IOX1 Fails to Reduce α-Globin and Mediates γ-Globin Silencing in Adult β^0-Thalassemia/Hemoglobin E Erythroid Progenitor Cells

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The accumulation of unbound α-globin chains in red blood cells is a crucial pathophysiology of β-thalassemia. IOX1 (5-carboxy-8-hydroxyquinoline) is a broad-spectrum 2-oxoglutarate (2OG)-dependent oxygenase inhibitor that can reduce α-globin mRNA expression in human cord blood erythroid progenitor cells. Therefore, IOX1 has been proposed as a potential compound for β-thalassemia treatment through the decrease in α-globin chain synthesis. However, there is no empirical evidence regarding the consequences of IOX1 in β-thalassemia. In this study, the therapeutic effects of IOX1 were investigated in β^0-thalassemia/hemoglobin E (HbE) erythroid progenitor cells during in vitro erythropoiesis. The results indicated that IOX1 had no impact on α-globin gene expression, but it led instead to significant decreases in γ-globin and fetal hemoglobin (HbF, $\alpha_2\gamma_2$) production without affecting well-known globin regulators: *KLF1, BCL11A, LRF,* and *GATA1*. In addition, differential mRNA expression of several genes in the hypoxia response pathway revealed the induction of *EGLN1*, the PHD2-encoding gene, as a result of IOX1 treatment. These findings suggested that IOX1 fails to lower α-globin gene expression; on the contrary, it mediates γ-globin and HbF silencing in β^0-thalassemia/HbE erythroid progenitor cells. Because of the negative correlation of *EGLN1* and γ-globin gene expression after IOX1 treatment, repurposing IOX1 to study the hypoxia response pathway and γ-globin regulation may provide beneficial information for β-thalassemia.

HIGHLIGHTS:

- IOX1 did not reduce α-globin production in β^0-thalassemia/HbE erythroid progenitor cells.
- IOX1 treatments reduce γ-globin and HbF production in β^0-thalassemia/HbE erythroid progenitor cells.
- *EGLN1*, a hypoxia response gene, is upregulated after IOX1 treatments in β^0-thalassemia/HbE erythroid progenitor cells.

β-Thalassemia is an autosomal recessive blood disorder that presents with a quantitative abnormality of β-globin synthesis. Compound heterozygosity for β^0-thalassemia and the β-globin chain variant (*HBB*: c.79G>A, hemoglobin E), hereinafter referred to as β^0-thalassemia/HbE, has been characterized as one of the severe β-thalassemia genotypes caused by mutations within the β-globin gene [1]. This leads to the absence of β-globin chain and adult hemoglobin (HbA, $\alpha_2\beta_2$) production, leaving unpaired α-globin chains in erythroid lineage cells. Oxidized heme-bound α-globin chains precipitate in the cell membrane and generate reactive oxygen species (ROS) [2,3]. This eventually results in serious red blood cell membrane abnormalities, hemolytic anemia, and ineffective erythropoiesis in β-thalassemia [4,5]. Unless appropriate treatments are provided, the patients endure chronic anemia and life-threatening complications. Occasional or constant lifelong blood transfusions with iron chelation have been considered as the first-line therapy for improving anemia in β-thalassemia. Currently, hematopoietic cell transplantation and gene therapy are only the curative treatments for β-thalassemia [6,7]. Despite the significant clinical results, shortfalls in access to these approaches compound the

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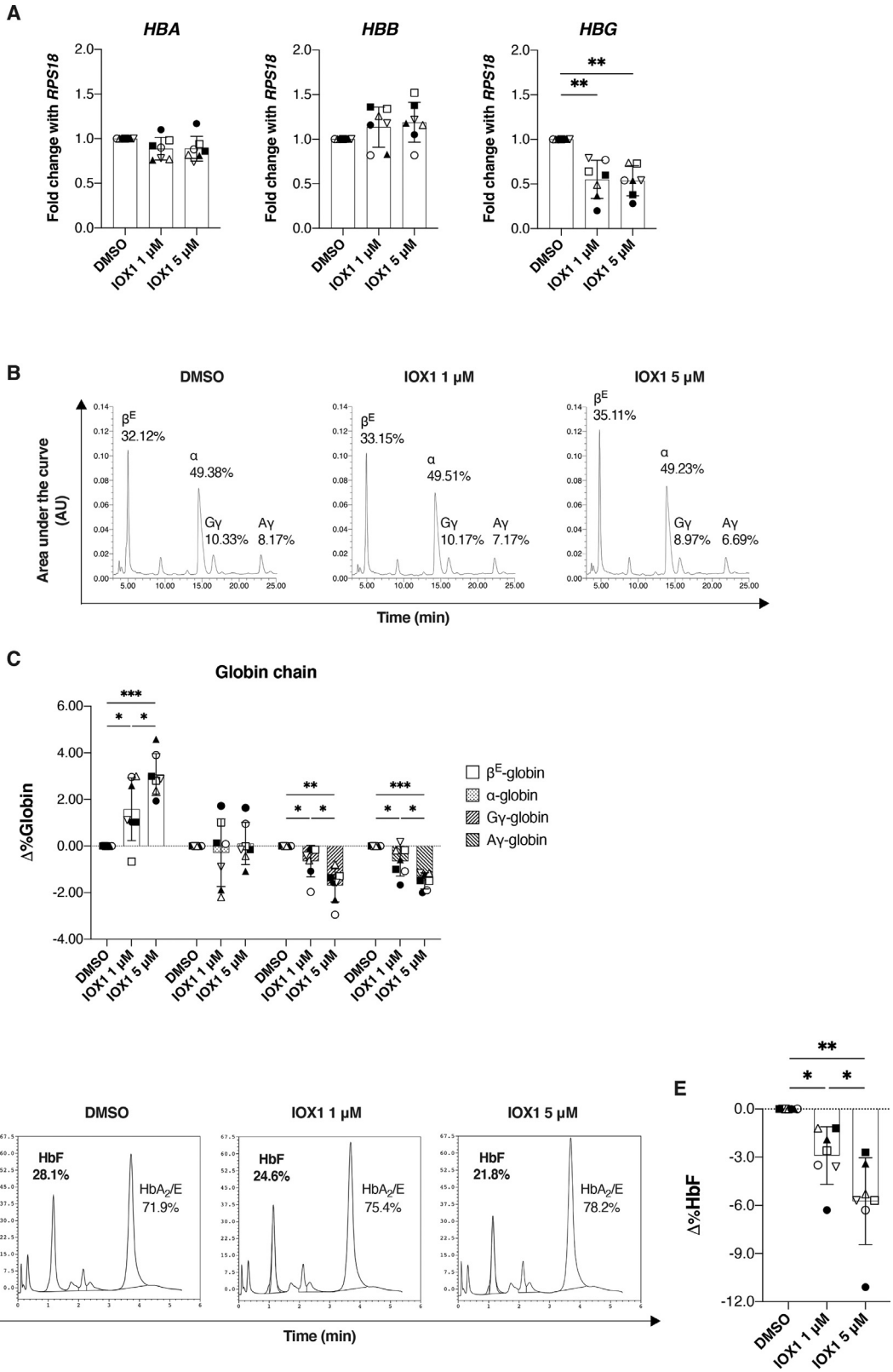

Figure 1 Reduction of γ-globin and HbF expression after IOX1 treatment in β^0-thalassemia/HbE. **(A)** Relative fold change in *HBA* (α-globin), *HBB* (β-globin), and *HBG* (γ-globin) mRNA expression normalized to *RPS18* from qPCR analysis at day 12 (n = 7). **(B)** Representative globin chain analysis investigated by reverse-phase HPLC at day 14. **(C)** Relative globin chain percentage is expressed

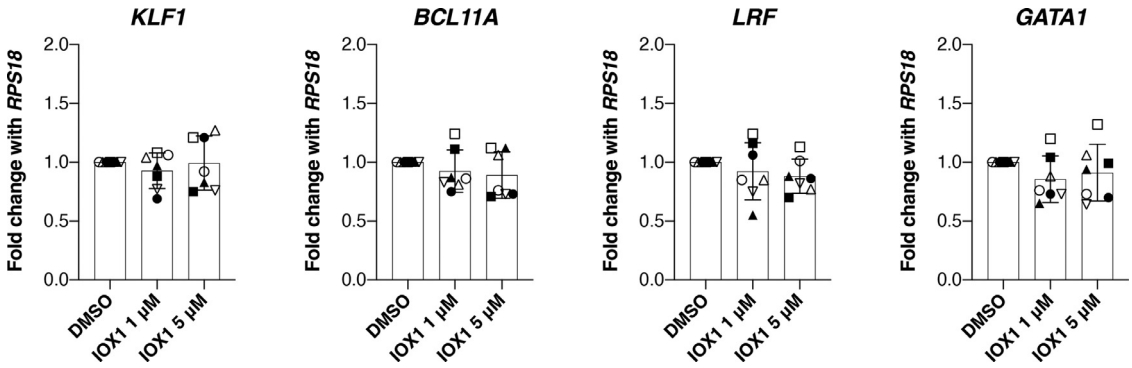

Figure 2 Insignificant change in mRNA expression levels of four selected well-characterized γ-globin gene regulators after IOX1 treatment in β^0-thalassemia/HbE. Relative fold change was normalized to *RPS18* from qPCR analysis at day 12 (n = 7). Data are expressed as the mean ± SD.

profound impact on the inequality of health opportunities in most patients.

Clinical observations and genetic evidence have indicated that the co-inheritance of α-thalassemia is one of the beneficial genetic modifying factors in β^0-thalassemia/HbE. The patients who inherited one (-α/$\alpha\alpha$) or two (−/$\alpha\alpha$ or -α/-α) α-globin gene(s) deletions clearly exhibit intermediate to very mild phenotypes in which a reduced transfusion burden is observed [8,9]. In addition, the experimental downregulation of α-globin expression using siRNA [10] and a gene editing approach [11] in β-thalassemia successfully decreased the degree of α/β-globin imbalance and revealed phenotypic improvements. This strongly suggested that the decrease in α-globin gene expression is a promising target for β-thalassemia treatment.

Screening of small-molecule epigenetic modulators revealed that IOX1, or 5-carboxy-8-hydroxyquinoline, a cell permeable compound with a broad spectrum 2-oxoglutarate (2OG)-dependent oxygenase inhibitor [12], is capable of inhibiting α-globin mRNA expression in human cord blood erythroid progenitor cells through the increase in H3K27me3 and H3K9me3 at the α-globin promoter [13]. This finding suggested the possibility of using IOX1 as a potent therapeutic pharmaceutical agent to reduce α-globin expression in β-thalassemia and further improve clinical appearance. However, there is no scientific evidence supporting the hypothesis that IOX1 is able to reduce the α-globin expression in β-thalassemia. A lack of evidence proving the potency and efficacy of IOX1 in β-thalassemia limits further applications. Thus, in this study, the effects of IOX1 in β^0-thalassemia/HbE erythroid progenitor cells were investigated in an erythroid liquid culture system.

METHODS

Methods are described in detail in the Supplementary Data (online only, available at www.exphem.org). Briefly, $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) were isolated from seven unrelated β^0-thalassemia/HbE volunteers who had no α-thalassemia co-inheritance after obtaining the written informed consent (Supplementary Table S1, online only, available at www.exphem.org). HSPCs were differentiated toward the erythroid lineage in a three-phase liquid culture system. During in vitro erythropoiesis, IOX1 was added to the cells and the effects of IOX1 on β^0-thalassemia/HbE erythroid progenitor cells were investigated. Protocols involving human subjects in this study were approved by the Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University (Study Code: AMSEC-63EX-110) according to good clinical practices and relevant international ethical guidelines, the applicable laws and regulations.

RESULTS AND DISCUSSION

IOX1 concentrations ranging from 1 to 100 μM were tested on β^0-thalassemia/HbE erythroid progenitor cells during days 4−14 and days 8−14 of the three-phase liquid culture. Concentrations >5 μM were removed from further studies because of cytotoxicity (Supplementary Figure S1, online only, available at www.exphem.org). Dose-dependent effects of relative changes in globin chains and hemoglobin profiles were observed when IOX1 was supplemented on days 4−14 (Supplementary Figure S2, online only, available at www.exphem.org). These effects did not differ at concentrations >5 μM. IOX1 exhibited greater potency during the early stage of erythroid differentiation. At this stage, baseline levels of globin gene expression were low because of the sophisticated mechanisms of regulation. Therefore, a pharmacological investigation of globin gene expression during the early stage was capable of providing greater efficacy. On the basis of this information, doses of 1 and 5 μM were chosen and added to the β^0-thalassemia/HbE erythroid progenitor cells during days 4−14 of culture to determine the effects of IOX1. No cytotoxicity was observed using 1 and 5 μM IOX1 (Supplementary Figure S3, online only, available at www.exphem.org). As a result of IOX1 treatment in β^0-thalassemia/HbE erythroid progenitor cells, γ-globin mRNA expression was significantly reduced (1 μM = 0.6 ± 0.2, $p <$ 0.005, and 5 μM = 0.5 ± 0.2, $p < 0.005$), and α- and β-globin mRNA expression levels were not significantly changed (Figure 1A). Relative globin chain and hemoglobin analysis were in accordance

as the Δ%globin (%area under the curve of IOX1-treated − %area under the curve of DMSO−vehicle control, n = 7). **(D)** Representative hemoglobin analysis investigated by HPLC at day 14. **(E)** Relative HbF percentage was represented as Δ% HbF (%HbF of IOX1-treated − %HbF of DMSO−vehicle control, n = 7). Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

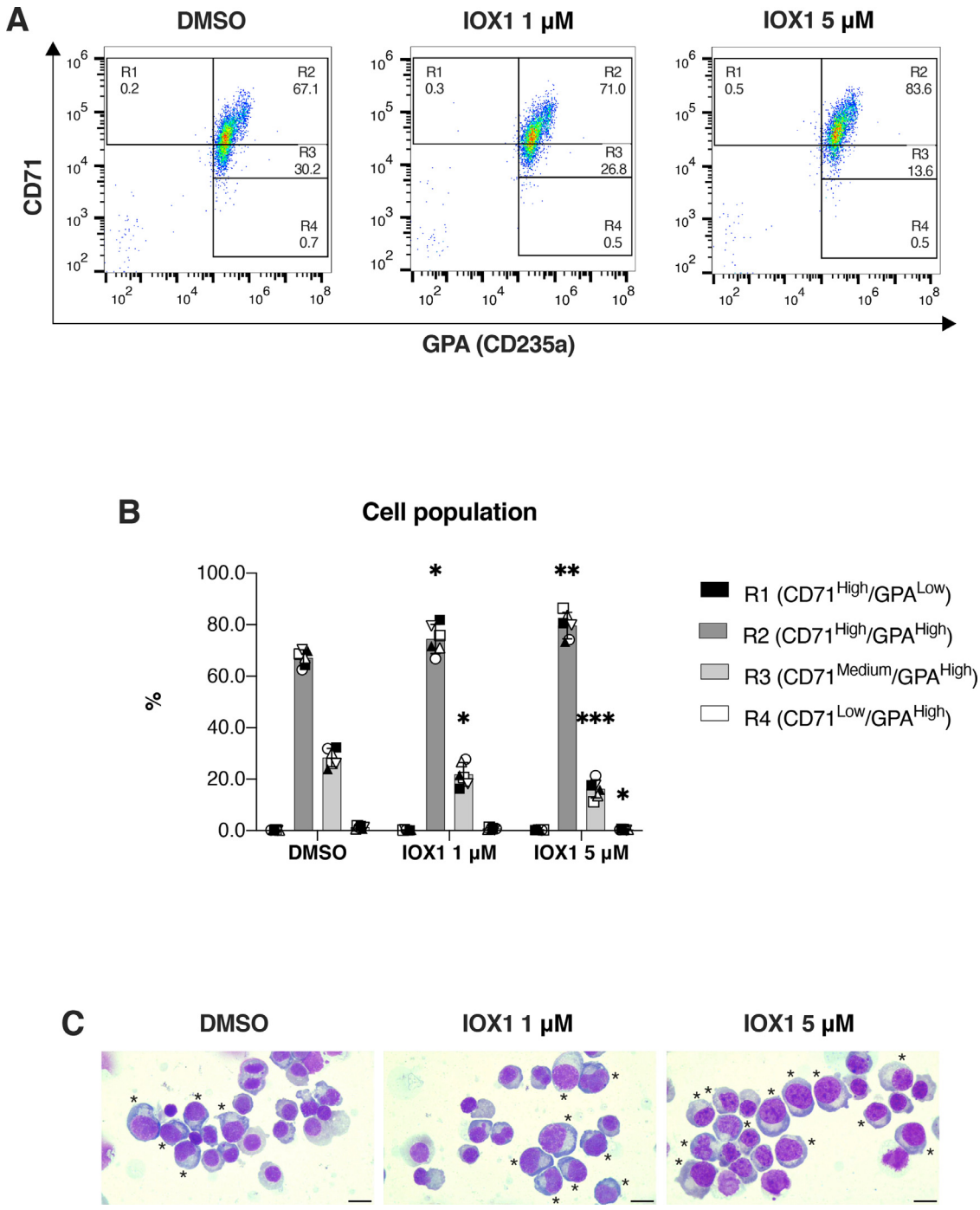

Figure 3 Inhibition of erythroid maturation after IOX1 treatment in β^0-thalassemia/HbE. **(A)** Representative flow cytometry analysis by measuring the expression levels of CD71 and GPA (CD235a) on the cell membrane at day 12. **(B)** Quantitative analysis of gated cell populations (n = 6). **(C)** Representative Wright–Giemsa-stained cells at day 12 (*asterisk* indicates erythroid cell in early stage of differentiation; scale bar = 10 μm,). Data are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

with the mRNA results. Decreases in Δ%Gγ (1 μM = 0.65 ± 0.68, $p < 0.05$, and 5 μM = 1.69 ± 0.71, $p < 0.005$), Δ%Aγ (1 μM = 0.65 ± 0.64, $p < 0.05$, and 5 μM = 1.49 ± 0.35, $p < 0.001$) and Δ%HbF (1 μM = 2.9 ± 1.8, $p < 0.05$, and 5 μM = 5.7 ± 2.7, $p < 0.005$) were significant (Figure 1B–E; Supplementary Table S2 and S3, online only, available at www.exphem.org), suggesting that IOX1 mediates γ-globin and HbF silencing in β^0-thalassemia/HbE erythroid progenitor cells. In contrast to the previous IOX1 study in cord blood

erythroid progenitor cells [13], significant changes in α-globin gene expression were not observed in the current study (Figure 1). This finding was also comparable to that in the study in transgenic mice in which IOX1 failed to reduce α-globin gene expression [14]. This may be due to a dynamic epigenetic landscape within human globin genes that specifically controls globin expression during development [15,16]. In contrast to human, mouse erythroid cells behave differently from the human cells [17]. Therefore, dissimilar sources of erythroid progenitor cells may affect globin expression patterns.

The mRNA expression levels of well-known γ-globin regulators including *KLF1, BCL11A, LRF,* and *GATA1* were investigated because the alterations of γ-globin expression were noticed in β^0-thalassemia/HbE erythroid progenitor cells after IOX1 treatment. However, no significant change was observed among these transcripts (Figure 2). Furthermore, correlation analysis of the changes between well-known γ-globin regulators and globin gene expression revealed no considerable relationship (Supplementary Table S4, online only, available at www.exphem.org). IOX1 effects also exhibited the inhibition of erythroid differentiation as marked by a greater cell accumulation within the R2 population ($CD71^{High}/GPA^{High}$) and a lower cell differentiation into the R3 population ($CD71^{Medium}/GPA^{High}$) when compared with the control on culture day 12 (Figure 3A,B). Erythroid morphology assessment confirmed the delay in erythroid differentiation after IOX1 treatment (Figure 3C). Although there was a marked delay in differentiation on IOX1 treatment in this study, gene expression alterations of master γ-globin regulators were unremarkable. These phenomena were observed in all β^0-thalassemia/HbE patients regardless of genotypes or hematological variables. Together, these observations suggested that IOX1 presumably silenced γ-globin and HbF production in β^0-thalassemia/HbE erythroid progenitor cells through independent mechanisms of these four well-known γ-globin regulators.

The inhibitory attributes of IOX1 on 2OG-dependent oxygenases are possibly associated with the downregulation of γ-globin and HbF synthesis in β^0-thalassemia/HbE erythroid progenitor cells after IOX1 treatment. Prolyl hydroxylases (PHDs) are members of the 2OG-oxygenase family that play important roles in the cellular hypoxia response pathway [18]. In humans, PHD2 is encoded by egg-laying defective nine 1 gene (*EGLN1*) and efficiently hydroxylates the α-subunit of hypoxia-inducible factors (HIF-α) under normoxic conditions [19,20]. The hydroxylated HIF-α is recognized by the tumor suppressor protein von Hippel–Lindau (pVHL), allowing proteasomal degradation. In contrast to normoxia, HIF-α accumulates, translocates into the nucleus, forms a heterodimeric transcription factor with HIF-β (aryl hydrocarbon receptor nuclear translocator [ARNT]) and activates hundreds of genes by recognizing hypoxia response elements (HREs) within the promoters or enhancers under hypoxic conditions. In addition to an oxygen, PHD2 requires 2OG as a co-substrate for functioning. Therefore, cell treatment with a broad-spectrum inhibitory 2OG-oxygenase, such as IOX1, can mimic hypoxia because PHD2 is inhibited and incapable of hydroxylating HIF-α. Despite the regulation of PHD2 on HIF-α stability, *EGLN1* is targeted to feedback upregulation by HIFs as it contains an HRE element [21]. Therefore, *EGLN1* is upregulated under hypoxic conditions and under treatment with PHD inhibitors [22–24]. With this, mRNA expression levels of *HIF1A, HIF2A, HIF1B, VHL, FIH, EGLN1, EGLN2,* and *EGLN3* were investigated after IOX1 treatment in β^0-thalassemia/HbE erythroid progenitor cells. Of these, *HIF2A, EGLN2,* and *EGLN3* failed to be detected by quantitative PCR (qPCR) in this study because of C_t values >30, comparable to the results of previous studies in which the expression of these genes was poor in most adult tissues, especially bone marrow cells [25,26]. As expected, a significant increase in *EGLN1* expression was observed (1 μM = 1.5 ± 0.3, $p < 0.005$, and 5 μM = 1.4 ± 0.2, $p < 0.005$) in β^0-thalassemia/HbE erythroid progenitor cells after IOX1 treatment, while the *HIF1A, HIF1B, VHL,* and *FIH* were not significantly changed (Supplementary Figure S4, online only, available at www.exphem.org). These findings initially demonstrated the inverse association between *EGLN1* and γ-globin gene expression. Recently, many studies have reported that PHD2 harnesses crucial functions in erythroid cell production [25,27,28], and the pharmacological inactivation of PHDs results in the upregulation of HbF levels [29,30]. However, the role of PHD2 in human globin regulation is uncertain and needs to be finely dissected.

CONCLUSIONS

The present study found that IOX1 is unable to suppress α-globin gene expression in β^0-thalassemia/HbE erythroid progenitor cells. Instead, IOX1 treatment significantly reduces γ-globin and HbF production in β^0-thalassemia/HbE erythroid progenitor cells. IOX1 also upregulates *EGLN1,* which mimics hypoxic conditions and correlates negatively with γ-globin expression in β^0-thalassemia/HbE erythroid progenitor cells. Therefore, repurposing IOX1 to study the hypoxia response pathway and γ-globin gene regulation in β-thalassemia may be worthwhile.

Conflict of Interest Disclosure

The authors declare no competing interests.

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Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.07.004.

REFERENCES

1. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. Blood 2010;115:4331–6.
2. Nagababu E, Rifkind JM. Heme degradation by reactive oxygen species. Antioxid Redox Signal 2004;6:967–78.
3. Nienhuis AW, Nathan DG. Pathophysiology and clinical manifestations of the beta-thalassemias. Cold Spring Harb Perspect Med 2012;2:a011726.

4. Schrier SL. Pathophysiology of thalassemia. Curr Opin Hematol 2002;9:123–6.
5. Rund D, Rachmilewitz E. Beta-thalassemia. N Engl J Med 2005;353:1135–46.
6. Lucarelli G, Galimberti M, Polchi P, et al. Bone marrow transplantation in patients with thalassemia. N Engl J Med 1990;322:417–21.
7. Thompson AA, Walters MC, Kwiatkowski J, et al. Gene therapy in patients with transfusion-dependent beta-thalassemia. N Engl J Med 2018;378:1479–93.
8. Winichagoon P, Fucharoen S, Chen P, Wasi P. Genetic factors affecting clinical severity in beta-thalassemia syndromes. J Pediatr Hematol Oncol 2000;22:573–80.
9. Sripichai O, Munkongdee T, Kumkhaek C, Svasti S, Winichagoon P, Fucharoen S. Coinheritance of the different copy numbers of alpha-globin gene modifies severity of beta-thalassemia/Hb E disease. Ann Hematol 2008;87:375–9.
10. Voon HP, Wardan H, Vadolas J. siRNA-mediated reduction of alpha-globin results in phenotypic improvements in beta-thalassemic cells. Haematologica 2008;93:1238–42.
11. Mettananda S, Fisher CA, Hay D, et al. Editing an alpha-globin enhancer in primary human hematopoietic stem cells as a treatment for beta-thalassemia. Nat Commun 2017;8:424.
12. Hopkinson RJ, Tumber A, Yapp C, et al. 5-Carboxy-8-hydroxyquinoline is a broad spectrum 2-oxoglutarate oxygenase inhibitor which causes iron translocation. Chem Sci 2013;4:3110–7.
13. Mettananda S, Fisher CA, Sloane-Stanley JA, et al. Selective silencing of alpha-globin by the histone demethylase inhibitor IOX1: a potentially new pathway for treatment of beta-thalassemia. Haematologica 2017;102:e80–4.
14. Mettananda S. Genetic and epigenetic therapies for beta-thalassaemia by altering the expression of alpha-globin gene. Front Genome Ed 2021;3:752278.
15. Yin W, Barkess G, Fang X, et al. Histone acetylation at the human beta-globin locus changes with developmental age. Blood 2007;110:4101–7.
16. Chang KH, Fang X, Wang H, et al. Epigenetic modifications and chromosome conformations of the beta globin locus throughout development. Stem Cell Rev Rep 2013;9:397–407.
17. Pishesha N, Thiru P, Shi J, Eng JC, Sankaran VG, Lodish HF. Transcriptional divergence and conservation of human and mouse erythropoiesis. Proc Natl Acad Sci USA. 2014;111:4103–8.
18. Fletcher SC, Coleman ML. Human 2-oxoglutarate-dependent oxygenases: nutrient sensors, stress responders, and disease mediators. Biochem Soc Trans 2020;48:1843–58.
19. Appelhoff RJ, Tian YM, Raval RR, et al. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J Biol Chem 2004;279:38458–65.
20. Fong GH, Takeda K. Role and regulation of prolyl hydroxylase domain proteins. Cell Death Differ 2008;15:635–41.
21. Metzen E, Stiehl DP, Doege K, Marxsen JH, Hellwig-Burgel T, Jelkmann W. Regulation of the prolyl hydroxylase domain protein 2 (phd2/egln-1) gene: identification of a functional hypoxia-responsive element. Biochem J 2005;387(Pt 3):711–7.
22. D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C. Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem 2003;278:38183–7.
23. Marxsen JH, Stengel P, Doege K, et al. Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J 2004;381(Pt 3):761–7.
24. Stiehl DP, Wirthner R, Koditz J, Spielmann P, Camenisch G, Wenger RH. Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels: evidence for an autoregulatory oxygen-sensing system. J Biol Chem 2006;281:23482–91.
25. Takeda K, Aguila HL, Parikh NS, et al. Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. Blood 2008;111:3229–35.
26. Liu H, Tang F, Su J, et al. EPAS1 regulates proliferation of erythroblasts in chronic mountain sickness. Blood Cells Mol Dis 2020;84:102446.
27. Ladroue C, Carcenac R, Leporrier M, et al. PHD2 mutation and congenital erythrocytosis with paraganglioma. N Engl J Med 2008;359:2685–92.
28. Gardie B, Percy MJ, Hoogewijs D, et al. The role of PHD2 mutations in the pathogenesis of erythrocytosis. Hypoxia (Auckl) 2014;2:71–90.
29. Hsieh MM, Linde NS, Wynter A, et al. HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. Blood 2007;110:2140–7.
30. Feng R, Mayuranathan T, Huang P, et al. Regulation of fetal hemoglobin expression by the VHL-HIF1α oxygen sensing system. Blood 2021;138(Suppl 1):574.

SUPPLEMENTARY DATA

Materials and Methods

Primary erythroid cell culture. Peripheral blood samples were taken from β^0-thalassemia/HbE volunteers with no α-thalassemia co- inheritance. $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) were purified from blood samples using the CD34 MicroBead Kit according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). Hematological data of the participants are described in Supplementary Table S1. Isolated $CD34^+$ HSPCs were subjected to a three-phase erythroid liquid culture system. Cells were allowed to proliferate and undergo erythroid lineage differentiation for 14 days in this culture system at 37°C and 5% CO_2 in a 100% humidified atmosphere. Iscove's Modified Dulbecco Medium (Cytiva, South Logan, UT) supplemented with 20% v/v fetal bovine serum (HyClone, Pasching, Austria), 300 μg/mL holo-transferrin (ProSpec, Ness Ziona, Israel) and 1% v/v penicillin/streptomycin (Gibco, Grand Island, NY) was prepared as a basal medium. Cytokines with different concentrations including human interleukin-3 (IL-3, PeproTech, Rehovot, Israel), human stem cell factor (SCF; PeproTech) and erythropoietin (EPO; Janssen-Cilag, Bangkok, Thailand) were supplemented to the basal medium during each phase of cell culture as follows: phase I (days 0-4) included 10 ng/mL IL-3, 50 ng/mL SCF and 2 U/mL EPO; phase II (days 4-8) included 10 ng/mL SCF and 2 U/mL EPO; and phase III (days 8-14) included 4 U/mL EPO. Later, cell density was maintained at 1-2x10^6 cells/ml.

Compound. IOX1 (SML0067, Sigma-Aldrich, St. Louis, MO) was freshly dissolved in dimethyl sulfoxide (DMSO; D8418, Sigma-Aldrich) and added to cells regarding specific conditions. A concentration of 0.1% v/v DMSO was used as the concentration-matched vehicle control.

Cell proliferation, viability, and morphology. Cells were stained with 0.4% trypan blue solution and applied to a hemocytometer. Cell number and viability were examined under a microscope. To investigate cell morphology, a total of 1x10^5 cells were harvested on day 12 and subjected to a cytocentrifugation (Cytospin 3, Thermo Shandon, Cheshire, UK) at 600 rpm for four minutes. Cells on a slide were briefly fixed with absolute methanol, stained with Wright-Giemsa stain and investigated under a microscope (Leica DM750, Leica Microsystems, Heerbrugg, Switzerland).

Quantitative PCR. Total RNA was extracted from at least 1x10^6 cells on day 12 by TRIzol Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocols. cDNA was synthesized from 1 μg total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Quantitative PCR (qPCR) was performed on CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, CA) using RBC ThermOne Real-Time Premix SYBR Green (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's protocols and specific primers listed in Supplementary Table S5. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ method against the expression of ribosomal protein S18 (*RPS18*).

Flow cytometry. Cell suspensions were prepared at a final concentration of 1x10^5 cells on day 12 in 100 μl of 1xDPBS (Cytiva). Flow cytometric staining was performed using mouse anti-human CD71 PE-conjugated antibody (CY1G4; Biolegend, San Diego, CA) and mouse anti-human CD235a APC-conjugated antibody (HIR2; Biolegend) according to the manufacturer's recommendations. Stained cells were analyzed on DxFLEX flow cytometer (Beckman Coulter, Indianapolis, IN). Data analysis was performed using FlowJo software version 10.7 (FlowJo LLC, Ashland, OR, USA).

Globin chain analysis. Cell pellets were prepared from at least 1x10^6 cells on day 14. The pellet was lysed in deionized water with two freeze-thaw cycles. Hemolysate was used in reverse-phase HPLC using the Waters HPLC alliance e2695 separations module (Waters Corporation, Milford, MA) and Aeris 3.6 μm WIDEPORE-C4 200 A° column (Phenomenex, Torrance, CA). HPLC analytical protocol for globin chain separation was performed as previously described [Hatzistavrou et al., 2009].

Hemoglobin analysis. Cell pellets were prepared from at least 1x10^6 cells on day 14. Each pellet was completely lysed in 500 μl of Wash/Diluent Solution (BioRad) and subjected to HPLC separation using VARIANT II β-Thalassemia Short Program (BioRad), following the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed on Prism version 9.0.1 (GraphPad Software, San Diego, CA) using unpaired Student's *t*-test. A *P*-value of less than 0.05 ($p < 0.05$) was considered as statistical significance.

Reference

Hatzistavrou T, Micallef SJ, Ng ES, Vadolas J, Stanley EG, Elefanty AG. ErythRED, a hESC line enabling identification of erythroid cells. Nat Methods. 2009;6(9):659-62.

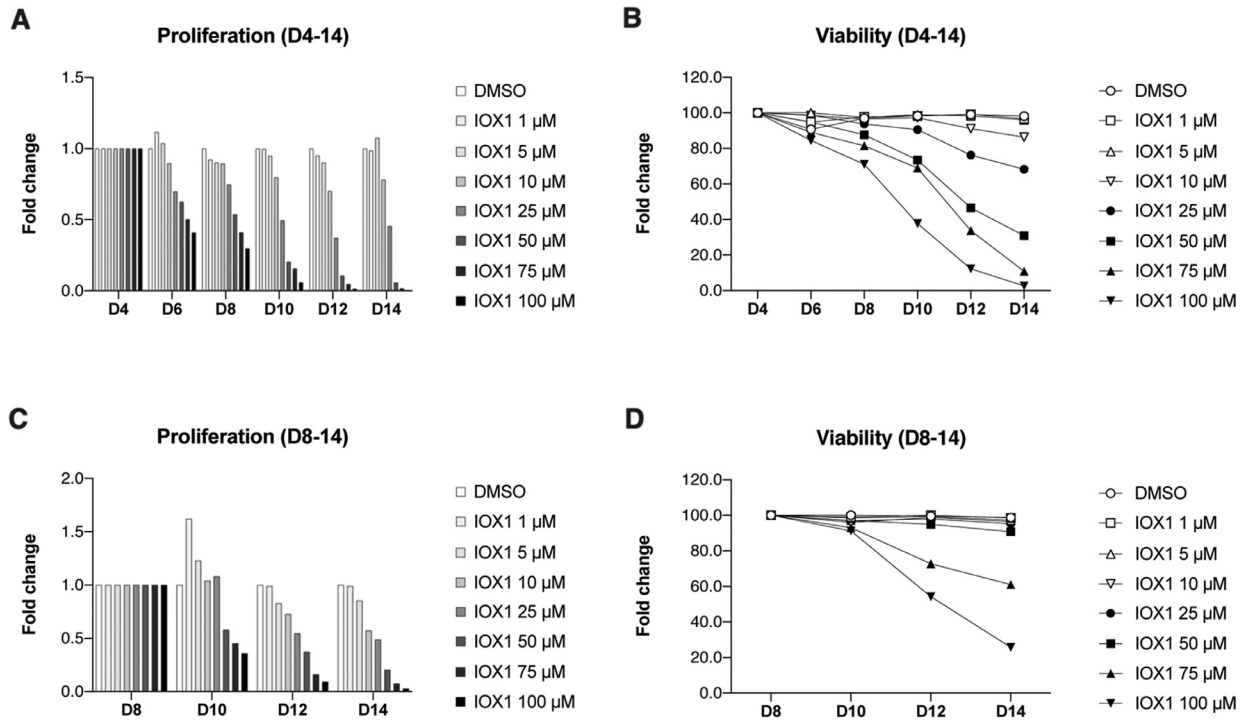

Figure S1 Different concentrations of IOX1 were treated on days 4-14 **(A-B)** and days 8-14 **(C-D)** during β^0-thalassemia/HbE primary erythroid cell culture (n = 2). Cell proliferation **(A-C)** and viability **(B-C)** were investigated and relatively compared to day 4 or day 8. Data are presented as average values.

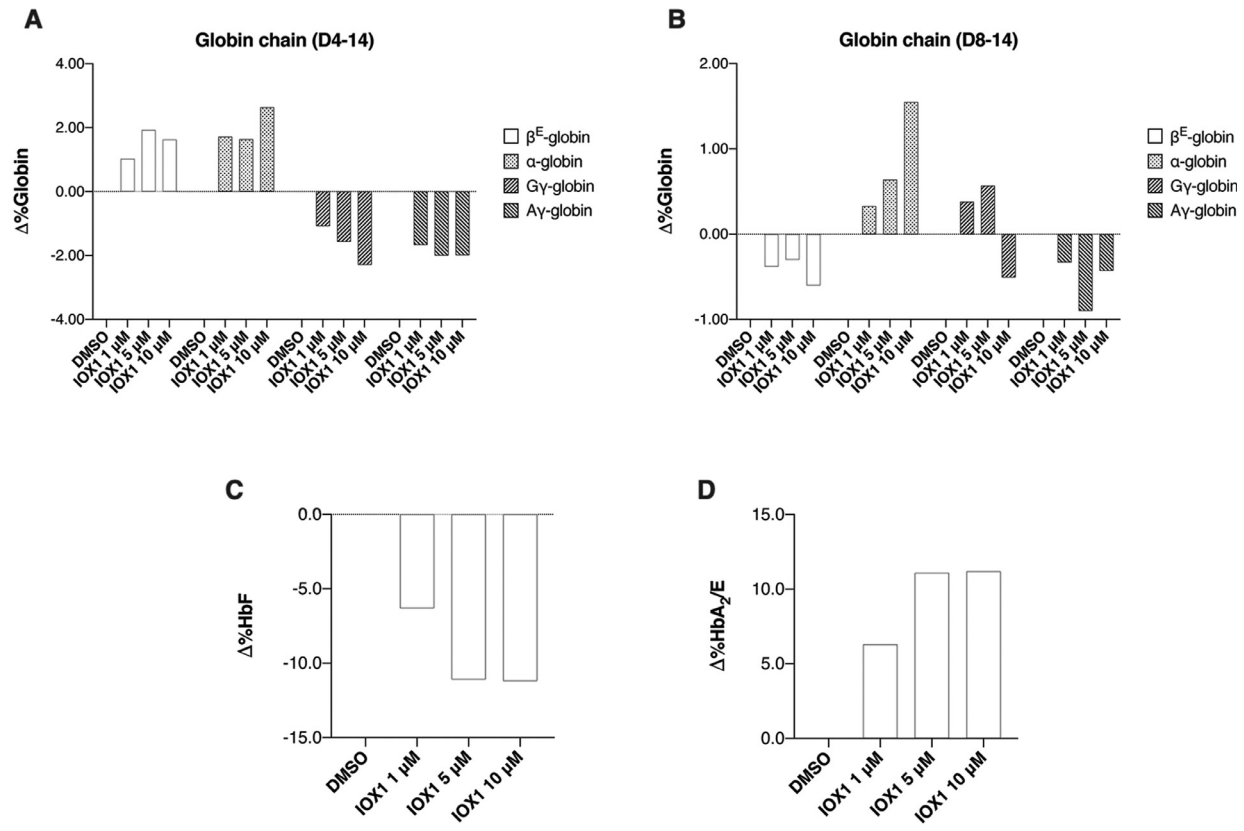

Figure S2 IOX1 concentrations ranging from 1 to 10 μM were treated during β^0-thalassemia/HbE primary erythroid cell culture at different time intervals (n = 1) **(A-D)**. Globin chain analysis was performed by reverse-phase HPLC at day 14 **(A-B)**. Bar graph represents Δ%globin after IOX1 treatment upon **(A)** days 4-14 and **(B)** days 8-14. Hemoglobin analysis was performed by HPLC at day 14 **(C-D)**. Bar graph represents **(C)** Δ%HbF and **(D)** Δ%HbA_2/E after IOX1 treatment upon days 4-14.

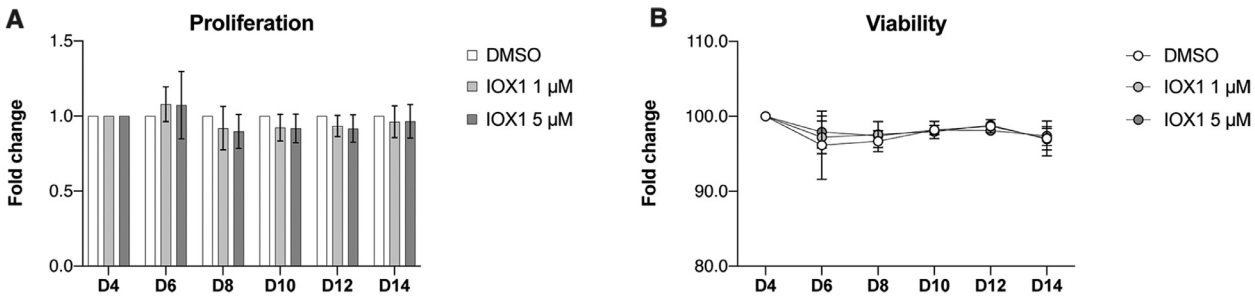

Figure S3 Cell proliferation **(A)** and cell viability **(B)** assessed by trypan blue assay during erythroid differentiation (n = 7). Data are presented as mean ± SD.

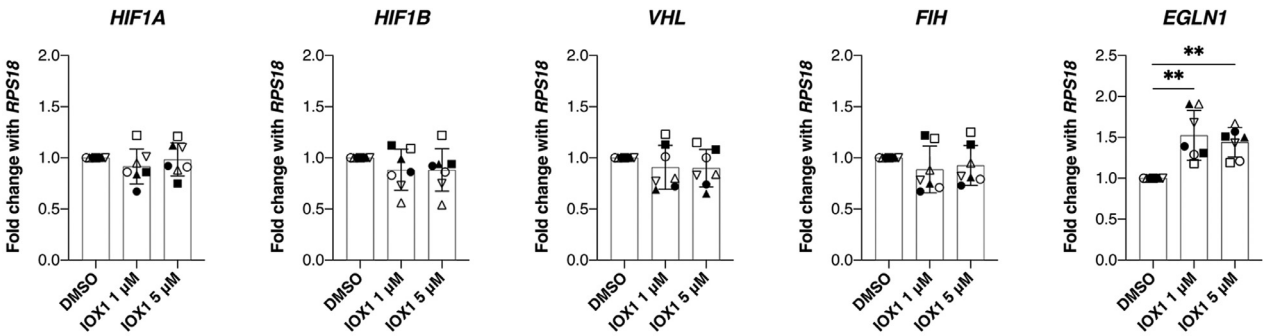

Figure S4 qPCR analysis showing relative mRNA expression fold-change of selected five hypoxia response genes. The expression level of each gene was normalized to *RPS18*. Data are presented as mean ± SD (n = 7). Data are presented as mean ± SD. $^{**}p < 0.005$

Table S1 Hematological descriptions of β^0-thalassemia/HbE patients participated in the IOX1 study.

Sample	Sex	Age (year-old)	*HBB**	HbF (%)	$HbA_2/E^{\dagger}$ (%)	RBC ($x10^6/\mu L$)	Hb (g/dL)‡	Hct (%)	MCV (fL)	MCH (pg)	RDW (%)	NRBC (/100WBC)
BE1	Female	24	CD41/42(-TCTT)/ CD26(HbE)	42.2	57.8	4.83	9.0	27.6	57.1	18.6	31.5	3.3
BE2	Female	29	CD17(A>T)/ CD26(HbE)	22.8	77.2	3.97	6.9	20.9	52.6	17.4	37.4	4.9
BE3	Female	30	CD41/42(-TCTT)/ CD26(HbE)	65.6	34.4	2.73	6.2	19.7	72.2	22.7	33.1	5.4
BE4	Male	30	CD17(A>T)/ CD26(HbE)	27.7	72.3	3.23	7.0	22.5	71.0	22.1	33.0	217.6
BE5	Male	20	CD71/72(+A)/ CD26(HbE	39.5	60.5	4.02	7.6	23.5	58.5	18.9	32.7	5.5
BE6	Male	32	CD41/42(-TCTT)/ CD26(HbE)	18.1	81.9	3.39	6.1	20.9	61.7	18.0	34.5	51.1
BE7	Female	24	CD17(A>T)/ CD26(HbE)	37.9	62.1	2.93	7.2	21.3	72.7	24.6	22.1	3.8

All patients were negative for four deletional α-thalassemias including $-\alpha^{3.7}$, $-\alpha^{4.2}$, $--^{SEA}$, and $--^{THAI}$.
* *HBB* was genotyped by Sanger sequencing;
† HbA_2 and HbE were coeluted and unable to be separated by HPLC;
‡ Hb was measured at steady state or prior an upcoming transfusion;Hb, hemoglobin; RBC, red blood cell; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; NRBC, nucleated red blood cell; WBC, white blood cell

Table S2 Globin chain analysis of erythroid progenitor cells from β^0-thalassemia/HbE patients after IOX1 treatments.

Sample	Treatment	β^E-globin (AU)	α-globin (AU)	Gγ-globin (AU)	Aγ-globin (AU)
BE1	DMSO 0.1% v/v	34.59	55.62	4.06	5.73
	IOX1 1 μM	35.62	57.34	2.98	4.06
	IOX1 5 μM	36.51	57.27	2.49	3.73
BE2	DMSO 0.1% v/v	31.49	52.16	8.87	7.48
	IOX1 1 μM	34.45	52.25	6.90	6.40
	IOX1 5 μM	35.38	53.12	5.92	5.58
BE3	DMSO 0.1% v/v	32.12	49.38	10.33	8.17
	IOX1 1 μM	33.15	49.51	10.17	7.17
	IOX1 5 μM	35.11	49.23	8.97	6.69
BE4	DMSO 0.1% v/v	31.62	54.85	7.20	6.33
	IOX1 1 μM	30.95	55.86	7.04	6.15
	IOX1 5 μM	34.42	54.84	5.91	4.83
BE5	DMSO 0.1% v/v	31.97	56.68	6.52	4.83
	IOX1 1 μM	34.97	54.49	5.90	4.64
	IOX1 5 μM	34.35	56.25	5.73	3.67
BE6	DMSO 0.1% v/v	28.02	60.59	6.68	4.71
	IOX1 1 μM	30.61	58.71	6.56	4.12
	IOX1 5 μM	32.60	59.51	4.38	3.51
BE7	DMSO 0.1% v/v	27.66	57.50	6.98	7.86
	IOX1 1 μM	28.78	56.61	6.57	8.04
	IOX1 5 μM	30.54	57.35	5.41	6.70

Table S3 Hemoglobin analysis of erythroid progenitor cells from β^0-thalassemia/HbE patients after IOX1 treatments.

Sample	Treatment	HbF (%)	HbA_2E (%)
BE1	DMSO 0.1% v/v	29.7	70.3
	IOX1 1 μM	23.5	76.5
	IOX1 5 μM	18.6	81.4
BE2	DMSO 0.1% v/v	28.1	71.9
	IOX1 1 μM	24.6	75.4
	IOX1 5 μM	21.8	78.2
BE3	DMSO 0.1% v/v	34.4	65.6
	IOX1 1 μM	33.2	66.8
	IOX1 5 μM	31.6	68.4
BE4	DMSO 0.1% v/v	23.1	76.9
	IOX1 1 μM	20.6	79.4
	IOX1 5 μM	17.4	82.6
BE5	DMSO 0.1% v/v	22.7	77.3
	IOX1 1 μM	21.5	78.5
	IOX1 5 μM	17.4	82.6
BE6	DMSO 0.1% v/v	23.3	76.7
	IOX1 1 μM	21.4	78.6
	IOX1 5 μM	19.9	80.1
BE7	DMSO 0.1% v/v	29.4	70.6
	IOX1 1 μM	25.8	74.2
	IOX1 5 μM	23.7	76.3

Table S4 Correlation analysis of the changes between well-known γ-globin regulators and globin gene expression.

	HBA		*HBB*			*HBG*
Correlation	IOX1 1 μM	IOX1 5 μM	IOX1 1 μM	IOX1 5 μM	IOX1 1 μM	IOX1 5 μM
KLF1	-0.143	0.451	0.036	-0.342	0.321	0.505
BCL11A	0.036	-0.036	0.393	0.073	0.321	0.698
LRF	0.829*	0.393	0.649	-0.071	-0.018	0.222
GATA1	0.432	0.214	0.739	0.464	0.288	0.593

*$p = 0.028$

Table S5 Primers used in qPCR.

Target	Primer name	Sequence (5' to 3')
HBA	hHBA-Fwd hHBA-Rev	TGG ACC CGG TCA ACT TCA AG TCA CAG AAG CCA GGA ACT TGT
HBB	hHBB-Fwd hHBB-Rev	GAA GGC TCA TGG CAA GAA AG CAC TGG TGG GGT GAA TTC TT
HBG	hHBG-Fwd hHBG-Rev	TCA CAG AGG AGG ACA AGG CTA GCT TTA TGG CAT CTC CCA AG
KLF1	hKLF1-Fwd hKLF1-Rev	CAC ACA GGG GAG AAG CCA TA GAA AAA GCA CGT GGG CAG AG
BCL11A	hBCL11A-Fwd hBCL11A-Rev	GGG AAT TCT CGC CCG AG GGG AAG TTC ATC TGG CAC T
LRF	hLRF-Fwd hLRF-Rev	CTT CAC CAG GCA GGA CAA GGT TCT TCA GGT CGT AGT TGT G
GATA1	hGATA1-Fwd hGATA1-Rev	CAG GAC AGG CCA CTA CCT AT CTG ACA ATC AGG CGC TTC TT
HIF1A	hHIF1A-Fwd hHIF1A-Rev	TTT GGC AGC AAC GAC ACA GA CGT TTC AGC GGT GGG TAA TG
HIF2A	hHIF2A-Fwd hHIF2A-Rev	CAC CTC GGA CCT TCA CCA CC TCC TCT CCG AGC TAC TCC TTT T
HIF1B	hHIF1B-Fwd hHIF1B-Rev	CAG CCA TTG CCT CTG GAA AC CAT CAA AAT CCA GCC CTG GTC
VHL	hVHL-Fwd hVHL-Rev	ATC CAC AGC TAC CGA GGT CA GGC AAA AAT AGG CTG TCC GTC
FIH	hFIH-Fwd hFIH-Rev	TGA ATC CCA GTT GCG CAG TTA T CAG CAC CAC AGG CTC CTC ATT
EGLN1	hEGLN1-Fwd hEGLN1-Rev	CTG GGA TGC CAA GGT AAG TG TAC TTC ATG AGG GTT GCG ACG
EGLN2	hEGLN2-Fwd hEGLN2-Rev	CTA CTG CGC TCA GAA GGG G CTT CAG GGT GGC CGG AGA
EGLN3	hEGLN3-Fwd hEGLN3-Rev	GCG TCT CCA AGC GAC AC CAA GCC ACC ATT GCC TTA GAC
RPS18	hRPS18-Fwd hRPS18-Rev	GGA TGA GGT GGA ACG TGT CTA GGA CCT GGC TGT ATT TTC