Research article

Genetic modifications of *EGLN1* reactivate HbF production in β⁰-thalassemia/HbE

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ABSTRACT

Reactivation of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) potentially alleviates clinical presentation in β-thalassemia. Prolyl hydroxylase domain enzymes (PHDs) play roles in the canonical oxygen-sensing pathway and maintain the stability of cellular hypoxia-inducible factor α (HIF-α) in response to low oxygen levels or hypoxia. Pharmacological inhibition of PHDs has been shown to increase HbF production in erythroid progenitors derived from healthy donors. Here, we demonstrated the relationship between PHD2, the main PHD isoform, and clinical phenotypes in β⁰-thalassemia/HbE disease. Although the targeted sequencing annotated several common variants within *EGLN1*, the gene encoding PHD2, none of these variants were located in the functional domains of PHD2 and were irrelevant to the clinical phenotypes. CRISPR-mediated *EGLN1* modifications at the functional regions; however, led to significantly reduce PHD2 expression and increase HbF expression levels in severe β-thalassemia erythroblasts. Moreover, these beneficial phenotypes were independent to the two well-known HbF regulators including BCL11A and GATA1. Our findings introduce an additional mechanism for HbF regulation in β-thalassemia and propose that targeting the canonical oxygen-sensing pathway, particularly PHD2 functional domains, might offer a promising therapeutic strategy to β-thalassemia diseases.

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1. Introduction

β-thalassemia is a common autosomal recessive blood disorder caused by mutations within the β-globin gene. Currently, greater than 200 variants are identified with clinical significance according to the quantitative effect on β-globin production [1]. Compound heterozygous β^0-thalassemia with the common structural variant hemoglobin E or β^0-thalassemia/HbE disease is one of the most severe β-thalassemia genotypes worldwide [2–4]. The patients are unable to produce adult hemoglobin (HbA, $\alpha_2\beta_2$) due to absence of the β-globin subunit, resulting in severe chronic anemia. Postnatal reactivation of γ-globin and fetal hemoglobin (HbF, $\alpha_2\gamma_2$) by genetic modifications in *cis* or *trans* of the β-globin gene has been recognized as crucial modifying factors in β-thalassemia major [5–9]. The observations evidenced that the patients with high HbF levels had relatively mild clinical phenotypes. BCL11A is the well-known γ-globin regulator that has proven as effective therapeutic target for β-thalassemia disease [10–13]. However, new potential candidates have been discovered and *in vivo* verified [14,15], suggesting various γ-globin regulatory pathways.

Stress erythropoiesis is defined by the increase of erythroid production typically in respond to anemic or hypoxic stress. Although the precise understanding of this response is unclear, integration of experimental data suggested distinct mechanisms from steady state erythropoiesis [16–18]. Hypoxia is a physiological condition in which oxygen concentration is insufficient at the tissue level. The oxygen-sensing pathway responds to hypoxia by controlling the stability of hypoxia-inducible factor α (HIF-α) subunits, including HIF1-α and HIF2-α [19–22]. At normal oxygen levels, oxygen dependent enzymes known as HIF-specific prolyl hydroxylase domain enzymes (PHDs) hydroxylate HIF-α at the oxygen-dependent degradation (ODD) domain. Hydroxylated HIF-α is consequently ubiquitinated by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and targeted for degradation by the proteasome in the cytoplasm. Decrease of oxygen levels restrains the function of PHDs, leading to the accumulation of HIF1-α and dimerization with HIF1-β in the cytoplasm. HIF-α/β complex relocates toward the nucleus and activates the downstream expression of genes involving in cellular metabolism, angiogenesis, proliferation, and erythropoiesis. Previous studies demonstrated that pharmacologic inhibition of PHDs and genetic repression of VHL increased HbF production through the HIF1-α pathway in non-thalassemia models [23–25], suggesting the association between hypoxia response pathway and HbF regulation.

PHDs consist of three main isoforms including PHD1 encoded from *EGLN2* gene, PHD2 encoded from *EGLN1* gene and PHD3 encoded from *EGLN3* gene. Of these three isoforms, PHD2 is the primary oxygen sensor that abundantly expresses in almost cell type [26–28]. In this study, genotype-phenotype correlations of *EGLN1* and clinical presentations were investigated in β^0-thalassemia/HbE patients. In addition, CRISPR-Cas9 mediated *EGLN1* knockdown and knockout were evaluated in erythroid progenitors derived from β^0-thalassemia/HbE patients. Despite the insignificant relevance of common *EGLN1* variants to HbF levels, disruption of *EGLN1* significantly increased γ-globin and HbF expression in the β^0-thalassemia/HbE. These findings supported the previous studies and indicated that PHD2 plays a role in HbF regulation in β^0-thalassemia/HbE.

2. Materials and methods

2.1. Targeted sequencing

EDTA blood samples were collected from 57 Thai β^0-thalassemia/HbE without common α-thalassemia coinheritance during December 2022 to December 2023 at Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Genotypes were confirmed and described in Supplementary Table S1. Disease severity was classified into mild and severe using the previous scoring system [29]. Genomic DNA was extracted using Genomic DNA Mini Kit (Geneaid, New Taipei City, Taiwan) and was estimated for the concentration using Qubit dsDNA BR Assay Kit (Invitrogen, Eugene, OR). PCR were set to amplify coding sequences within five exons of human *EGLN1* gene using Platinum *Taq* DNA Polymerase (Invitrogen, São Paulo, Brazil) with 5%v/v DMSO supplementation for the exon 1 and Quick Taq HS DyeMix (Toyobo, Osaka, Japan) for the rest, and specific primers as listed in Supplementary Table S2. Amplicons were purified using GenepHlow Gel/PCR Kit (Geneaid) and the sequences were verified by Sanger DNA sequencing (1st BASE, Selangor, Malaysia). Of these amplicons, the exon 1 was analyzed using difficult template sequencing. To confirm the *cis* inheritance of Tibetan variant, purified amplicon was cloned into pLB vector using Lethal Based Fast Cloning Kit (Tiangen, Beijing, China) following the manufacturer's protocol. Plasmid DNA were purified from a total of six transformants using Presto Mini Plasmid Kit (Geneaid) and the inserted fragment was verified by Sanger DNA sequencing. Data were analyzed against reference human *EGLN1* gene (NC_000001.11: 231,363,756–231,422,287). *Xmn*I polymorphism in the *HBG2* promoter ($-158C > T$, rs7482144) was genotyped using Platinum *Taq* DNA Polymerase and primers listed in Supplementary Table S2 and *Xmn*I restriction enzyme (Vivantis, Selangor, Malaysia) following the principle of restriction fragment length polymorphism (RFLP). Functional relevance of *EGLN1* variants was *in silico* predicted by three different algorithms including Polymorphism Phenotyping-2 (PolyPhen-2) [30], Sorting Intolerant from Tolerant (SIFT) [31] and Protein Variation Effect Analyzer (PROVEAN) [32].

2.2. Erythroid cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from ACD preserved blood sample using Lymphoprep (Serumwerk, Bernburg, Germany) and gradient centrifugation. Hematopoietic stem/progenitor cells (HSPCs) were purified from PBMCs using CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and were cultured following the previous protocol [33] with some modifications. Briefly, 48 h before transfection, HSPCs were cultured in expansion medium consisting of X-VIVO 15 (04-418Q; Lonza, Walkersville, MD) supplemented with 100 ng/ml recombinant human stem cell factor (SCF; Peprotech, Rehovot, Israel), 100 ng/ml recombinant human Flt3-Ligand (Flt3-L; Peprotech), 100 ng/ml recombinant human thrombopoietin (TPO; Peprotech), 2 mM

GlutaMAX (Gibco, Grand Island, NY), and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). Cell viability was estimated by 0.4 % trypan blue and should be greater than 80 % at the day of transfection. A total of 2×10^5 HSPCs were transfected with Cas9-sgRNA ribonucleoprotein and maintained in the expansion medium for 48 h. HSPCs were subsequently transferred into erythroid differentiation medium (EDM) consisting of IMDM (SH30259.02; Cytiva, Logan, UT) supplemented with 5%v/v male AB serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM GlutaMAX, 330 μg/ml human holo-transferrin (Prospec-Tany, Ness Ziona, Israel), 10 μg/ml recombinant human insulin (Prospec-Tany), 2 IU/ml heparin (Leo, Ballerup, Denmark) and 3 IU/ml erythropoietin (Cilag AG, Schaffhausen, Switzerland). To induce erythroid differentiation and maturation, HSPCs were culture in 3-phase erythroid culture media for 16 days. During phase I (EDM I; days 0–7), EDM was supplemented with 1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO), 100 ng/ml SCF and 5 ng/ml recombinant human IL-3 (IL-3; Peprotech). During phase II (EDM II; days 7–11), EDM was supplemented with only 100 ng/ml SCF. During phase III (EDM III; days 11–16), EDM had no additional supplements. Culture condition was maintained at 37 °C with 5 % CO_2 in a humidified atmosphere.

2.3. Cas9 production

pET-21a_3xNLS_SpCas9_protein_expression was provided by Scot Wolfe (#114365; Addgene, Watertown, MA) and transformed into *E. coli* Rosetta 2(DE3) (#70954; Novagen, Darmstadt, Germany). SpCas9 expression was induced by IPTG for overnight. Bacterial cell pellet was harvested and lysed using Vibra-Cell VCX 500 sonicator (Sonics & Materials, Newtown, CT). Cell lysate was filtered through a 0.22 μm PES membrane and total proteins were purified using the ÄKTA Pure 25 M1 FPLC system (GE Healthcare, Uppsala, Sweden). Briefly, the His-tagged SpCas9 was purified on a HisTrap HP column (#17524802; GE Healthcare). Peak fractions were collected, filtered through a 0.22 μm PES membrane, and loaded into a HiTrap SP High Performance cation exchange column (#17115201; GE Healthcare). The protein was eluted with ion exchange buffer containing increasing salt gradient. Fractions containing SpCas9 protein were combined and concentrated using Amicon Ultra-15 Centrifugal Unit with 30 kDa cutoff (#UFC903024; Merck, Darmstadt, Germany) following the manufacturer's protocol. The protein solution was supplemented with 30 % v/v glycerol and 2 mM DTT, and stored at −80 °C.

2.4. Ribonucleoprotein transfection

Chemically modified single guide RNAs (sgRNAs) targeting human *EGLN1* including sgEGLN1#1: CGUCAUGUUGAUAAUCCAAA and sgEGLN1#2: GUACUUCAUGAGGGUUGCGA were *in silico* designed using http://crispor.tefor.net and ordered from Synthego (Redwood City, CA). In addition, a chemically modified sgRNA targeting human adeno-associated virus integration site 1, sgAAVS1: CUCCCUCCCAGGAUCCUCUC, was used as a non-target control. sgRNA (200 pmol) and Cas9 (200 pmol) were prepared as a ribonucleoprotein complex prior to electroporation into cells using P3 Primary Cell 4D X Kit and DZ100 program on 4D-Nucleofector X Unit (Lonza, Cologne, Germany).

2.5. Editing efficiency

Genomic DNA was extracted from a total of 1×10^6 cells at culture day 11. PCR were set to amplify regions of interest using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems, Wilmington, MA) and specific primers as listed in Supplementary Table S2. Amplicon was purified and the sequences were verified by Sanger DNA sequencing. Inference of CRISPR Edits (ICE) algorithm [34] was used to determine editing outcomes of sgEGLN1#1 and sgEGLN1#2 against sgAAVS1. To estimate the editing efficiency of the dual gRNA, PCR reactions were set to amplify three different regions within the editing windows using specific primers as listed in Supplementary Table S2. Amplicons were investigated by agarose gel electrophoresis and the results were analyzed relative to the sgAAVS1 control.

2.6. Erythroid phenotyping

Flow cytometry and Wright-Giemsa staining were used to examine erythroid differentiation and erythroid morphology. At least 1×10^5 cells were harvested and washed with DPBS for each experiment. For the flow cytometry, cells were stained with 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 protocol. Flow cytometry was performed using DxFLEX Flow Cytometer (Beckman Coulter, Indianapolis, IN) and data were analyzed using FlowJo software version 10.7 (FlowJo LLC, Ashland, OR). For the Wright-Giemsa staining, cells were captured on a glass slide using Cytospin 3 (Thermo Shandon, Cheshire, UK) at 600 rpm for 4 min and stained with diluted Wright-Giemsa stain for 30 min. Cell morphology was investigated and captured under a microscope (Leica DM750, Leica Microsystems, Heerbrugg, Switzerland).

2.7. Quantitative real-time PCR

Total RNA were extracted from at least 5×10^5 cells at culture day 14 using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). At least 500 ng of total RNA were reverse transcribed to cDNA using FastKing RT Kit (Tiangen) according to the manufacturer's protocol. Quantitative real-time PCR was carried out in duplicate on CFX Opus 96 Real-Time PCR System (Biorad, Hercules, CA) using specific primers as listed in Supplementary Table S2 and FastReal qPCR PreMix (Tiangen) according to the manufacturer's protocol. Gene

expression was normalized to the *GAPDH* and relatively compared with the sgAAVS1 using $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot analysis

At least 5×10^6 cells at culture day 14 were harvested and washed with cold DPBS. Cell pellet was lysed with 200 μl RIPA buffer supplemented with protease inhibitors. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. A total of 20 μg protein was mixed with loading buffer and was boiled at 95 °C for 5 min. Proteins were separated by size using 12 % SDS-PAGE and were transferred to 0.45 μm Immobilon-P PVDF membrane (Merck Millipore, Cork, Ireland) at 240 mA for 90 min. Transferred membrane was blocked with 5 % skim milk in PBS-T buffer and was incubated with primary antibodies including rabbit anti-human PHD2 (#4835; Cell Signaling, Danvers, MA), rabbit anti-human GATA1 (#4591; Cell Signaling), mouse anti-human BCL11A (ab19487; Abcam, Cambridge, MA) and rabbit anti-human GAPDH (ABS16; Merck). HRP-conjugated secondary antibodies including goat anti-rabbit IgG (W401B; Promega, Madison, WI) and goat anti-mouse IgG (W402B; Promega) were probed against the primary antibodies. Chemiluminescent detection was carried out by Immobilon Forte Western HRP Substrate (EMD Millipore, Burlington, MA). X-ray film photography was developed using Polycon developer and Fixaplus fixer (Champion, Kuala Lumpur, Malaysia) according to the manufacturer's protocol. Target proteins were visualized based on their size on the X-ray photography.

2.9. Hemoglobin analysis

Qualitative and quantitative analysis of hemoglobin were performed following the principle of cation exchange HPLC with VARIANT II β-Thalassemia Short Program (Biorad). Briefly, cells (at least 1×10^6) were harvested at culture day 16 and were washed with cold DPBS. Cell pellet was lysed with 500 μl Wash/Diluent Solution (Biorad) and the hemolysate was injected to the system for investigations.

2.10. Statistical analysis

Data were statistical analyzed using unpaired Student's *t*-test by GraphPad Prism version 8.2.0 (GraphPad Software, San Diego, CA) and presented as mean ± standard deviation (SD). *P*-value of less than 0.05 ($P < 0.05$) was considered as a statistical significance.

3. Results

3.1. Naturally occurring variants of EGLN1 have neutral effects in β^0-thalassemia/HbE

Inherited secondary erythrocytosis is a rare disease defined as an increase in red blood cell (RBC) mass due to the overproduction of erythropoietin. This mainly caused by mutations in genes involved in the canonical oxygen-sensing pathway [35–37]. To date, there are at least 10 natural *EGLN1* variants has been identified in patients with erythrocytosis [38,39]. To investigate the association between *EGLN1* variants, RBC parameters and clinical phenotypes in β-thalassemia diseases, *EGLN1* targeted sequencing was performed in a total of 57 Thai β^0-thalassemia/HbE who had no α-thalassemia coinheritance (Supplementary Table S1). We classified these patients into severe (n = 50) and mild (n = 7) according to the previous scoring system [29]. The results revealed three *EGLN1*
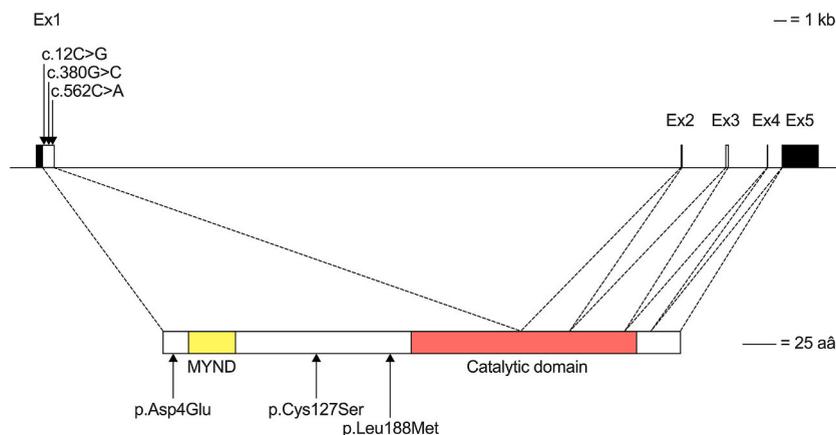

Fig. 1. Targeted sequencing reveals three PHD2 variants in 57 β^0-thalassemia/HbE patients. Schematic diagram representing the human *EGLN1* gene (58,532 bp; NC_000001.11: 231,363,756–231,422,287) and PHD2 protein (426 aâ; UniProtKB: Q9GZT9). MYND, MYND zinc finger domain; Catalytic domain, ferrous iron and 2-oxoglutarate (2OG)-dependent HIF prolyl hydroxylase domain. Arrow lines indicate variants identified in this study.

variants including rs186996510 (*EGLN1*: c.12C > G, p.Asp4Glu), rs12097901 (*EGLN1*: c.380G > C, p.Cys127Ser), and rs755928135 (*EGLN1*: c.562C > A, p.Leu188Met) (Fig. 1 and Supplementary Fig. S1). These missense variants were identified in 42 individuals (39 severe and 3 mild), and contributed to four different genotypes which were heterozygous for c.380G > C (29 severe and 3 mild), homozygous for c.380G > C (7 severe), heterozygous for c.562C > A (1 severe) and compound heterozygous for c.12C > G with c.380G > C (2 severe). The later genotype was confirmed as heterozygous for *cis* inheritance or Tibetan variant (*EGLN1*: c.12C > G; 380G > C, p.Asp4Glu; p.Cys127Ser) (Supplementary Figs. S2–S3). In this population, the minor allele frequency (MAF) of rs12097901, rs755928135 and Tibetan variant was 0.403, 0.009 and 0.017, respectively. These MAF were consistent with data from Genome Aggregation Database (gnomAD) and Allele Frequency Aggregator (ALFA) in which the allele frequency of c.380G > C and c.562C > A was common (gnomAD = 0.447 and ALFA = 0.464) and rare (gnomAD <0.001 and ALFA <0.001) in Asian population, respectively. Additionally, the allele frequency of Tibetan variant was common (MAF = 0.021) in Asian population and significantly increased (MAF = 0.808) in Tibetan highlanders [40]. All of the variants identified in this study were located outside the two main PHD2 functional regions, MYND zinc finger and catalytic domain. *In silico* functional prediction suggested that c.380G > C and c.562C > A were neutral variants (Supplementary Table S3). Homozygous Tibetan variant was previously described to modify the oxygen binding to the PHD2 [40]. This gained the PHD2 function and impaired erythroid proliferation through the degradation of HIF-α under hypoxic conditions. However, heterozygous Tibetan variant reacted relatively normal to wild-type PHD2, suggesting neutral phenotypes in heterozygote. Although these variants were mostly identified in the severe β^0-thalassemia/HbE, hematological parameters were insignificant difference between individuals (Supplementary Table S1).

3.2. PHD2 disruptions induce HbF production in β^0-thalassemia/HbE

PHD2 is the main PHD that plays role in oxygen-sensing pathway [26–28]. Previous studies demonstrated that pharmacological inhibition of PHDs increased HbF levels in non-thalassemia models [23,24]. Therefore, PHD2 may serve as a promising target for inducing HbF in β-thalassemia disease. To investigate the effect of PHD2 suppression in severe β-thalassemia model, CD34$^+$ HSPCs derived from three independent severe β^0-thalassemia/HbE were transfected with either AAVS1 or *EGLN1* specific sgRNA, and Cas9 as ribonucleoprotein complex. These patients had different *HBB* genotype and HbF baseline (Supplementary Table S4). ICE analysis revealed that the %indel of sgAAVS1, sgEGLN1#1 and sgEGLN1#2 was 85.7 ± 17.9, 96.7 ± 0.6 and 85.0 ± 11.1 (Fig. 2A and Supplementary Fig. S4), respectively. *EGLN1* mRNA and PHD2 protein expression were relatively decreased when compared to the sgAAVS1 (Fig. 2B and C; Supplementary Fig. S7), confirming efficiency of these sgRNAs. Moreover, sgEGLN1#1 and sgEGLN1#2 mediated PHD2 suppression relatively increased *HBG* (γ-globin) mRNA (sgEGLN1#1 = 1.3 ± 0.1-fold and sgEGLN1#2 = 1.0 ± 0.1-fold) and HbF levels (sgEGLN1#1 = 10.4 ± 3.7 % and sgEGLN1#2 = 2.8 ± 1.2 %) when compared to the sgAAVS1 (Fig. 3A–C). We also assessed the additional effects of co-transfection using sgEGLN1#1 and sgEGLN1#2 (dual gRNA) in the CD34$^+$ HSPCs. The results showed that the dual gRNA induced a large deletion with 93.6 ± 1.4 % (Supplementary Figs. S5–S6). Furthermore, *EGLN1* mRNA and PHD2 protein expression levels decreased to nearly undetectable (Fig. 2B and C; Supplementary Fig. S7), indicating knockout genotype. However, elevated *HBG* mRNA (1.4 ± 0.3-fold) and HbF levels (10.3 ± 2.1 %) observed after dual gRNA treatment were comparable to the sgEGLN1#1 (Fig. 3A–C). These findings supported the efficacy of sgEGLN1#1 in HbF reactivation in severe β^0-thalassemia/HbE erythroblasts. We next determined the effects of PHD2 disruption on erythroid differentiation using flow cytometry and Wright-Giemsa staining. The results revealed that PHD2 manipulations using sgEGLN1#1 and dual gRNA slightly delayed erythroid differentiation as shown by a relative decrease of the CD71^{Low}/GPA^{High} cell population (Fig. 4A) and late-stage erythroid cells (Fig. 4B). Although recent insights have described many transcription factors involving in HbF regulation, functional relevance to the PHD2 is unknown. We then investigated the expression levels of the two well-known HbF regulators, BCL11A
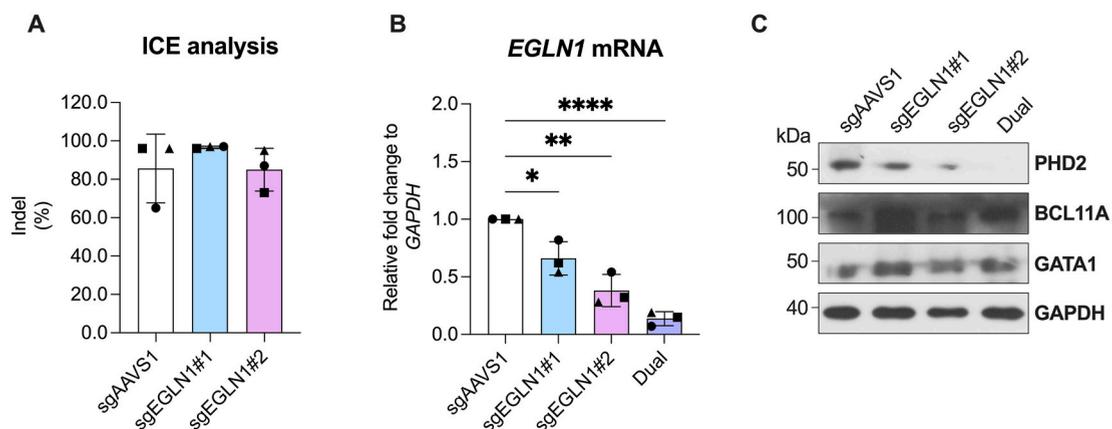
Fig. 2. EGLN1-specific gRNA mediated PHD2 knockdown. (A) Editing efficiency of sgRNAs used in this study. (B) Quantitative real-time PCR demonstrating the relative *EGLN1* mRNA expression normalized to the *GAPDH* mRNA expression at day 14 of culture. (C) Representative western blot analysis showing the expression of PHD2, BCL11A, GATA1 and GAPDH at day 14 of culture. Data are presented as mean ± SD. *, $P < 0.05$; **, $P < 0.005$; ****, $P < 0.0001$.
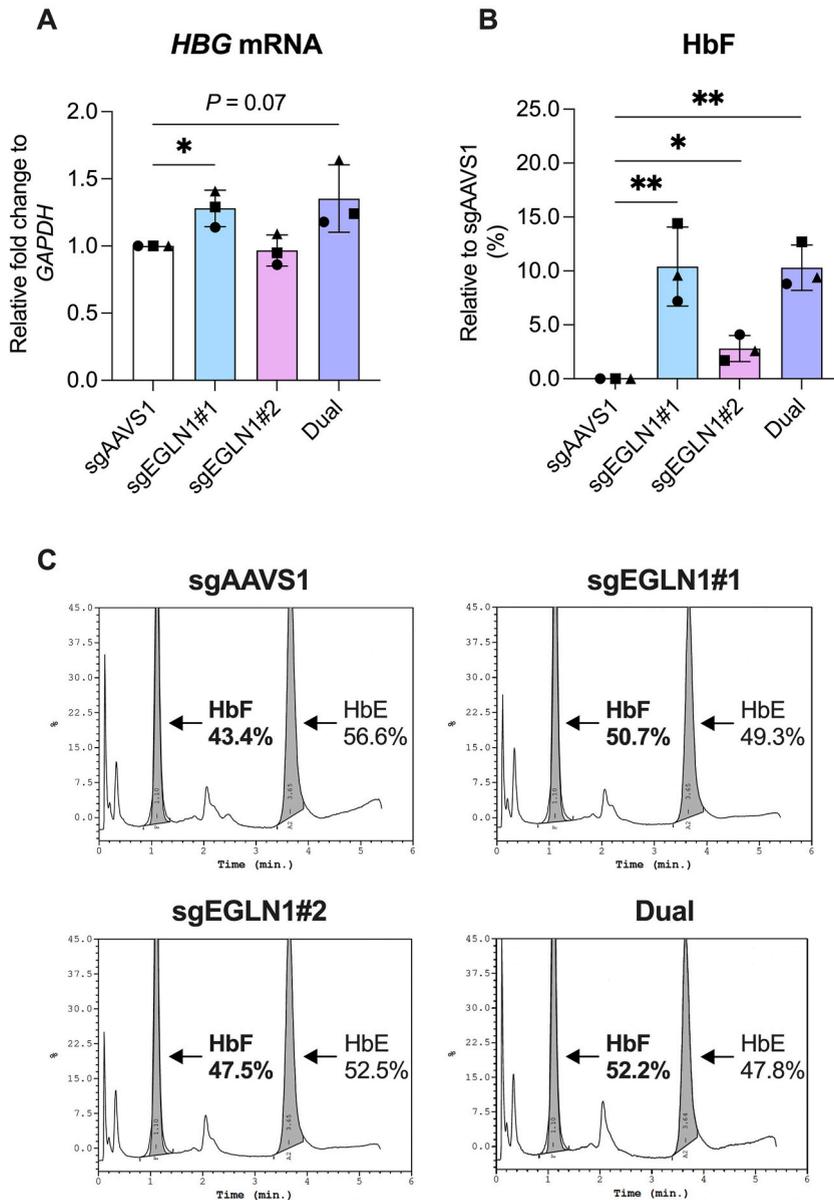
Fig. 3. HbF induction after PHD2 manipulations. (A) Quantitative real-time PCR demonstrating the relative *HBG* mRNA expression normalized to the *GAPDH* mRNA expression at day 14 of culture. (B) Relative HbF levels compared to the control (sgAAVS1) at day 16 of culture. (C) Representative hemoglobin analysis by HPLC. Data are presented as mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.005$.

[10] and GATA1 [41], after experimental downregulation of PHD2. The results showed that the γ-globin repressor, BCL11A, was noticeably upregulated after PHD manipulations using sgEGLN1#1 and dual gRNA, while the γ-globin activator, GATA1, was unchanged (Fig. 2C and Supplementary Fig. S7). Previous study demonstrated that BCL11A expression level was gradually decreased upon erythroid differentiation in the 3-phase erythroid culture media [33]. Therefore, the upregulation of BCL11A in sgEGLN1#1 and dual gRNA may be due to delayed erythroid differentiation and the accumulation of early-stage erythroid cells.

4. Discussion

Postnatal induction of γ-globin and HbF expression has a significant modifying effect on the disease severity of β-thalassemia diseases, leading to milder clinical presentations. Increased γ-globin chains compensate for the defective β-globin chains through the production of HbF and, consequently, decrease the relative excess of α-globin chains in β-thalassemia. Molecular strategies aimed at inhibiting BCL11A have shown promise in clinical studies for increasing HbF levels and ameliorating the clinical symptoms of β-thalassemia diseases. However, new potential HbF regulators have been discovered and suggest independent regulatory mechanisms
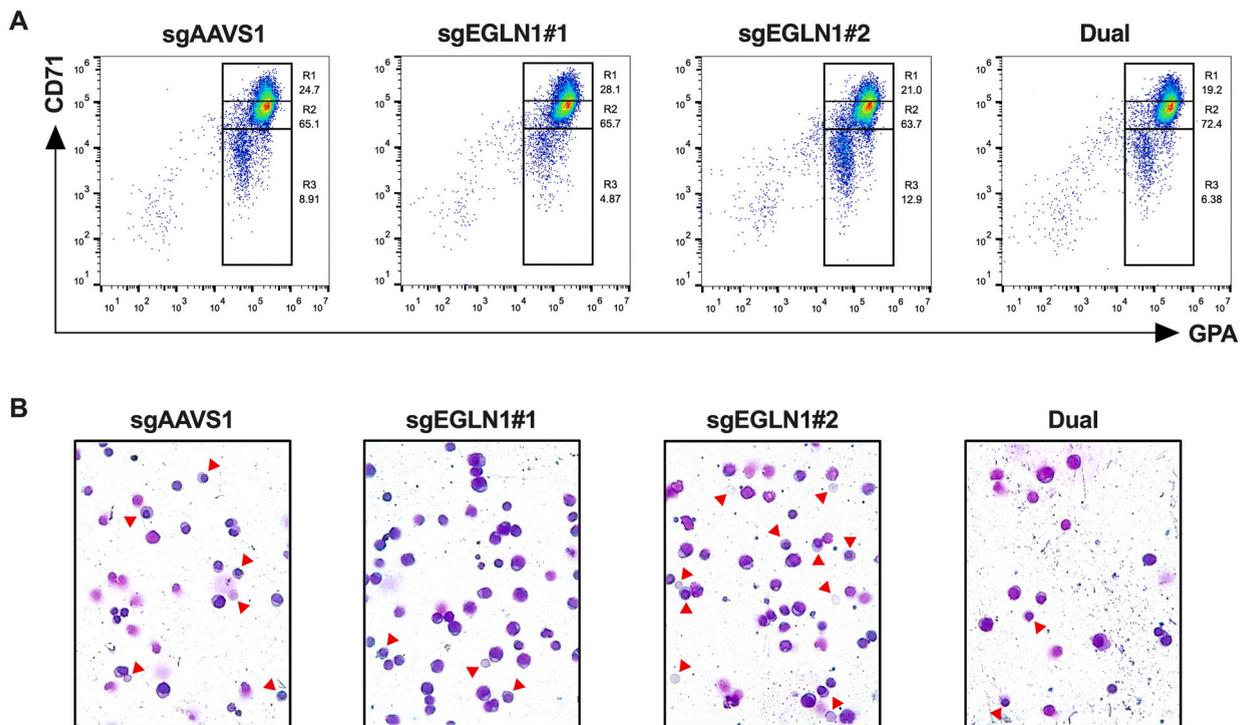
Fig. 4. Effects of PHD2 manipulations on erythroid maturation. (A) Representative erythroid differentiation analysis by flow cytometry at day 14 of culture. (B) Representative images of β^0-thalassemia/HbE erythroid progenitor cells obtained from cytocentrifugation and Wright-Giemsa staining at day 14 of culture. Arrows indicate late-stage erythroid cells.

to BCL11A. Disruption of key factors in the canonical oxygen-sensing pathway, including PHDs and VHL, has shown to significantly induce HbF production in erythroid progenitors derived from non-thalassemia individuals by maintaining the stability of HIF-α and activating γ-globin expression. Moreover, natural variants of effectors involving the canonical oxygen-sensing pathway were associated with secondary erythrocytosis, suggesting the linkage of the canonical oxygen-sensing pathway, HbF regulation, and increased erythropoiesis.

In this study, we demonstrated the function of PHD2 in β-thalassemia using targeted sequencing and experimental gene silencing. The main pathophysiology of β-thalassemia is caused by imbalanced globin synthesis, leading to ineffective erythropoiesis due to the accumulation of excess α-globin chains. Therefore, efforts to reduce these excess α-globin chains and improve ineffective erythropoiesis are of substantial interest for treating β-thalassemia. Previous studies revealed that PHD2 variants are associated with increased red blood cell production [38,39]. Thus, these variants may be considered as ameliorating factors for β-thalassemia. Our findings; however, revealed that the naturally occurring variants of PHD2 did not associate with red blood cell parameters in β-thalassemia. Although the frequency of minor alleles found in this study was comparable to the global population variation database, some rare or novel alleles may have been missed because of the relatively small sample size. Further investigation integrating large-scale cohorts to address *EGLN1* variations in β-thalassemia would provide great value for studying genotype-phenotype interactions.

Since the functional significance *EGLN1* variants were not identified, we then investigated the effects of PHD2 inhibition on HbF induction by CRISPR-mediated *EGLN1* gene silencing in erythroid progenitor cells derived from β^0-thalassemia/HbE. We intentionally designed sgEGLN1#1 and sgEGLN1#2 to target the catalytic relevant dioxygenase domain of PHD2. However, sgEGLN1#1 was specific to the sequence encoding a β4β5 connecting loop and supposed to interfere the HIF-1α binding while sgEGLN1#2 was specific to the sequence encoding a putative polar binding surface and supposed not to interfere the HIF-1α binding [38,42–44]. Manipulations of PHD2 were capable of inducing HbF expression in β-thalassemia regardless of *HBB* genotype and HbF baseline levels. Of these two sgRNAs, sgEGLN1#1 was the most effective in stimulating HbF production. Although dual transfection of sgEGLN1#1 with sgEGLN1#2 revealed knockout effects, this combinatorial treatment did not provide additive effects on HbF induction, suggesting the dominant effects of sgEGLN1#1. However, modest retardation of erythroid differentiation was noticed after PHD2 inhibition using sgEGLN1#1 alone or in combination with sgEGLN1#2. This finding corresponded to the previous study in which a loss-of-function PHD2 mutation impaired erythroid colony formation [40]. While these findings suggest that the increased HbF observed after PHD2 manipulations may be affected by the stage of erythroid differentiation, the expression of key γ-globin regulators, including BCL11A and GATA1, remained relatively unchanged. This indicated that the induction of γ-globin and HbF synthesis was independent to BCL11A and GATA1, and was not caused by delayed erythroid differentiation. Similarly, pharmacological inhibition of pan-PHD using the FG4592 compound relatively increased HbF levels to approximately 5 % in normal erythroid progenitors compared to the control [24]. Interestingly, additive effects on HbF reactivation were observed with cotreatment using hydroxyurea, suggesting

additional HbF regulatory pathways beyond those of hydroxyurea. HIF1-α is the downstream effector of PHDs, where inhibition of PHDs leads to the accumulation of HIF1-α in erythroid progenitor cells. HbF induction capacity was suspended when FG4592 was applied to HIF1-α compromised erythroid progenitor cells. The previous study also showed that increased HIF1-α activated *BGLT3* long non-coding RNA and remodeled the β-globin gene cluster to activate *HBG* gene expression [24]. These findings were comparable to our study, in which downregulation of the main cellular PHD isoform, PHD2, reactivated HbF production in erythroid progenitor cells derived from β-thalassemia. However, our study did not dissect the downstream effectors of PHD2, which may demonstrate different pathways due to the relatively high HbF baseline in β-thalassemia. Therefore, the regulation of HbF through PHD2 in β-thalassemia warrants further investigation and may enhance the understanding of unclear HbF regulatory mechanisms in humans.

Although the genetic inhibition of PHD2 led to significantly elevated HbF production compared to the control, the incremental HbF was approximately 10 % of the total hemoglobin, which is less than that achieved by the manipulation of BCL11A [10]. However, clinical findings have demonstrated that the induction of HbF to at least 10 % of the total hemoglobin can ameliorate clinical presentations in β-thalassemia patients [13,14]. In addition to the increased HbF levels, prolonged elevation of total hemoglobin is a crucial factor in substituting for anemia in β-thalassemia patients. Nowadays, combinatorial therapy is an alternative approach for curing many blood disorders. The complementary combination of PHD2 suppression and other potential compounds to induce HbF and total hemoglobin may be considered and requires further investigation to achieve beneficial outcomes for treating β-thalassemia.

In conclusion, this study provides empirical evidence that common *EGLN1* variants did not affect clinical phenotypes in β^0-thalassemia/HbE patients. Nonetheless, *in vitro* downregulation of a key regulator in the canonical oxygen sensing pathway, PHD2, exhibits HbF reactivation in β^0-thalassemia/HbE erythroid progenitors. Taken together, pharmacologic or genetic inhibition of PHD2 may hold an alternative therapeutic option for β-thalassemia.

Data availability

Data is provided within the manuscript or supplementary information files.

Ethical approval

This research project was approved by Faculty of Medicine, Chiang Mai University (PED-2565-09152). Blood sample was collected after obtaining a written informed consent. Research involving human research participants was performed in accordance with the Declaration of Helsinki.

Funding information

CRediT authorship contribution statement

Varit Jan-ngam: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Siriraj Boontha:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alisa Tubsuwan:** Writing – review & editing, Resources, Methodology. **Somsakul Pop Wongpalee:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Kanda Fanhchaksai:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Data curation. **Adisak Tantiworawit:** Writing – review & editing, Resources. **Pimlak Charoenkwan:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Pinyaphat Khamphikham:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38020.

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