNAs are members of a large class of non-coding RNAs that control gene expression and regulate a wide array of biological processes. miRNAs target mRNAs and induce translational repression or mRNA degradation (12). Several miRNAs, including miR-451a and miR-210-3p, profoundly alter the erythroid phenotype by regulating early cell maturation and proliferation, the expression level of fetal γ-globin genes and enucleation (13). miR-210-3p, known as 'hypoxamiR,' contributes to the cellular adaptation to hypoxia (14). Moreover, miR-210-3p is associated with an elevated expression level of fetal γ-globin in mithramycin-induced K562 cells (13). miR-210-3p expression is also reportedly elevated during murine fetal liver erythroid cell differentiation *in vitro* (15). Thus, miR-210-3p may mediate erythropoiesis during hypoxia.

K562 cells, a myelogenous leukemia cell line derived from the highly undifferentiated progenitor of the erythrocytic and megakaryocytic lineages (16), have the potential for megakaryocyte and erythroid cell differentiation, thereby

providing an excellent model system for investigating cellular differentiation-related mechanisms (17).

The present study used a K562 cellular erythroid differentiation model under hypoxia to investigate the expression level of GATA-1 and the mechanism underlying its effects on erythroid cell differentiation and miR-210-3p expression regulation, which ultimately affects SMAD2 expression.

Materials and methods

Cell lines and lentivirus vectors. K562 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences and were maintained in RPMI-1640 medium (Hyclone; Cytiva) supplemented with 10% FBS (Zhejiang Tianhang Biotechnology Co., Ltd.) and 1% penicillin/streptomycin solution (Beijing Solarbio Science & Technology Co., Ltd.), at 37˚C and 5% CO_2. Lentivirus vectors (LV-GATA1, cat. no. 26211-1; LV-GATA1-RNAi, cat. no. 18817-1; LV-hsa-mir-210, cat. no. 41113-2; LV-hsa-miR-210-3p-inhibition, cat. no. 5212-1; LV-SMAD2-RNAi, cat. no. 15901-1) and transfection reagent (HitransG A&P, cat. no. REVG003-1) were purchased from Shanghai GeneChem Co., Ltd.

Experimental grouping. K562 cells were divided into a normoxic group (21% O_2, 5% CO_2, 37˚C, saturation humidity) and a hypoxic group (1% O_2, 5% CO_2, 94% N_2, 37˚C, saturation humidity). Hemin was added (40 mM/l; 37˚C; Beijing Solarbio Science & Technology Co., Ltd.), and incubation was performed for 96 h.

Western blot analysis. K562 cells were lysed with mammalian cell lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) containing protease and phosphatase (both Nanjing KeyGen Biotech Co., Ltd.) inhibitors. The BCA protein determination method was used to detect the amount of protein. The total protein content was extracted with a 10% Tris-HCl gradient gel (Bio-Rad Laboratories, Inc.) and transferred onto PVDF membranes, which was blocked using 5% non-fat milk in TBS/Tween-20 (0.1%) for 2 h at room temperature. The membrane was then probed with antibodies for GATA-1 (monoclonal rabbit anti-human; 1:10,000; cat. no. ab181544; Abcam), SMAD2 (monoclonal rabbit anti-human; 1:10,000; cat. no. ab40855; Abcam) and α-tubulin (monoclonal mouse anti-human; 1:10,000; cat. no. ab7291; Abcam) and incubated overnight at 4˚C. The following day, the PVDF membrane was taken out of the refrigerator, reheated at room temperature for 1 h and washed in TBS/Tween-20 (0.1%). The secondary antibodies (Goat anti-mouse IgG, HRP conjugate, cat. no. SA00001-1; Goat Anti-Rabbit IgG, HRP conjugate, cat. no. SA00001-2; ProteinTech Group, Inc.) were added and incubated at room temperature for 1 h, followed by washing with TBS/Tween-20 (0.1%). The visualization reagent (cat. no. 34094; Thermo Fisher Scientific, Inc.) was prepared according to the instructions of the kit. Quantity One software (4.6.2; Bio-Rad Laboratories, Inc.) was used for density analysis of protein bands.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cells harvested using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. RNA was quantified using an Ultra-microspectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Inc.) at 260 nm. cDNA was synthesized using a reverse transcriptase kit (60 min at 42˚C and then 5 min at 70˚C; cat. no. K1691; Thermo Fisher Scientific, Inc.) from 1 μg total RNA. For mRNAs, RT-qPCR was performed using the ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the QuantiNova SYBR Green PCR kit (Qiagen Sciences, Inc.), according to the manufacturer's instructions (95˚C, 2 min for pre-degeneration; followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec). The relative quantification of the transcripts was performed using the $2^{-\Delta\Delta Cq}$ method (18). The primer sequences used were as follows: γ-globin forward, 5'-GCA GCTTGTCACAGTGCAGTTC-3' and reverse, 5'-TGGCAA GAAGGTGCTGACTTC-3'; and β-actin forward, 5'-CCT GGCACCCAGCACAAT-3' and reverse, 5'-GCTGATCCA CATCTGCTGGAA-3'.

miRNA isolation and RT-qPCR. miRNA was isolated using a miRcute miRNA isolation kit (Tiangen Biotech Co., Ltd.) and transcribed into cDNA, according to the manufacturer's instructions (42˚C for 60 min, 95˚C for 3 min; cat. no. KR211; Tiangen Biotech Co., Ltd.). RT-qPCR was performed using ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a miRcute Plus miRNA qPCR Detection kit (FP411-02, Tiangen Biotech Co., Ltd.), according to the manufacturer's instructions (initial denaturation at 95˚C for 15 min; followed by 40 cycles at 94˚C for 20 sec and 60˚C for 34 sec). The relative quantification of the transcripts was performed using the $2^{-\Delta\Delta Cq}$ method (18). Primers for internal reference U6 (cat. no. CD201-0145) and miR-210-3p (cat. no. CD201-0293) were purchased from Tiangen Biotech Co., Ltd.

Benzidine staining. A total of $1x10^6$ cells were collected into a 1.5-ml EP tube with 500 μl PBS, following which 15 μl 0.4% benzidine solution and 10 μl 3% H_2O_2 were added and the suspension was incubated for 3 min at room temperature. Then, 1 μl 5% sodium nitroprusside solution was added. Then, 10 min later at room temperature, 10 μl suspension was collected for observation (magnification, x200) using an inverted microscope (Olympus Corporation). The positive cells were blue-black in color, and 200 cells were counted to calculate the positive cell rate.

Wright's-Giemsa staining. A 10-μl cell suspension was coated on the slide. After drying at room temperature, the staining solution A (Wright's-Giemsa Staining Solution) was added for 1 min and the staining solution B (PBS) was added and mixed for 8-10 min at room temperature. After rinsing the excess dye with running water, the cells were observed under an inverted microscope (magnification, x1,000; Olympus Corporation). In total, 200 cells were counted in each preparation, and the proportion of cells with increased volume, nuclear migration and nuclear shrinkage was determined.

Lentivirus transfection. For the GATA-1 overexpression assay, K562 cells were transfected with either the GV358 vector with a full-length GATA-1 [multiplicity of infection (MOI)=30] or

with an empty vector, and a transfection reagent was added into medium. For the GATA-1 knockdown assay, K562 cells were transfected with either a GV248 vector with the sequence 5'-ACAGAGCATGGCCTCCAGA-3' (MOI=80) or with an empty vector, and a transfection reagent was added into medium. FLAG was inserted into the lentivirus vector of GATA-1 overexpression, and the high-molecular-weight bands in western blotting results were FLAG protein bands. After adding lentivirus reagent in the cell medium (operates at room temperature), the culture was maintained in the incubator at 37˚C with 5% CO_2. After 96 h, the cells were observed using an inverted fluorescence microscope (magnification, x100; Olympus Corporation). Transfection efficiency was determined using western blotting.

For the miR-210-3p overexpression and knockdown assay, K562 cells were transfected with either a GV309 vector with a full-length cDNA of miR-210-3p (MOI=30) or a GV280 vector with a reverse complementary sequence (5'-TCAGCCGCT GTCACACGCACAG-3'; MOI=50), and a transfection reagent was added into medium. K562 cells transfected with lentiviruses with the sequence, 5'-TTCTCCGAACGTGTCACG T-3' (MOI=30), were used as negative controls (NC) for both reactions. After adding lentivirus reagent in the cell medium (operates at room temperature), the culture was continued in the incubator at 37˚C with 5% CO_2. After 96 h, the cells were observed using an inverted fluorescence microscope (magnification, x100; Olympus Corporation). Transfection efficiency was confirmed via RT-qPCR.

For the SMAD2 inhibition assay, K562 cells were transfected with either a GV248 vector with the sequence 5'-CGA TTAGATGAGCTTGAGAAA-3' (MOI=80) or with an empty vector, and a transfection reagent was added into medium. After adding lentivirus reagent in the cell medium (operates at room temperature), the culture was continued in the incubator at 37˚C with 5% CO_2. After 96 h, the cells were observed using an inverted fluorescence microscope (magnification, x100; Olympus Corporation). Transfection efficiency was confirmed using western blotting.

Flow cytometry analysis of CD235a. K562 cells were harvested at the indicated time points (induced by hemin for 0 and 96 h) and washed twice at 4˚C with PBS. Cells were incubated with APC-Cy7-conjugated anti-CD235a antibodies for 15 min (cat. no. 349116; BioLegend, Inc.). Flow cytometry was conducted in K562 cells using the fluorescence-activated cell sorter Beckman CytoFLEX flow cytometer (Beckman Coulter, Inc.) to analyze the primary erythroid cell surface marker CD235a.

Luciferase reporter assay. Cells in the logarithmic growth stage were resuspended, counted, inoculated in a 24-well culture plate (~10^5 cells, depending on the size of the cells) and cultured at 37˚C in a 5% CO_2 incubator until the degree of cell fusion reached ~60%. PGL3 plasmid (Shanghai GeneChem Co., Ltd.) containing firefly and adrenal luciferase reporter genes was transfected into cells at room temperature using the X-tremeGENE HP transfection reagent (cat. no. 06366236001; Roche Diagnostics) for 24-48 h to observe the fluorescent marker gene expression on the plasmid and to determine the transfection efficiency, or cells were transfected for 48 h to detect luciferase activity using a Dual-Luciferase® Reporter Assay system (cat. no. E2910; Promega Corporation), according to the manufacturer's instructions (19-21).

miRNA target prediction. The downstream target genes of miR-210-3p were predicted by using databases such as miRBase (miRbase, http://www.mirbase.org/) and TargetScan (TargetScanHuman 7.2, http://www.targetscan.org/vert_72/). Finally, from a literature review, the target genes related to erythroid development were screened and sequence matching analysis was carried out.

Statistical analysis. SPSS 19.0 software (IBM, Corp.) was used for data processing. Normally distributed data are presented as mean ± SD from three independent experiments. One-way ANOVA was used to compare multiple groups. When the variances were homogeneous, the Tukey's test was used for post hoc analysis. When the variances were uneven, Tamhane's T2 test was used for post hoc analysis. An unpaired Student's t-test was used to compare two groups. $P<0.05$ was considered to indicate a statistically significant difference. Experiments was repeated for three times.

Results

K562 cells successfully differentiate into erythroid cells under hypoxia. An erythroid cell differentiation model under hypoxia was established using hemin-induced K562 cells. Benzidine staining was performed to identify hemoglobin-containing cells as an indicator of erythrocyte differentiation. The results demonstrated that hemin treatment significantly increased the proportion of benzidine-positive K562 cells under hypoxia (Fig. 1A and B). Moreover, under hypoxia, a 2.6-fold elevation of γ-globin expression was observed at 96 h compared with the level at 0 h (Fig. 1C). The results also indicated that the 96-h hemin treatment increased the proportion of $CD235a^+$ K562 cells compared with the 0-h treatment group (Fig. 1D and E). Thus, it was found that K562 cells successfully differentiated into erythroid cells under hypoxia.

GATA-1 expression is upregulated in hemin-induced K562 cells under hypoxia. To assess the influence of hypoxia on GATA-1 expression during erythroid cell differentiation, GATA-1 expression levels were determined in hemin-treated K562 cells using western blotting. The results demonstrated that the expression level of GATA-1 was significantly increased in the hypoxia group compared with that in the normoxia group (Fig. 2).

GATA-1 promotes the erythroid differentiation of K562 cells under hypoxia. To further validate the role of GATA-1 in erythroid differentiation under hypoxia, the corresponding erythroid differentiation index was detected after GATA-1 overexpression or knockdown. Under fluorescence microscope, the cells showed green fluorescence, indicating successful lentivirus transfection (Fig. 3A and B). The results of western blot analysis of GATA-1 protein expression showed that its expression level was higher in the overexpression group compared with that of the NC group (Fig. 3C); In addition, the results of western blot analysis of GATA-1 protein expression

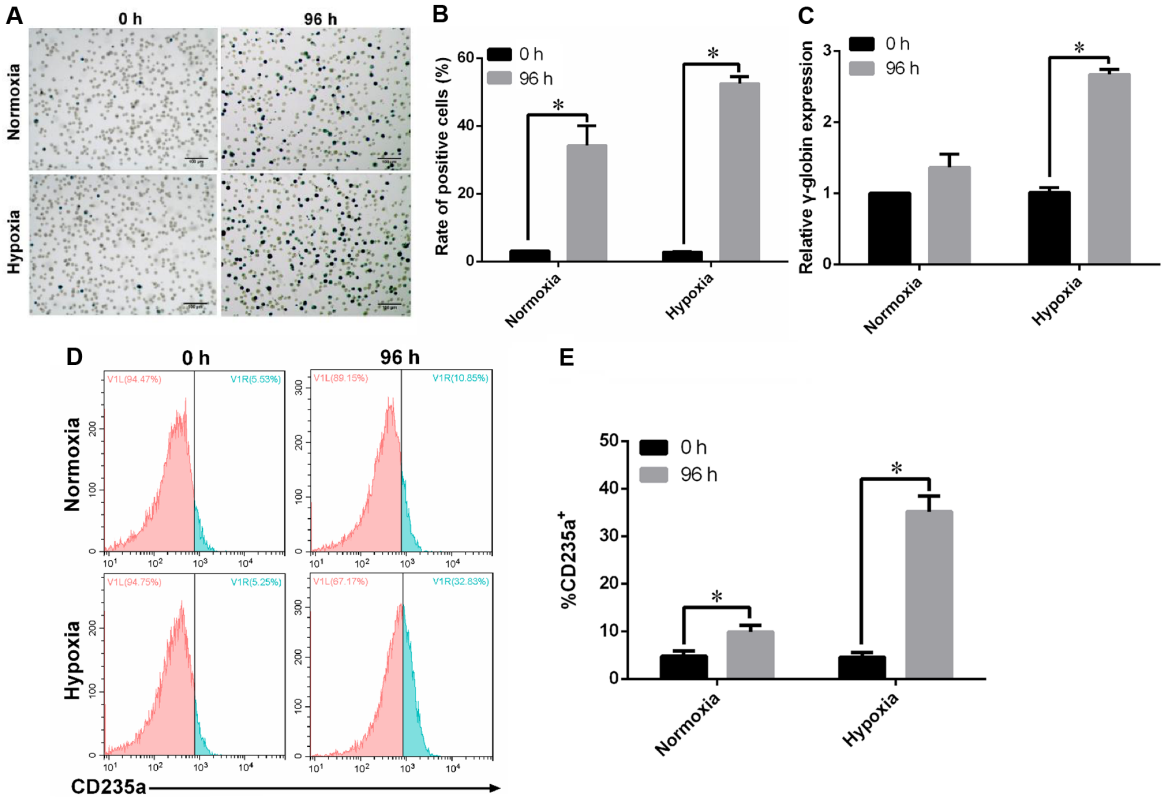

Figure 1. K562 cells successfully differentiated into erythroid cells under hypoxia. (A and B) Benzidine staining of K562 cells after hemin treatment for 0 and 96 h under hypoxic and normoxic conditions. Hemoglobinized cells were stained dark blue/black. *P<0.05, two-tailed Student's t-test, n=3, hypoxia vs. normoxia. Scale bar, 100 μm. (C) Reverse transcription-quantitative PCR analysis of γ-globin expression in K562 cells after hemin treatment for 0 and 96 h under hypoxia and normoxia. *P<0.05, two-tailed Student's t-test, n=3. (D and E) Fluorescence-activated cell sorter analysis of K562 cells after hemin induction for 0 and 96 h under hypoxia and normoxia. *P<0.05, two-tailed Student's t-test, n=3.

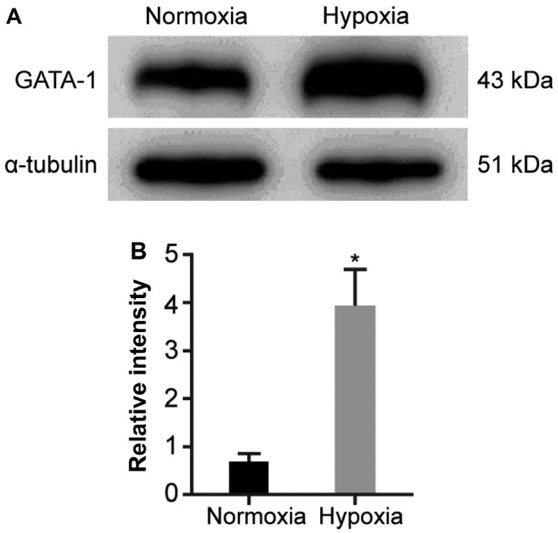

Figure 2. GATA-1 expression is upregulated in the K562 erythroid differentiation model under hypoxia. (A and B) Western blot analysis of GATA-1 expression in cells after hemin treatment. Signals are normalized to α-tubulin, and the results of the semi-quantification are presented in the graph. *P<0.05 vs. normoxia, two-tailed Student's t-test, n=3.

indicated that the relative expression level in the knockdown group was significantly lower compared with that of the NC group (Fig. 3D).

An elevation in the percentage of benzidine-positive cells (Fig. 4A; left panel) and γ-globin expression (Fig. 4B; left panel) was observed at 96 h in GATA-1-overexpressing K562 cells compared with the NC group. Additionally, the percentage of CD235a$^+$ (Fig. 4C; left panel) in GATA-1-overexpressing K562 cells showed an expected increase at 96 h under hypoxia. Wright's-Giemsa staining also demonstrated that the proportion of cells with a larger cell volume and a smaller nucleus accumulated at one side was significantly higher in the GATA-1-overexpressing group compared with that in the NC group (Fig. 4D; upper panel). Conversely, transfection with the GV248 vector (knockdown group) inhibited the expression of GATA-1 in K562 cells, as indicated by the decrease in benzidine-positive cells (Fig. 4A; right panel) and repressed γ-globin accumulation (Fig. 4B; right panel) compared with the phenotype observed in the NC group. Concurrently, the reduced percentage of CD235a$^+$ cells (Fig. 4C; right panel), as well as the proportion of cells with an increased volume and a lopsided nucleus were significantly lower in the GATA-1-knockdown group compared with in the NC group; however, no significant difference was observed with hypoxia (Fig. 4D; bottom panel). These results indicated that GATA-1 promoted erythroid differentiation in K562 cells under hypoxia.

miR-210-3p is a direct target gene of GATA-1 in erythroid differentiation. qPCR was conducted to validate the expression level of miR-210-3p in K562 cells during erythroid

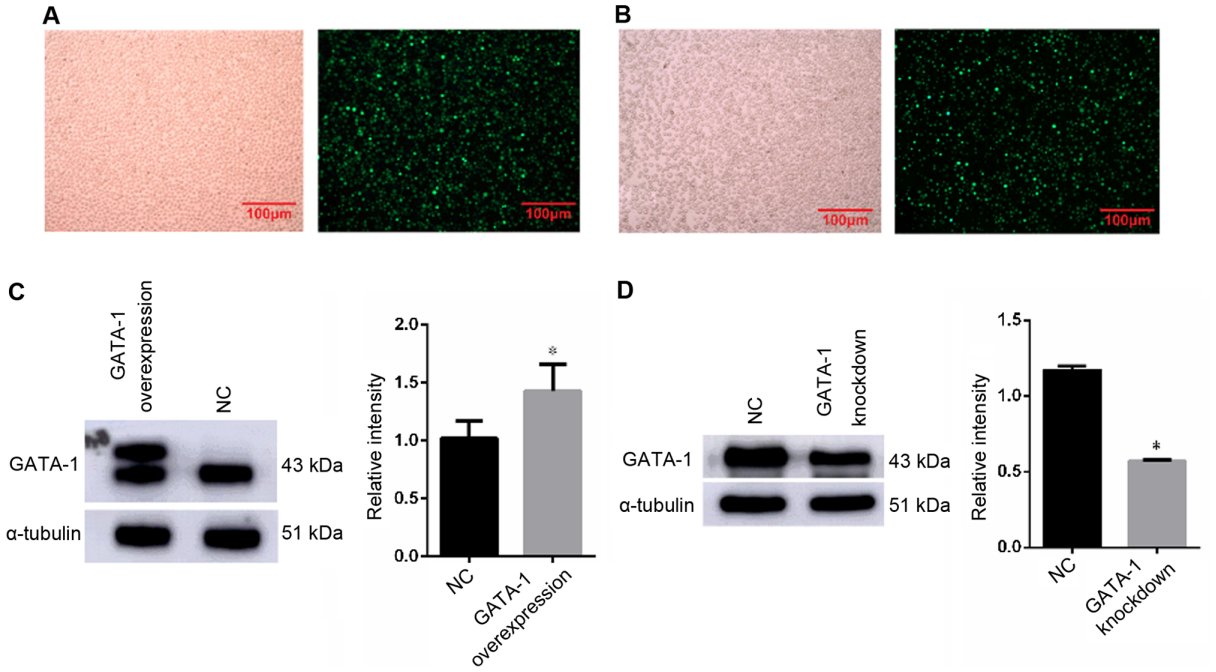

Figure 3. Successful transfection of GATA-1-overexpressing and GATA-1-knockdown lentivirus vectors. (A) The fluorescence results of GATA-1-overexpressing lentivirus transfection efficiency were detected. The field of view under an optical microscope (left). The field of view under a fluorescence microscope (right). Scale bar 100 μm. (B) The fluorescence results of GATA-1-knockdown lentivirus transfection efficiency were detected. The field of view under an optical microscope (left). The field of view under a fluorescence microscope (right). Scale bar, 100 μm. (C) Western blot analysis of GATA-1 expression in K562 cells at 96 h after treatment with GATA-1-overexpressing lentivirus. GATA-1, GATA binding protein 1; NC, negative control. (D) Western blot analysis of GATA-1 expression in K562 cells at 96 h after treatment with GATA-1-knockdown lentivirus. *P<0.05, two-tailed Student's t-test, n=3.

differentiation under hypoxia. The expression level of miR-210-3p in the hypoxia group was higher compared with that in the normoxia group (Fig. 5A). To further determine the direct relevance of miR-210-3p upregulation in response to GATA-1 activation during erythroid cell differentiation, qPCR was conducted to analyze miR-210-3p expression in K562 cells after GATA-1 overexpression or knockdown. The results demonstrated that miR-210-3p showed a significant increase in GATA-1-overexpressing K562 cells under hypoxia (Fig. 5B). Moreover, miR-210-3p was downregulated after the GATA-1 knockdown (Fig. 5C), which revealed the mechanism via which GATA-1 could activate miR-210-3p during erythroid cell differentiation under hypoxia.

To confirm these findings, a dual-luciferase reporter assay was performed to determine the binding site of miR-210-3p and GATA-1. The binding site prediction of the miR-210-3p promoter region is shown in Fig. 5D. The reporter assays revealed a GATA-1-dependent activation of the miR-210-3p promoter. Notably, mutations of the GATA-1-binding site abolished this upregulation, as evidenced by the luciferase activity assay (Fig. 5E).

miR-210-3p promotes the erythroid differentiation of K562 cells under hypoxia. To examine the role of miR-210-3p in erythroid cell differentiation under hypoxia, a miR-210-3p-overexpressing and knockdown lentivirus was transfected into K562 cells. Under fluorescence microscope, the cells showed green fluorescence, indicating successful lentivirus transfection (Fig. 6A and B), and RT-qPCR was performed to assess the transfection efficiency (Fig. 6C and D). The effect of NC lentivirus on the expression level of miR-210-3p was also detected via RT-qPCR. The results demonstrated that, compared with the blank control group, the NC lentivirus did not affect the expression level of miR-210-3p itself, which was convenient for conducting subsequent experiments (Fig. 6E).

The benzidine staining results demonstrated that miR-210-3p overexpression increased the proportion of benzidine-positive cells after 96 h of hemin treatment in K562 cells under hypoxia (Fig. 7A; left panel). Furthermore, a 1.7-fold increase in γ-globin expression was observed at 96 h in miR-210-3p-overexpressing K562 cells compared with that in the NC group under hypoxia (Fig. 7B; left panel). It was found that miR-210-3p overexpression increased the percentage of $CD235a^+$ cells compared with that in the NC group under hypoxia (Fig. 7C; left panel). Wright's-Giemsa staining also showed that miR-210-3p served a vital role in erythroid cell differentiation by increasing the cell volume, nuclear shrinkage and incidence of a lopsided nucleus under hypoxic conditions (Fig. 7D; left panel). In agreement with the gain-of-function data, a significant decrease was observed in the results of the erythroid cell markers γ-globin and CD235a, combined with the benzidine staining, under normoxia, which demonstrated that loss of miR-210-3p function impaired erythroid cell maturation (Fig. 7; right panels of A-D). Thus, it was suggested miR-210-3p promoted erythroid cell differentiation.

SMAD2 is a potential downstream target gene for miR-210-3p. As miRNAs function by translationally repressing their targets (22), the current study aimed to examine the targets of miR-210-3p. To this end, TargetScan was used to predict the downstream target genes of miR-210-3p (Fig. 8A). When further establishing the interaction between miR-210-3p and

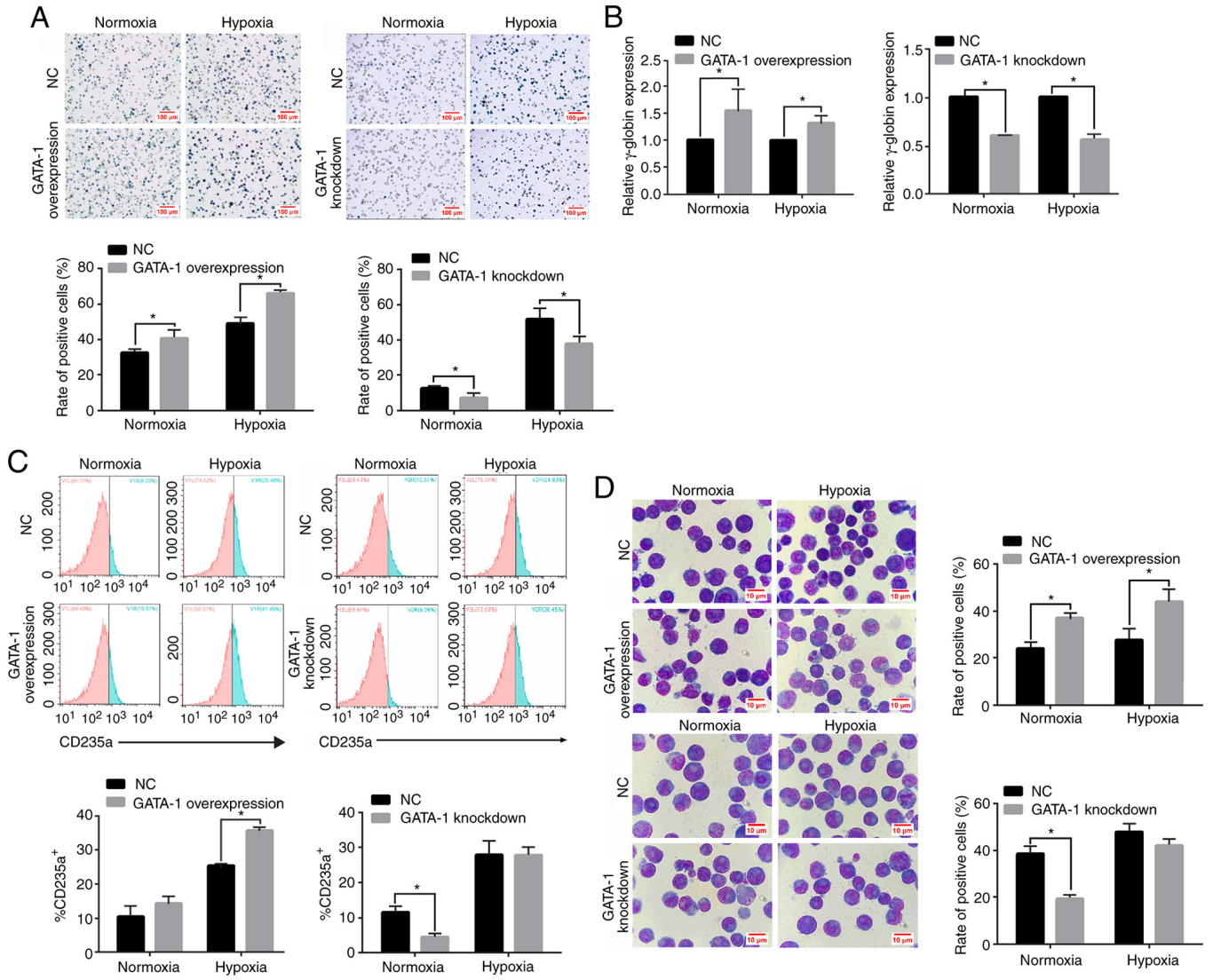

Figure 4. GATA-1 promotes erythroid differentiation under hypoxia. (A) Benzidine staining of K562 cells in the context of GATA-1 overexpression or knockdown after hemin treatment for 96 h. Hemoglobinized cells were stained dark blue/black. Scale bar, 100 μm. Data are expressed as the mean ± SD values from three independent experiments. (B) Reverse transcription-quantitative PCR analysis of γ-globin expression in K562 cells in the context of GATA-1 overexpression or knockdown, following hemin induction. The expression levels of γ-globin were significantly increased in GATA-1-overexpressing vs. NC-transfected K562 cells under hypoxia, while its expression in GATA-1 knockdown K562 cells was lower compared that in the NC group. (C) Fluorescence-activated cell sorter analysis of K562 cells in the context of GATA-1 overexpression or knockdown after hemin treatment for 96 h. Data are expressed as the mean ± SD values from three independent experiments. The analysis of data obtained in FACS showing the percentage of $CD235a^+$ cells is presented in the lower panel. (D) Representative Wright's-Giemsa staining of K562 cells in the context of GATA-1 overexpression or knockdown after hemin treatment for 96 h. Scale bar, 10 μm. *P<0.05, two-tailed Student's t-test, n=3. GATA-1, GATA binding protein 1; NC, negative control.

SMAD2, a significant decrease in SMAD2 expression was observed in the presence of lentivirus-mediated miR-210-3p overexpression in K562 cells under hypoxia compared with the NC group, in which no effect was observed (Fig. 8B). Conversely, SMAD2 expression was increased after the endogenous knockdown of miR-210-3p by the inhibition lentivirus under normoxia (Fig. 8C).

SMAD2 acts as a negative regulator of erythroid cell differentiation under hypoxia. To date, the function of SMAD2 in erythropoiesis remains unknown. Thus, to investigate its biological role in erythroid cell differentiation under hypoxia, a loss-of-function experiment was conducted using lentivirus-mediated SMAD2-inhibition in K562 cells. Under fluorescence microscope, the cells showed green fluorescence, indicating successful lentivirus transfection, western blotting was performed to assess transfection efficiency (Fig. 9A). The percentage of $CD235a^+$ cells (Fig. 9B) and benzidine-positive cells (Fig. 9C) was increased in SMAD2 inhibition lentivirus-transfected K562 cells under normoxic and hypoxic conditions. Furthermore, Wright's-Giemsa staining revealed that, under normoxic and hypoxic conditions, the percentage of cells with larger volumes, lopsided nucleus and nuclear shrinkage was higher compared with that in the NC group (Fig. 9D). As expected, SMAD2 knockdown increased the expression level of γ-globin in K562 cells at 96 h under normoxic and hypoxia (Fig. 9E). The aforementioned results suggested that the increase was more significant under hypoxia. Therefore, it was indicated SMAD2 negatively regulated erythroid cell differentiation under hypoxia.

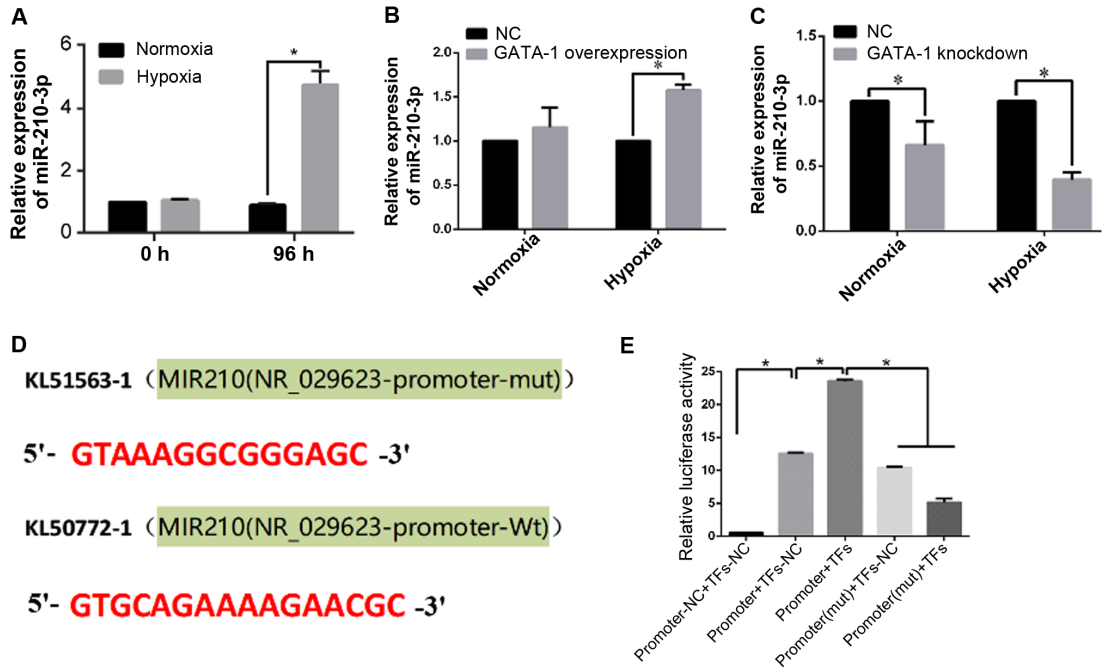

Figure 5. miR-210-3p is a direct target of GATA-1 in erythroid differentiation. (A) Reverse transcription-quantitative PCR analysis of miR-210-3p expression in K562 cells after hemin treatment for 0 and 96 h under hypoxia and normoxia. *P<0.05, two-tailed Student's t-test, n=3. Reverse transcription-quantitative PCR analysis of miR-210-3p expression in hemin-induced K562 cells with (B) GATA-1 overexpression or (C) GATA-1 knockdown. Data are presented as the mean ± SD from three independent experiments. *P<0.05, two-tailed Student's t-test, n=3. (D) Prediction of binding sites between the miR-210-3p promoter region and GATA-1. (E) Relative luciferase activity of the indicated reporter constructs. Firefly luciferase activity was normalized to the activity of co-expressed *Renilla* luciferase. Data are presented as the mean ± SD from three independent experiments. *P<0.05, univariate ANOVA, n=3. GATA-1, GATA binding protein 1; NC, negative control; miR, microRNA; mut, mutant; Wt, wild-type; TFs, transcription factors.

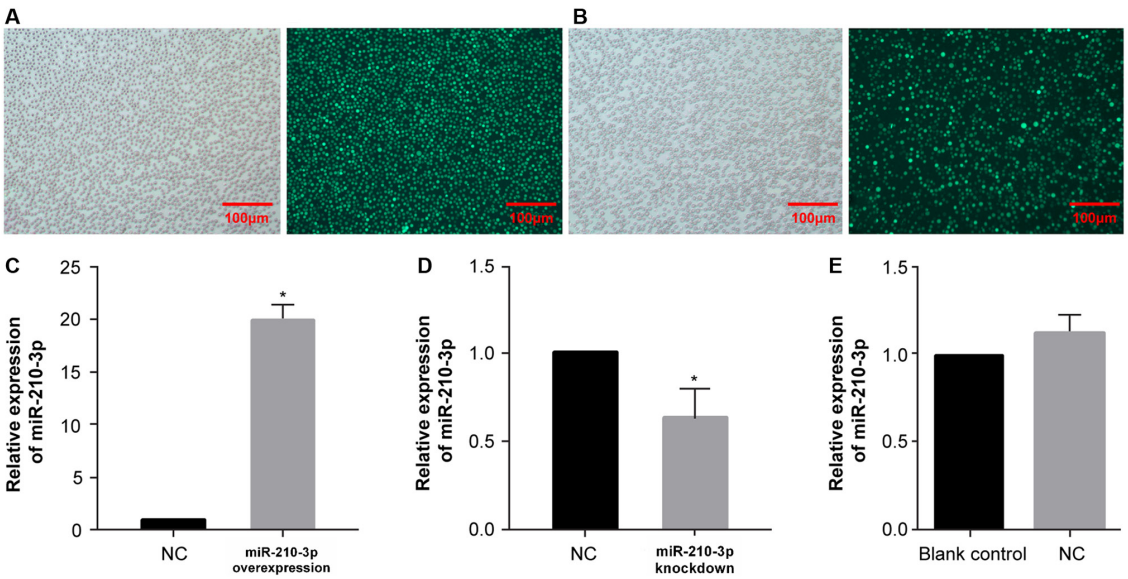

Figure 6. Successful transfection of miR-210-3p-overexpressing and miR-210-3p-knockdown lentivirus vectors. (A) The fluorescence results of miR-210-3p-overexpressing lentivirus transfection efficiency were detected. The field of view under an optical microscope (left). The field of view under a fluorescence microscope (right). Scale bar 100 μm. (B) The fluorescence results of miR-210-3p-knockdown lentivirus transfection efficiency were detected. The field of view under an optical microscope (left). The field of view under a fluorescence microscope (right). Scale bar 100 μm. (C) RT-qPCR analysis of miR-210-3p in K562 cells at 96 h after treatment with miR-210-3p-overexpressing lentivirus. (D) RT-qPCR analysis of miR-210-3p in K562 cells at 96 h after treatment with miR-210-3p-knockdown lentivirus. (E) RT-qPCR analysis of miR-210-3p expression in K562 cells in the blank control group or NC group. *P<0.05 vs. NC, two-tailed Student's t-test, n=3. RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; miR, microRNA.

SMAD2 does not bind to miR-210-3p directly. To further determine the regulatory relationship between miR-210-3p and SMAD2, direct binding sites between miR-210-3p and SMAD2 were detected using a dual-luciferase assay. The results demonstrated that the relative luciferase activity of miR-210-3p-overexpressing vectors combined with SMAD2

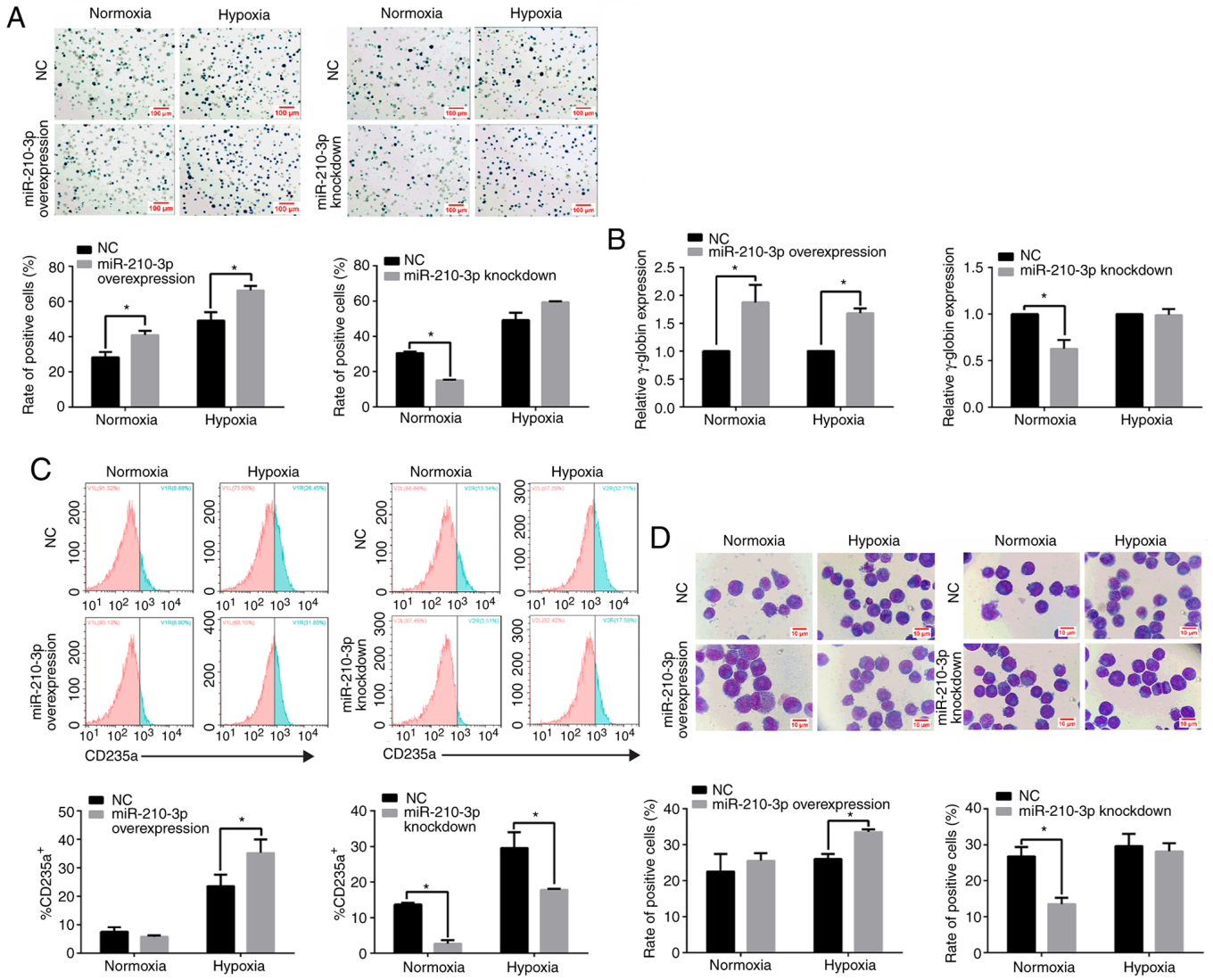

Figure 7. miR-210-3p promotes erythroid differentiation under hypoxia. (A) Benzidine staining of K562 cells with miR-210-3p overexpression or knockdown after hemin treatment for 96 h. Hemoglobinized cells were stained dark blue/black. Scale bar, 100 μm. (B) Reverse transcription-quantitative PCR analysis of γ-globin expression in K562 cells with miR-210-3p overexpression or knockdown, following hemin induction. The expression level of γ-globin was significantly increased in miR-210-3p-overexpressing vector-transfected K562 cells compared with NC group under hypoxia. There was no significant difference between the miR-210-3p downregulated group and the NC group with regards to the expression level of γ-globin in K562 cells under hypoxia. (C) FACS analysis of K562 cells with miR-210-3p overexpression or knockdown and hemin induction for 96 h. Data are presented as the mean ± SD from three independent experiments. (D) Representative Wright's-Giemsa staining of K562 cells with miR-210-3p overexpression or knockdown and hemin induction for 96 h. Scale bar, 10 μm. *P<0.05, two-tailed Student's t-test, n=3. NC, negative control; miR, microRNA.

wild-type vectors was not significantly different compared with that of SMAD2 mutant vectors. Moreover, miR-210-3p could not bind directly to the 3' untranslated region (UTR) of SMAD2 (Fig. 9F).

Discussion

Erythropoiesis, the process of erythroid cell production, is controlled by several factors, including oxygen levels (23). Hypoxia occurs at high-altitude areas and in several physiological and pathological processes, such as rapid tissue growth and acute and chronic ischemia (24). Recently, studies have been performed from the perspective that various geographical and specific environmental differences may influence erythroid cell differentiation. In fact, it has been reported that 'special environments' (e.g., high-altitude areas) significantly influence various physiological functions of the human body, including erythroid cell differentiation (25). The effect of high-altitude hypoxia on erythroid cell development continues to gain increased attention from researchers. Of note, hypoxia may affect the core regulatory factors of healthy erythroid cell differentiation (26).

GATA-1 regulates numerous erythroid cell differentiation-specific genes by binding to its target protein via double zinc finger domains. By activating target genes, GATA-1 helps to establish and maintain erythroid phenotypes (27). Moreover, it has been reported that the maturation of GATA-1-deficient erythroid progenitor cells was inhibited, and apoptosis was induced. However, the erythroid cells matured when GATA-1 activity was restored in GATA-1

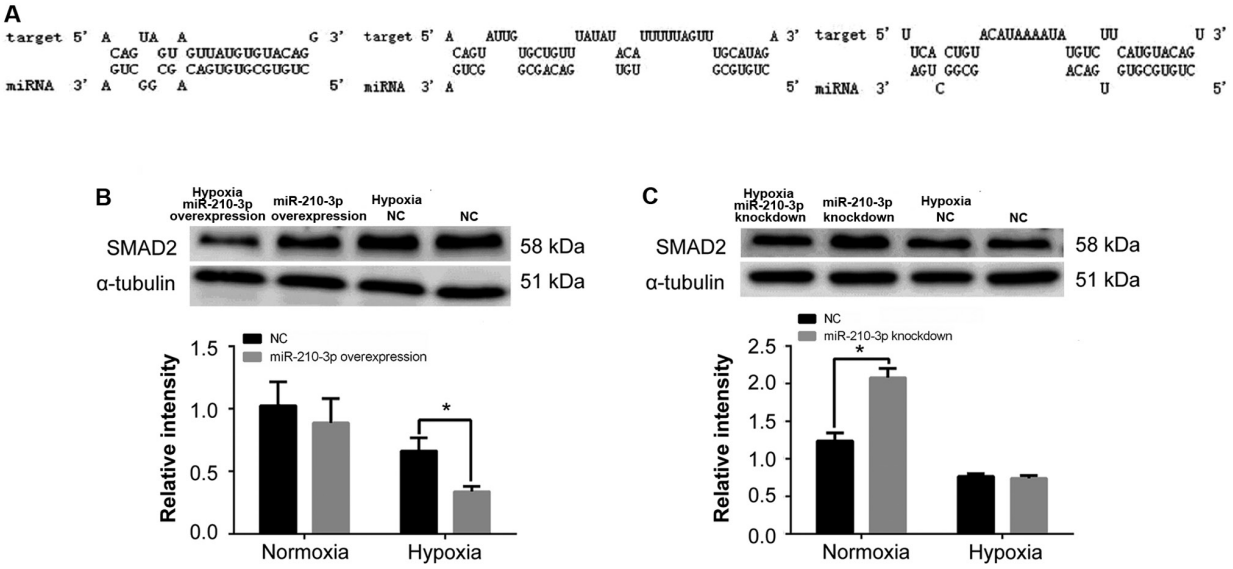

Figure 8. A possible regulatory mechanism of miR-210-3p in erythroid differentiation. (A) SMAD2 was a potential downstream target gene for miR-210-3p. Prediction data of possible binding sites between SMAD2 and miR-210-3p are shown. Western blot analysis of SMAD2 expression in K562 cells after transfection with (B) miR-210-3p-overexpressing lentivirus or (C) miR-210-3p inhibition lentivirus. *P<0.05, two-tailed Student's t-test, n=3. NC, negative control; miR, microRNA.

knockout erythroid lines (28). GATA-1 can modulate erythroid cell differentiation by modulating critical miRNAs, and the mechanism underlying gene regulation via these post-transcriptional inhibitors is being gradually revealed by experimental observations (11). Previous studies have confirmed that miRNAs are essential regulators of all stages of hematopoiesis and hematopoietic disorders (29,30). Some miRNAs reportedly prevent the differentiation of early-stage progenitor cells or regulate the terminal stages of hematopoietic development (31).

In the present study, an erythroid differentiation model of K562 cells under hypoxia was used to investigate the effects and functional relationship of GATA-1 and miR-210-3p on erythroid differentiation and elucidate the possible regulatory mechanism of erythroid differentiation under hypoxia. The expression level of GATA-1 protein in the K562 cell erythroid differentiation model was significantly higher compared with that in the normoxic group. Additionally, the current study evaluated the GATA-1-mediated promotion of erythroid development in K562 cells during hypoxia through gain- and loss-of-function experiments. The results demonstrated that GATA-1 promoted erythroid cell differentiation under hypoxic conditions. Other regulatory pathways under hypoxia, however, may also influence erythroid cell differentiation (32). Hypoxia can upregulate the expression level of GATA-1 and accelerate erythroid cell differentiation; this may be why some indicators of erythroid differentiation after inhibiting GATA-1 under hypoxia showed no significant difference compared with the NC group.

The present study demonstrated that the expression level of miR-210-3p was associated with the upregulation and downregulation of GATA-1 during erythroid differentiation of K562 cells under hypoxia. A dual-luciferase assay was used to verify the relationship between GATA-1 and miR-210-3p. The results demonstrated that the transcription factor could bind to the wild-type miR-210-3p vector, increasing the level of fluorescence expression, thus suggesting the presence of a direct binding site between GATA-1 and the miR-210-3p promoter.

miR-210-3p reportedly mediates hypoxia-induced K562 and erythroid progenitor cell differentiation (33). miR-210-3p also participates in the regulation of erythrocytic maturation, proliferation (13) and γ-globin gene expression in early erythrocytes. Previous research has reported that miR-210-3p enhanced $CD34^+$ erythroid progenitor cell differentiation (34). Moreover, a notable increase in miR-210-3p expression during erythroid differentiation of a murine fetal liver cell culture has been observed (15), and the induction of erythropoiesis after phenylhydrazine-induced hemolytic anemia increased miR-210-3p levels (15). Based on these results, it was suggested that miR-210-3p could affect erythroid differentiation under hypoxia. Therefore, the current study aimed to upregulate and to inhibit miR-210-3p expression to detect the corresponding erythroid differentiation indexes. It was found that miR-210-3p positively regulated erythroid cell differentiation under hypoxia.

To determine the possible underlying mechanism, a previous study investigated miR-210-3p using miRBase/Targetscan/KEGG (Kyoto Encyclopedia of Genes and Genomes) (35) pathway analyses using bioinformatics software and identified SMAD2, which is involved in the proliferation (36), apoptosis (37) and differentiation (38) of several types of cells, as the possible downstream target gene involved in the regulation of erythroid differentiation. SMAD2, which exerts an inhibitory influence under normal steady-state conditions, has emerged as an important regulator of erythropoiesis (39-42). Additionally, overactivation or dysregulation of SMAD2 signaling has been implicated in diseases characterized by impaired erythroid cell differentiation (43-46). Histological examination has shown that SMAD2 was activated in hematopoietic progenitor cells and participated in the regulation of TGF-β-mediated

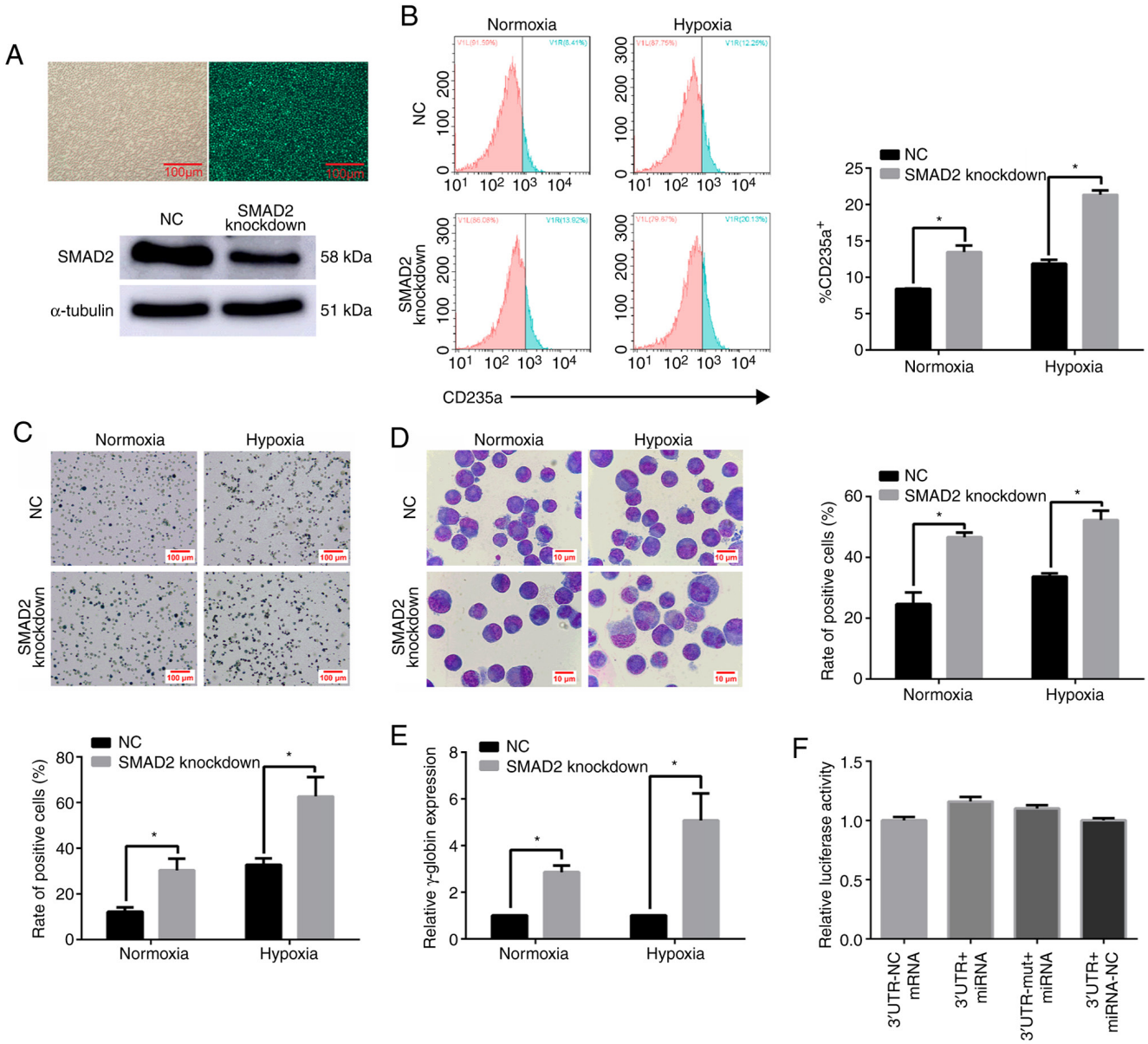

Figure 9. SMAD2 acts as a negative regulator of erythroid differentiation under hypoxia. (A) The fluorescence results of SMAD2 knockdown lentivirus transfection efficiency were detected. The field of view under an optical microscope (left). The field of view under a fluorescence microscope (right). Scale bar, 100 μm. Western blot analysis of SMAD2 expression in K562 cells at 96 h after treatment with SMAD2-knockdown lentivirus. (B) FACS analysis of K562 cells after SMAD2 knockdown and hemin induction for 96 h. (C) Benzidine staining of K562 cells after SMAD2 knockdown and hemin treatment for 96 h. Hemoglobinized cells were stained dark blue/black. Scale, bar 100 μm. (D) Representative Wright's-Giemsa staining of K562 cells with SMAD2 knockdown and hemin induction for 96 h. Scale bar, 10 μm. (E) Reverse transcription-quantitative PCR analysis of γ-globin expression in K562 cells with SMAD2 knockdown, following hemin induction. γ-globin expression was significantly increased in SMAD2 knockdown K562 cells compared with the NC under hypoxia. *P<0.05, two-tailed Student's t-test, n=3. (F) Relative luciferase activity of the indicated reporter constructs. Firefly luciferase activity was normalized to the activity of co-expressed *Renilla* luciferase, univariate ANOVA, n=3. NC, negative control; miRNA, microRNA; UTR, untranslated region; UTR-mut, UTR-mutant; Wt, wild type.

proliferation and erythroid differentiation (43). Accumulating evidence has also suggested that luspatercept-mediated inhibition of SMAD2 signaling promotes erythroid differentiation (47). Moreover, inhibition of SMAD2 increased the level of hepatocyte growth factor, an effective angiogenic factor, in patients with squamous cell carcinoma (48,49). Taken together, these results indicate that SMAD2 could regulate the development of erythrocytes. It has also been reported that miR-210-3p has a direct binding site for members of the SMAD family, which regulates its expression (50). Therefore, we hypothesized that miR-210-3p may serve a role in promoting erythroid cell differentiation by inhibiting the gene expression of SMAD2.

To verify whether SMAD2 was directly regulated by miR-210-3p, a dual-luciferase assay was conducted. However, it was found that miR-210-3p did not bind directly to the 3'UTR of SMAD2, and it was identified that it negatively affected SMAD2 expression, as determined detecting SMAD2 protein expression after the overexpression and knockdown of miR-210-3p expression. Further analysis demonstrated that SMAD2 knockdown enhanced erythroid cell differentiation. A previous study revealed that miR-210-3p could inhibit

the activity of the TGF signaling pathway during osteoblast differentiation (51). However, SMAD-1/5/8 competitive inhibition may interfere with SMAD2/3 binding to Co-SMAD (52), thereby accelerating osteoblast differentiation (53). Therefore, we hypothesized the existence of other sequences in the 3'UTR of SMAD2 that could directly bind to miR-210-3p. Alternatively, miR-210-3p may not bind directly to the 3'UTR of SMAD2 but may indirectly inhibit SMAD2 by suppressing the activity of the TGF signaling pathway, thus facilitating erythroid differentiation.

In conclusion, the present data suggested that, under hypoxia, GATA-1 overexpression significantly promoted erythroid differentiation, possibly by modulating miR-210-3p expression. Furthermore, the expression level of miR-210-3p increased with the degree of differentiation as it regulated several erythroid differentiation-related genes. Thus, with increasing information regarding miRNA profiles and transcription factor regulation, integrating these data may improve the current understanding of the molecular mechanisms underlying human adaptation and pathophysiology under hypoxic conditions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CH and LF conceptualized and designed this study. CH and LF are responsible for confirming the authenticity of the raw data. CY, CF, YY and JD acquired the data. CH, CF, JD, TL and SW analyzed and interpreted the data. CH drafted the manuscript, and CH, YY and LF edited the original draft. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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