

Oridonin enhances γ -globin expression in erythroid precursors from patients with β -thalassemia via activation of p38 MAPK signaling

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Abstract. Upregulation of fetal hemoglobin expression can alleviate the severity of β -thalassaemia. This study aimed to investigate the effects of Oridonin (ORI, a diterpenoid compound) on γ -globin expression in human erythroid precursor cells and the potential underlying mechanisms. Erythroid precursor cells were enriched from 12 patients with β -thalassaemia by two-phase culture. The cells were then treated with different doses of ORI and the survival of erythroid precursor cells was determined. In addition, the expression levels of γ -globin and potential mechanisms were analyzed by reverse transcription-quantitative PCR, western blotting and chromatin immunoprecipitation. Treatment with 0.5 μ M ORI preferably enhanced γ -globin expression and exhibited little cytotoxicity. Similar to sodium butyrate (NaB, a histone deacetylase inhibitor), ORI significantly increased p38 mitogen-activated protein kinase (MAPK) activation, γ -globin expression, histone H3 and H4 acetylation at the G γ - and A γ -globin promoters, and cAMP-response element binding protein 1 (CREB1) phosphorylation. These effects were significantly mitigated by treatment with SB23580, a p38 MAPK inhibitor, in erythroid precursor cells. Therefore, ORI may effectively enhance γ -globin expression by activating p38 MAPK and CREB1, leading to histone modification in γ -globin gene promoters during the maturation of erythroid precursor cells. These findings suggested that ORI may be a novel and potential therapeutic agent for the treatment of β -thalassaemia.

Introduction

β -thalassemia is an inherited disease that affects several organ systems, leading to a relatively short lifespan (1,2). β -thalassemia is particularly prevalent in the Mediterranean, Africa and Southeast Asia (3). Pathophysiologically, β -thalassemia is characterized by insufficient hemoglobin β -chain expression and excess α -chain accumulation, resulting in ineffective erythropoiesis and chronic hemocytolysis (4). Patients with β -thalassemia are dependent on frequent blood transfusions, which leave them at risk of transfusion-transmitted infections and iron overload-related tissue damage (5). Currently, allogeneic bone marrow transplantation can cure the disease; however, the limited availability of suitable bone marrow donors, high cost and potential graft-versus-host responses limit its clinical application (6). Gene editing and therapy may be promising approaches for treatment of β -thalassemia, but they still are at an early stage (6). Therefore, the development of novel therapies for intervention of β -thalassemia is urgently required.

Epidemiological surveys have demonstrated that high levels of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) are inversely associated with the severity of clinical symptoms in patients with β -thalassemia (7). Therefore, reactivation of γ -globin gene expression is a therapeutic strategy for the treatment of β -thalassemia (8). There are three categories of HbF inducers: Ribonucleotide reductase inhibitors, short-chain fatty acids and cytotoxic agents (9). Hydroxyurea (HU), a ribonucleotide reductase inhibitor, has been approved by the United States Food and Drug Administration (FDA) for the treatment of sickle cell disease (SCD) (7). However, its therapeutic efficacy in β -thalassemia remains contentious (8,10,11). Several compounds have been discovered to induce HbF or γ -globin gene expression in human erythroleukemic K562 cells, erythroid cells, primates and even in patients (12-15). However, these substances either have a short half-life *in vivo*, cytotoxicity, apparent carcinogenicity or induce hematopoietic suppression (12). Therefore, the discovery of new safe reagents for inducing HbF or γ -globin gene expression may be of significance in the management of patients with β -thalassemia.

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Previous studies have reported that some natural compounds from medical herbs can induce γ -globin gene expression with little toxicity (16-19). Oridonin (ORI) is a tetracycline diterpenoid from the traditional Chinese medicine *Rabdosia rubescens*, which has anti-tumor, immunomodulatory and neuroprotective activities (20-26). Previous studies have demonstrated that induction of HbF expression is associated with activation of p38 mitogen-activated protein kinase (MAPK) (27-29) and activated p38 MAPK can further activate cAMP response element binding 1 (CREB1), which can bind to specific sites in the γ -globin gene promoters to induce its expression (30,31). In addition, histone modification is a known regulator of gene expression (32). Given that ORI can activate p38 MAPK in some types of cancer cells (20,25), it was hypothesized that ORI may also activate p38 MAPK and CREB1 to induce histone modification and γ -globin gene expression in differentiating human erythroid precursor cells.

The present study purified and enriched human erythroid precursor cells from patients with β -thalassemia. Two-phase culture was employed to induce the differentiation of erythroid precursor cells, and the effects of ORI on γ -globin expression and the potential underlying mechanisms were analyzed.

Materials and methods

Subjects and cell culture. A total of 12 patients with β -thalassemia were recruited at the outpatient service of the Guangdong Women and Children Hospital between January 2015 and January 2016. The demographic and clinical characteristics of subjects are presented in Table I. Patients with β -thalassemia were diagnosed by laboratory blood tests and genetic examination. Patients were excluded from the study if they had received a blood transfusion during the past 3 months. Written informed consent was obtained from the patients' guardians and the experimental protocol was approved by the Ethical Committee of Guangdong Women and Children Hospital.

Peripheral venous blood samples were collected from the subjects. Blood mononuclear cells were isolated by Ficoll-Hypaque density gradient (GE Healthcare) centrifugation for 20 min at 400 x g and room temperature, and CD34⁺ cells were purified using immunomagnetic beads, as previously described (33). The purified CD34⁺ cells were subjected to two-phase liquid culture, as previously described (34). Briefly, CD34⁺ cells were cultured for 7 days (phase I culture) in minimal essential media (MEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Biological Industries), 1 μ g/ml cyclosporin A (Sandoz AG) and 10% conditioned medium from cultures of bladder carcinoma 5.637 cells (American Type Culture Collection) at 37°C in an atmosphere containing 5% CO₂ and 95% humidity. The non-adherent cells were harvested, washed and cultured (phase II culture) for 7 days in fresh medium supplemented with 30% FBS, 10⁻⁵ M β -mercaptoethanol, 10⁻⁶ M dexamethasone, 300 μ g/ml holo-transferrin (Sigma-Aldrich; Merck KGaA), 10 ng/ml human recombinant stem cell factor and 1 U/ml human recombinant erythropoietin (Ortho Pharmaceutical Corp.).

On day 6 of phase II culture, the cells were treated in triplicate with vehicle (0.1% DMSO), ORI (Chengdu Must Bio-Technology Co., Ltd.) at 0.1, 0.2, 0.5 or 1.0 μ M, or 500 μ M

sodium butyrate (NaB, a histone deacetylase inhibitor; Sigma-Aldrich; Merck KGaA). The number of viable cells in the different groups was counted by trypan blue dye exclusion assay daily from day 6 to day 14 in a blinded manner. Some cells were collected on day 12 and the relative mRNA expression levels of γ -globin and protein expression levels of HbF were determined by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis, respectively. For analysis of p38 MAPK signaling, some cells were pretreated with vehicle (control), 0.5 μ M ORI for 12, 24, 48, 72 or 96 h, or 500 μ M NaB for 48 h (as a positive control) to analyze the time-course of p38 MAPK activation. Some cells were pretreated with 10 μ M SB203580 (a p38 MAPK inhibitor; Promega Corporation) (35) for 30 min at room temperature and treated with ORI (0.5 μ M) or NaB (500 μ M) for 48 h. To evaluate the levels of CREB1, cells were treated with vehicle (control) or ORI (0.5 μ M) for 24, 48, 72 or 96 h, or with 500 μ M NaB for 72 h (as a positive control) to analyze CREB1 time-dependent activation. Some cells were pretreated with 10 μ M SB203580 for 30 min at room temperature and treated with ORI (0.5 μ M) or NaB (500 μ M) for 72 h. In addition, western blot analysis and chromatin immunoprecipitation (ChIP) were performed in cells treated with ORI (0.5 μ M) and NaB (500 μ M) for 72 h, alone or in combination with SB203580 pre-treatment.

Trypan blue dye exclusion assay. The number of viable cells was determined by trypan blue dye exclusion assay. Briefly, human erythroid progenitor cells during phase II culture were treated with the indicated agents between days 6 and 14, and cell samples were collected daily or every other day. The cells were stained with 0.5% trypan blue at room temperature for 2 min and the number of unstained viable cells in the different groups was counted in a blinded manner.

RT-qPCR. Total RNA was extracted from different groups of cells using the RNeasy kit (Qiagen, Inc.). Following qualification and quantification, each RNA sample (1 μ g) was reverse transcribed into cDNA using oligo(dT)₁₈ and Moloney murine leukemia virus reverse transcriptase (Promega Corporation). The RT reactions were performed at 42°C for 60 min, followed by 95°C for 5 min and 0-5°C for 5 min. The relative levels of target gene mRNA transcripts normalized to control GAPDH were determined by RT-qPCR using the SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) and specific primers in a RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: GAPDH, forward 5'-GCACCGTCAAGGCTGAGA AC-3', reverse 5'-TGGTGAAGACGCCAGTGG-3'; α -globin, forward 5'-TCCCCACCACCAAGACCTAC-3', reverse 5'-CCT TAACCTGGGCAGAGCC-3'; β -globin, forward 5'-CTCATG GCAAGAAAGTGCTCG-3', reverse 5'-AATTCCTTTGCCA AAGTGATGGG-3'; and γ -globin, forward 5'-GGCAAC CTGTCCTCTGCCTC-3' and reverse 5'-GAAATGGATTGC CAAAACGG-3'. PCR was performed in triplicate at 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec, 60°C for 20 sec and 72°C for 15 sec. PCR was conducted using an ABI7500 PCR system (cat. no. 4351104; Applied Biosystems; Thermo Fisher Scientific, Inc.). The data were normalized to GAPDH and analyzed by 2^{- $\Delta\Delta$ C_q} (36).

Table I. Demographic and clinical characteristics of subjects.

No.	Sex	Age (years)	Genotype	Subgroup
1	M	7.2	CD41-42/IVS-II-654	Major
2	F	13.0	CD41-42/CD17	Major
3	F	12.3	CD17/IVS-II-654	Intermedia
4	M	2.5	CD41-42/IVS-I-1	Major
5	F	8.0	CD41-42/CD41-42	Major
6	F	2.3	IVS-II-654/-28	Intermedia
7	F	8.0	IVS-II-654/-28	Intermedia
8	F	6.5	CD17/IVS-II-654	Intermedia
9	M	10.0	CD41-42/CD41-42	Major
10	M	3.5	β E/-28	Intermedia
11	M	10.5	-28/-28	Intermedia
12	F	6.6	β E/IVS-II-654	Intermedia

M, male; F, female.

Western blot analysis. The harvested cells were lysed on ice for 20 min in 50 mmol/l Tris-HCl (pH 8), 150 mmol/l NaCl, 2% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% sodium azide and 0.1% SDS supplemented with 10 mmol/l PMSF. This was followed by centrifugation at 12,000 x g for 15 min at 4°C. Following determination of protein concentrations using a Bradford assay (Bio-Rad Laboratories, Inc.), the cell lysate samples (30-50 μ g/lane) were separated by SDS-PAGE on 15% gels and transferred to nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk powder in TBS buffer containing 0.1% Tween for 2 h at room temperature and were incubated with the following primary antibodies: Sheep polyclonal anti-HbF (cat. no. ab19364; 1:1,000; Abcam), rabbit monoclonal anti-total (t)-p38 MAPK (cat. no. ab170099; 1:1,000; Abcam), mouse monoclonal anti-phosphorylated (p)-p38 MAPK (cat. no. ab45381; 1:1,000; Abcam), rabbit monoclonal anti-p-CREB1 (Ser 133; cat. no. 9198; 1:1,000; Cell Signaling Technology, Inc.), rabbit monoclonal anti-t-CREB1 (cat. no. 9197; 1:1,000; Cell Signaling Technology, Inc.) and mouse monoclonal anti- β -actin (cat. no. TA336770; 1:1,000; OriGene Technologies, Inc.) overnight at 4°C. The bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-sheep IgG (H+L) (cat. no. 14-23-06; 1:5,000; KPL, Inc.), goat anti-rabbit IgG (H+L) (cat. no. ZB 2301; 1:5,000; OriGene Technologies, Inc.) and goat anti-mouse IgG (H+L) (cat. no. ZB 2305; 1:5,000; OriGene Technologies, Inc.) and visualized using an enhanced chemiluminescent kit (Merck & Co., Inc.), followed by densitometric analysis in the Bio-BEST-140E gel image system (SIM Company). The data were normalized to the control β -actin and the relative levels of HbF, p38 MAPK and CREB1 expression and phosphorylation were determined using Glyko BandScan (Version 5.0; Glyko, Inc.) in the Bio-BEST-140E gel image system (SIM International).

ChIP. The impact of ORI on histone modification in the promoter regions of the γ - and α -globin genes was determined by ChIP using the EZ-ChIP kit (cat. no. 17-371; EMD

Millipore), according to the manufacturer's protocol (Upstate Biotechnology, Inc.; Thermo Fisher Scientific, Inc.). Briefly, 1×10^7 cells in the different groups were cross-linked with 1% formaldehyde for 10 min at room temperature under gentle agitation; cross-linking was terminated by the addition of 150 mM Glycine for 5 min. After the addition of SDS Lysis Buffer (cat. no. 20-163; EMD Millipore) containing Protease Inhibitor Cocktail II (cat. no. 20-238; EMD Millipore), according to the manufacturer's protocol, the cross-linked chromatin was sonicated five times at 100 W power in an ice bath (10 sec/sonication with 30 sec between them), in order to obtain DNA fragments averaging 200-1,000 bp in size. One part of the supernatant fraction of chromatin without primary antibody treatment was saved as the 'input sample'. According to the manufacturer's protocol, in order to minimize the risk of contamination, the 'input sample' was divided into three samples. These samples were incubated with the antibody of interest [input; against acetyl-histone H3 (cat. no. 06-599; 1:100; EMD Millipore) or H4 (cat. no. 06-866; 1:300; EMD Millipore)], anti-RNA polymerase (positive control; 1:1,000; cat. no. 05-623; EMD Millipore) or normal mouse IgG (negative control; 1:1,000; cat. no. 12-371; EMD Millipore) overnight at 4°C with agitation. One tube without DNA sample was referred to as the 'No DNA' PCR control. These purified DNA samples and the 'No DNA' PCR control then underwent PCR using Control Primers (included in the EZ-ChIP kit; cat. no. 22-004; EMD Millipore), which are specific for the human GAPDH gene. The size of the GAPDH PCR product was 166 bp. After verification, the chromatin was probed with antibodies against acetyl-histone H3 (cat. no. 06-599; 1:100; EMD Millipore) and H4 (cat. no. 06-866; 1:300; EMD Millipore) overnight at 4°C with agitation. The relative levels of the γ - and α -globin promoter DNA fragments to the control Necdin were determined by RT-qPCR. The primer sequences were as follows: Necdin (control), forward 5'-GTC CTCTGCCTCTGCCATCA-3', reverse 5'-ATACAGGGCACT GGCCACTC-3'; γ -globin, forward 5'-GTCCTCTGCCTC TGCCATCA-3', reverse 5'-ATACAGGGCACTGGCCACT TC-3'; and α -globin, forward 5'-TGTGGAAGATGCTGG AGGAG-3' and reverse 5'-ATACACAGGGCACTGGCCACT G-3'. The PCR products were visualized on a 2% agarose gel with ethidium bromide staining (Thermo Fisher Scientific, Inc.) and were semi-quantified; densitometric scanning was conducted using the Bio-BEST-140E gel image system (SIM International) and data were analyzed using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc.).

Statistical analysis. Data are presented as the mean \pm standard deviation. The difference among groups was analyzed by one-way ANOVA, or repeated ANOVA where appropriate, followed by the least significant difference post hoc test using SPSS 13.0 (SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference. When a P -value of 0.000 was achieved using the software it was designated as $P < 0.001$.

Results

ORI preferably enhances γ -globin expression during the maturation of erythroid precursor cells. To investigate the potential effect of ORI on the expression of γ -globin, human

CD34⁺ erythroid precursor cells were purified from patients with β -thalassaemia and subjected to two-phase culture. On day 6 of phase II culture, the cells were treated with vehicle alone (control), different doses of ORI or 500 μ M NaB (classic inducer of HbF expression, positive control). Subsequently, the number of viably cultured cells was counted longitudinally. As demonstrated in Fig. 1A, treatment with 0.5 μ M ORI temporarily reduced the number of cultured cells on day 4 (from $3.77 \pm 0.21 \times 10^6$ to $3.36 \pm 0.37 \times 10^6$ cells/ml) and day 6 (from $4.30 \pm 0.24 \times 10^6$ to $3.89 \pm 0.39 \times 10^6$ cells/ml) post-culture, whereas treatment with 1 μ M ORI, similar to that of NaB, further decreased the number of cultured cells 2 days after phase II culture. Cells were harvested on day 12, when the cells matured into orthochromatic normoblasts and enucleated erythrocytes (33). The relative levels of γ -globin mRNA transcripts normalized to the control GAPDH were determined by RT-qPCR (Fig. 1B). Treatment with different doses of ORI significantly increased the relative levels of γ -globin mRNA transcripts, which peaked following treatment with 0.5 μ M ORI (6.18 \pm 0.04-fold; $P < 0.05$) and were slightly reduced following treatment with 1.0 μ M ORI (4.57 \pm 0.20-fold; $P < 0.05$) in orthochromatic normoblasts and enucleated erythrocytes. Treatment with NaB also significantly increased the levels of γ -globin mRNA transcripts (4.22 \pm 0.05-fold; $P < 0.05$); its effects were significantly stronger than those of 0.2 μ M ORI (3.04 \pm 0.16-fold; $P < 0.05$), but less than those of 1.0 μ M ORI in erythroid precursor cells. These findings indicated that ORI treatment enhanced γ -globin expression during the maturation of erythroid precursor cells.

Given that treatment with 0.5 μ M ORI strongly enhanced γ -globin expression and exerted little cytotoxicity, this dose was selected for further experiments. Further RT-qPCR analysis revealed that treatment with ORI did not significantly affect the relative mRNA expression levels of α -globin (1.10 \pm 0.20-fold; $P = 0.419$) or β -globin (1.00 \pm 0.24-fold; $P = 0.971$), but did significantly increase the relative mRNA expression levels of γ -globin (6.42 \pm 0.57-fold; $P < 0.05$) in the cultured orthochromatic normoblasts and enucleated erythrocytes (Fig. 1C). However, treatment with NaB significantly increased the relative mRNA expression levels of α -globin (2.02 \pm 0.50-fold; $P < 0.05$) and γ -globin (4.27 \pm 0.76-fold; $P < 0.05$), but not β -globin (1.22 \pm 0.43-fold; $P = 0.061$) in the cultured cells. Western blotting indicated that treatment with ORI significantly increased the relative protein expression levels of HbF by 5.55 \pm 0.84-fold ($P < 0.05$), which was significantly higher than the 3.54 \pm 0.40-fold increase in HbF induced by NaB in the cultured cells ($P = 0.021$; Fig. 1D). Further western blot analysis indicated that treatment with NaB, but not ORI, significantly increased the levels of α -globin expression in the cultured cells (data not shown). Together, such data demonstrated that ORI treatment preferably enhanced γ -globin expression during the maturation of cultured erythroid precursor cells *in vitro*.

ORI enhances γ -globin expression, which is partially dependent on activation of p38 MAPK signaling in erythroid precursor cells. Previous studies have demonstrated that induction of HbF expression is associated with activation of p38 MAPK (26-29). To understand the molecular mechanisms underlying the action of ORI, erythroid precursor cells 1 day post-phase II culture were treated with ORI for 4 days or NaB

for 2 days, and the relative levels of p38 MAPK expression and phosphorylation in the cells were determined by western blotting. As demonstrated in Fig. 2A, 12 h post-treatment with ORI, the relative ratios of p38 MAPK phosphorylation to its expression were significantly increased (2.69 \pm 0.10; $P < 0.05$). The relative ratios of p38 MAPK phosphorylation to its expression peaked at 48 h post-treatment (6.19 \pm 0.09; $P < 0.05$) and declined gradually at later time points. Similarly, treatment with NaB for 48 h significantly increased the relative ratios of p-p38 MAPK to t-p38 MAPK in cultured cells (3.83 \pm 0.47; $P < 0.05$). Conversely, pretreatment with 10 μ M SB203580, a p38-specific inhibitor, significantly mitigated ORI- and NaB-induced p38 activation (Fig. 2B) and reduced γ -globin mRNA expression (Fig. 2C) and HbF protein expression in cultured cells (Fig. 2D). The levels of p38 phosphorylation, γ -globin mRNA expression and HbF protein expression in the SB203580-pretreated cells decreased to 34.82, 42.07 and 48.14% that of ORI-treated cells, respectively. Such data indicated that ORI-enhanced γ -globin expression was particularly dependent on activation of p38 MAPK in orthochromatic normoblasts and enucleated erythrocytes.

ORI treatment induces histone modification in γ -globin gene promoter regions by enhancing p38 MAPK signaling in erythroid precursor cells. Histone modification is a regulator of gene expression (31). To delineate the role of p38 MAPK-dependent epigenetic modifications in ORI-induced γ -globin gene expression, the cultured cells at 6 days post-phase II culture were treated with ORI or NaB in the presence or absence of SB23580 for 72 h. The different groups of cells were harvested and cross-linked, and DNA fragments were prepared by sonication. The DNA fragments were identified by ChIP, using anti-acetyl-histone H3 and 4 for targeting and anti-RNA polymerase as a control. Subsequently, the precipitated DNA fragments in the G γ - and A γ -globin promoters were amplified by PCR. As demonstrated in Fig. 3A, purified DNA was then analyzed by PCR using Control Primers specific for the GAPDH promoter. The PCR product was observed in the positive control group (lane 3) and scarcely in the negative control group (lane 2). GAPDH promoter-specific DNA was also observed in the Input (lane 4) but not in the 'No DNA' PCR control (lane 1). Quantitative analysis indicated that the levels of acetylated histone H3 (acH3) and acetylated histone H4 (acH4) in the G γ - and A γ -globin promoter regions in untreated cells were significantly higher than those of Necdin, a hallmark of specificity (Fig. 3B). The levels of G γ -acH3 and G γ -acH4 increased by 3.13 \pm 0.35- and 3.33 \pm 0.40-fold, respectively ($P < 0.05$), whereas A γ -acH3 and A γ -acH4 increased by 3.06 \pm 0.36- and 3.28 \pm 0.41-fold, respectively ($P < 0.05$; Fig. 3C and D). Conversely, the levels of acH3 and acH4 at the G γ - and A γ -globin gene promoter regions in SB203580-pretreated cells were decreased by 54.31 and 50.45% for the G γ -globin gene, and 51.93 and 47.88% for the A γ -globin gene, respectively, relative to cells treated with ORI alone. These findings indicated that ORI-enhanced γ -globin expression was partially mediated by p38 MAPK activation-dependent histone acetylation in cultured orthochromatic normoblasts and enucleated erythrocytes.

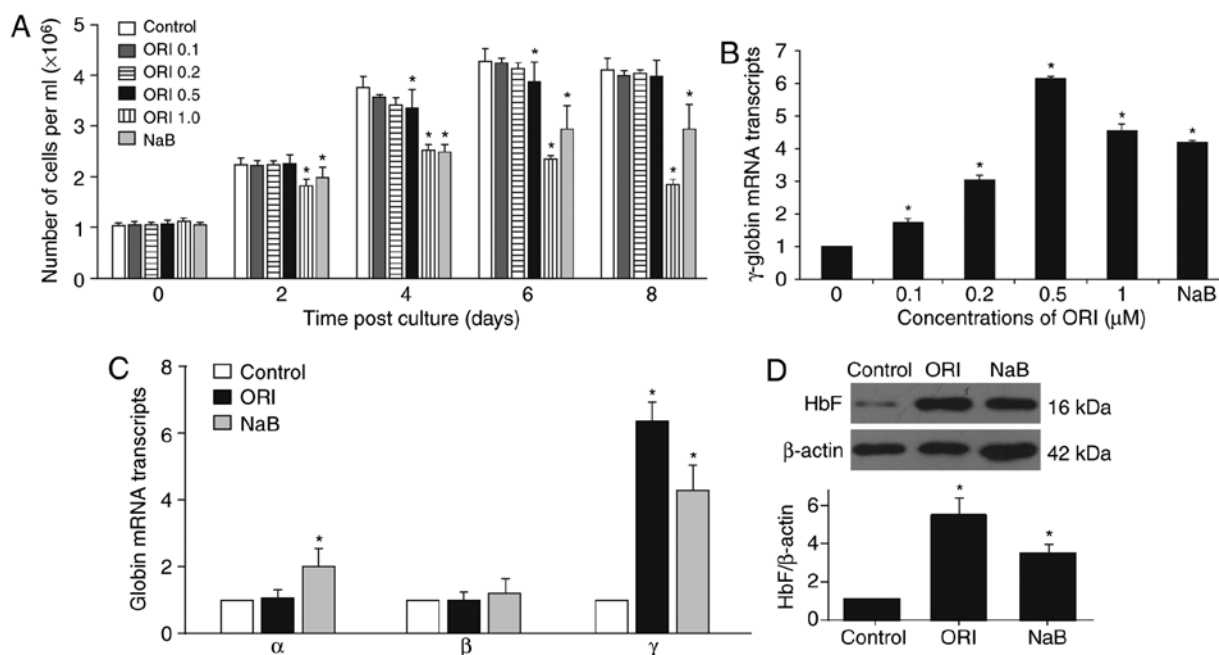


Figure 1. Treatment with ORI preferably induces γ -globin expression in cultured erythroid precursors from patients with human β -thalassemia. Peripheral blood mononuclear cells were cultured in a two-phase liquid culture system to enrich erythroid precursors and induce their differentiation. During phase II culture, the cells were treated with vehicle, or the indicated doses of ORI or NaB beginning on day 6 post-culture for varying time periods. The number of viable cells was counted longitudinally. On day 12 post-culture, the relative levels of α -globin, β -globin and γ -globin mRNA transcripts, and HbF protein expression were determined by reverse transcription-quantitative PCR and western blot analysis. Data are representative images or expressed as the mean \pm standard deviation of each group of cells from at least five biological samples from three separate experiments. (A) ORI exhibited little cytotoxicity against the cultured cells. (B) Treatment with ORI increased γ -globin mRNA transcripts. (C) ORI preferably induced γ -globin mRNA transcription. (D) ORI treatment increased HbF protein expression. * $P < 0.05$ vs. the control group. HbF, fetal hemoglobin; NaB, sodium butyrate; ORI, Oridonin.

ORI induces CREB1 phosphorylation by activating p38 MAPK signaling in erythroid precursor cells. Previous studies have demonstrated that activation of p38 MAPK signaling by NaB augments γ -globin gene transcription through CREB1 (29,30). The effects of ORI treatment on CREB1 activation in the cultured orthochromatic normoblasts and enucleated erythrocytes were analyzed by western blotting in this study. Treatment with ORI significantly increased the relative ratios of p-CREB1 to t-CREB1 in the cultured orthochromatic normoblasts and enucleated erythrocytes at 24 h post-treatment (2.13 ± 0.07 -fold; $P < 0.05$); its effects peaked at 72 h post-treatment (4.44 ± 0.07 -fold; $P < 0.05$) and then markedly declined to a level similar to the untreated control (1.10 ± 0.03 -fold; $P = 0.12$; Fig. 4A). Similarly, treatment with NaB for 72 h significantly increased CREB1 activation in the cultured cells (4.35 ± 0.11 -fold; $P < 0.05$). However, treatment with SB23580 significantly reduced ORI or NaB-mediated CREB1 activation by 51.65 and 29.16% in the cultured orthochromatic normoblasts and enucleated erythrocytes (Fig. 4B). These findings suggested that ORI treatment activated p38 MAPK and CREB1, contributing to histone modification, and enhanced γ -globin expression in the cultured orthochromatic normoblasts and enucleated erythrocytes *in vitro*.

Discussion

Several natural products have been demonstrated to induce HbF expression *in vitro* and *in vivo* (15-17,19); however, the mechanisms underlying the action of these compounds have yet to be elucidated. The present study revealed that treatment

with ORI significantly increased γ -globin expression with little cytotoxicity in cultured orthochromatic normoblasts and enucleated erythrocytes *in vitro*. In addition, treatment with ORI activated p38 MAPK and CREB1 signaling, which led to histone hyperacetylation and γ -globin expression in cultured orthochromatic normoblasts and enucleated erythrocytes. To the best of our knowledge, the present study was the first to demonstrate that ORI effectively induced γ -globin expression in cultured orthochromatic normoblasts and enucleated erythrocytes. This, together with little cytotoxicity, suggests that ORI may be a promising candidate for intervention in β -thalassemia.

An ideal HbF inducer should effectively induce HbF production and have no cytotoxicity against the differentiated erythrocytes (37). HU is a ribonucleotide reductase inhibitor, which has been approved by the FDA for treatment of SCD (7). However, this treatment fails to achieve a significant increase in the levels of HbF to prevent complications in patients with β -thalassemia (38,39). The notable hematopoietic suppression induced by HU has hindered the management of β -thalassemia (12). The present study demonstrated that treatment with ORI at 0.1-0.5 μ M significantly increased the expression levels of γ -globin in cultured primary erythroid progenitor cells obtained from patients with β -thalassemia in a dose-dependent manner. However, treatment with 1 μ M ORI decreased its effect on inducing γ -globin expression. This, together with a reduction of $\sim 50\%$ in the number of viable cells suggested that treatment with a higher dose of ORI may trigger apoptosis of erythroid progenitor cells. In addition, ORI treatment did not enhance α - and β -globin expression in cultured

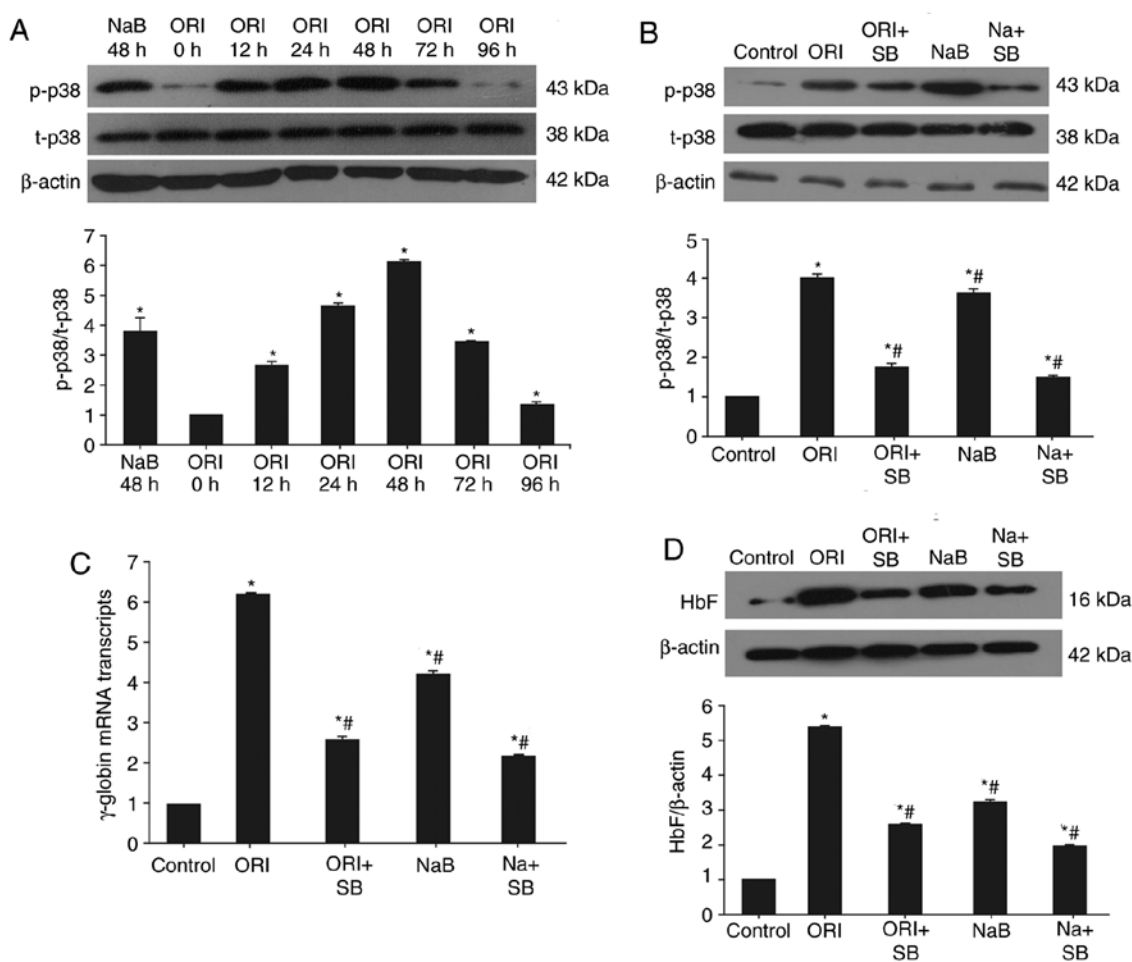


Figure 2. ORI treatment activates p38 MAPK to induce γ -globin expression in cultured erythroid precursor cells. (A) On day 6 of the phase II culture, the cells were treated with vehicle (control), 0.5 μ M ORI for the indicated time periods or 500 μ M NaB for 48 h. (B-D) Some cells were pretreated with, or without, SB203580 for 30 min and treated with 0.5 μ M ORI or 500 μ M NaB for 48 h. The relative levels of p38 MAPK expression and phosphorylation, γ -globin mRNA expression and HbF protein expression were determined by western blot analysis and reverse transcription-quantitative PCR. (B) ORI or NaB activated p38 MAPK, which was mitigated by SB203580. (C) ORI induced γ -globin and (D) HbF expression, which was partially dependent on p38 MAPK activation. Data are representative images or expressed as the mean \pm standard deviation of each group of cells from at least five biological samples from three separate experiments. * P <0.05 vs. the control group. # P <0.05 vs. 0.5 μ M ORI alone. MAPK, mitogen-activated protein kinase; NaB, sodium butyrate; ORI, Oridonin; SB, SB203580.

primary erythroid progenitor cells, and this preference may prevent the counteracting effect of some HbF inducers (15,40). Notably, ORI at 0.5 μ M exhibited little inhibition on the viability of cultured primary erythroid progenitor cells. *Rabdosia rubescens*, the herb from which ORI is extracted, has an excellent safety profile (41-44). Therefore, the present study suggested that ORI may be safe for intervention in patients with β -thalassemia in the clinic.

Previous studies have demonstrated that activation of the p38 MAPK signaling pathway is crucial for inducing γ -globin expression (27-30,45,46). MAPK kinase (MKK)3 and MKK6 are p38 MAPK activators, which can also independently induce HbF production (44). The present study revealed that ORI activated p38 MAPK signaling in a time-dependent manner, and inhibition of p38 MAPK signaling mitigated or abrogated ORI-induced increases in γ -globin expression in the cultured erythroid progenitors. Such data indicated that ORI-induced γ -globin expression partially depended on p38 MAPK activation in the cultured erythroid progenitors. Previous studies have demonstrated that some γ -globin inducers, such as

HU, thalidomide, butyrate and trichostatin A can promote reactive oxygen species (ROS) production to activate p38 MAPK signaling, increasing γ -globin expression (27,47,48). ORI has been reported to induce the apoptosis of some cancer cells by enhancing the ROS-mediated p38 MAPK signaling (49,50). Accordingly, it is possible that ORI may enhance ROS production to activate p38 MAPK and enhance γ -globin expression. More studies are required to determine the precise mechanisms underlying the pharmacological action of ORI.

It is notable that histone modification, particularly hyperacetylation, is important for enhancing gene expression. The present study demonstrated that ORI treatment significantly increased the levels of acetyl H3 and H4 in the A γ - and G γ -globin promoter regions and activated CREB1 in cultured orthochromatic normoblasts and enucleated erythrocytes; these effects were abrogated by the inhibition of p38 MAPK signaling. The findings of the present study extended previous observations and indicated that enhancement of histone acetylation or inhibition of histone deacetylases can promote γ -globin expression by activating p38 MAPK signaling (29,31,46). It is

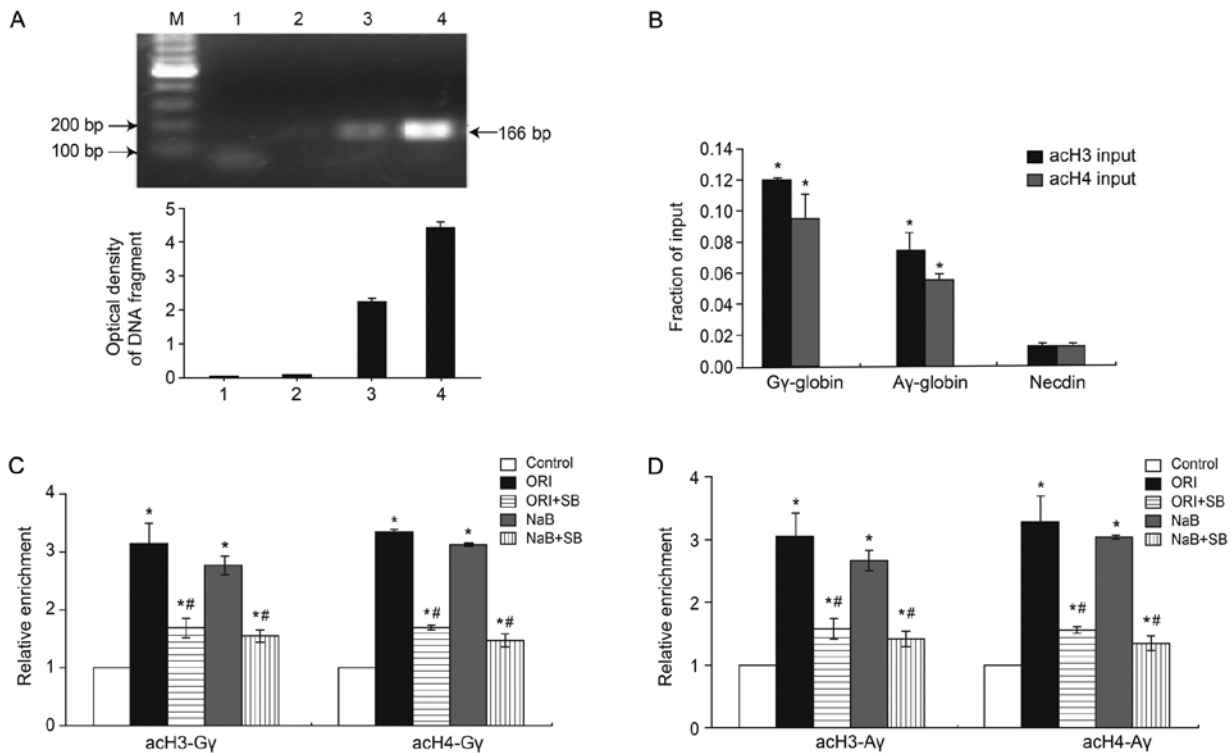


Figure 3. ORI induces hyperacetylation in the γ -globin promoter, which is partially dependent on p38 MAPK. On day 6 of the phase II culture, cells were pretreated with, or without, SB203580 for 30 min and treated with 0.5 μ M ORI or 500 μ M NaB for 72 h. Their chromatin was prepared and subjected to ChIP using acH3 and acH4 antibodies. The obtained DNA fragments were amplified by PCR using specific primers for the G γ -globin and A γ -globin promoters. Data are representative images or expressed as the mean \pm standard deviation of each group of cells from at least five biological samples from three separate experiments. (A) Identification of PCR products by agarose gel electrophoresis. 1: 'no DNA' PCR control; 2: normal mouse IgG ChIP; 3: anti-RNA polymerase II ChIP; 4: Input DNA. (B) Amplification of the G γ -globin and A γ -globin and control in the input samples. Amplification of the AcH3 and AcH4 in the (C) G γ -globin and (D) A γ -globin promoters. *P<0.05 vs. the control group. #P<0.05 vs. 0.5 μ M ORI alone. acH3, anti-acetyl-histone; acH4, anti-acetyl-histone H4; ChIP, chromatin immunoprecipitation; MAPK, mitogen-activated protein kinase; NaB, sodium butyrate; ORI, Oridonin; SB, SB203580.

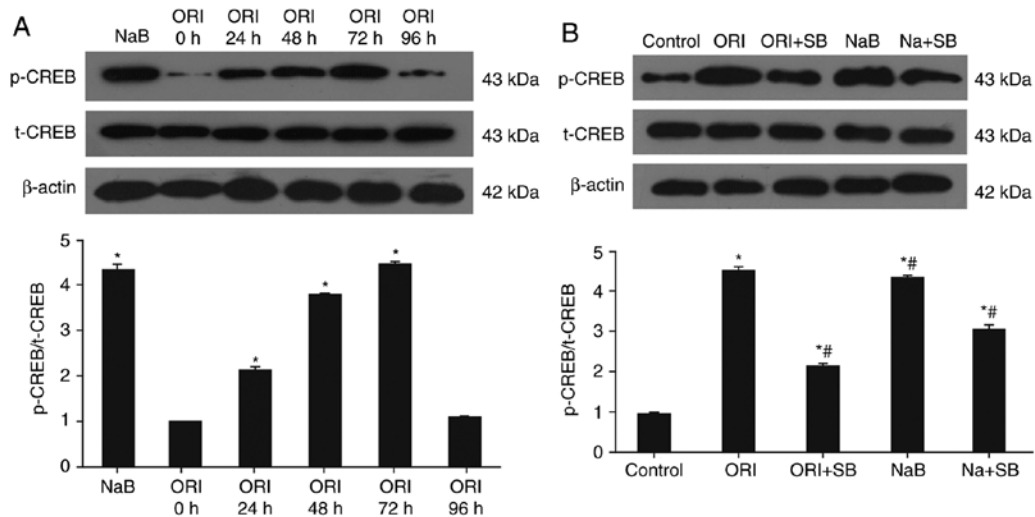


Figure 4. ORI activates CREB1, which is partially dependent on p38 MAPK activation. On day 6 of phase II culture, cells were pretreated with, or without, SB203580 for 30 min and treated with 0.5 μ M ORI for 96 h or 500 μ M NaB for 72 h. The relative levels of CREB1 phosphorylation and expression were determined longitudinally or tested after treatment by western blotting. Data are representative images or expressed as the mean \pm standard deviation of each group of cells from at least five biological samples from three separate experiments. (A) ORI activated CREB1 in a time-dependent manner. (B) ORI activated CREB1, which was partially dependent on p38 MAPK activation at 72 h post-treatment. *P<0.05 vs. the control group. #P<0.05 vs. 0.5 μ M ORI alone. CREB1, cAMP-response element binding protein 1; MAPK, mitogen-activated protein kinase; NaB, sodium butyrate; ORI, Oridonin; p-, phosphorylated; SB, SB203580; t-, total.

notable that activated CREB1 is crucial for γ -globin expression (29,30) and that the motif sequence for activated CREB1

binding is located in the G γ -globin promoter (G-CRE, 5'-TGA CGTCA-3', -1,222 to -1,229) (35,40). Accordingly, activation of

CREB1 by ORI suggests that ORI may activate p38 MAPK, which subsequently activates CREB1 to induce histone hyperacetylation, increasing γ -globin expression in cultured orthochromatic normoblasts and enucleated erythrocytes. Therefore, the findings of the present study may provide novel insights into regulation of the γ -globin expression in human orthochromatic normoblasts and enucleated erythrocytes. Further studies may investigate whether treatment with ORI can direct or indirectly increase the interaction of CREB1 with the G-CRE in the G γ -globin promoter.

In summary, the results of the present study indicated that ORI treatment selectively induced γ -globin expression with little cytotoxicity. ORI treatment induced epigenetic histone modification at the γ -globin promoter by activating p38 MAPK and CREB1. Therefore, ORI may be considered a promising therapeutic agent for intervention of β -thalassemia. Notably, the present study had limitations, including a small sample size, and a lack of *in vivo* studies and morphological investigation; in particular, morphological analysis was not conducted to confirm that cells developed into orthochromatic normoblasts and enucleated erythrocytes on day 12, and this study did not assess whether the addition of ORI arrested erythrocyte maturation. Although the present study provided data to indicate the therapeutic potentials of ORI, further studies with a larger population are warranted to validate the findings and explore the mechanisms underlying the pharmacological action of ORI in enhancing γ -globin expression.

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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

LSG conceived and designed the study and wrote the manuscript. JC performed the experiments, collected the data and wrote the manuscript. QYW, JLZ and WMH collected and analyzed the data. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from the patients' guardians. The experimental protocol was approved by the Ethical Committee of Guangdong Maternal and Child Health Hospital.

Patient consent for publication

The patients' guardians provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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