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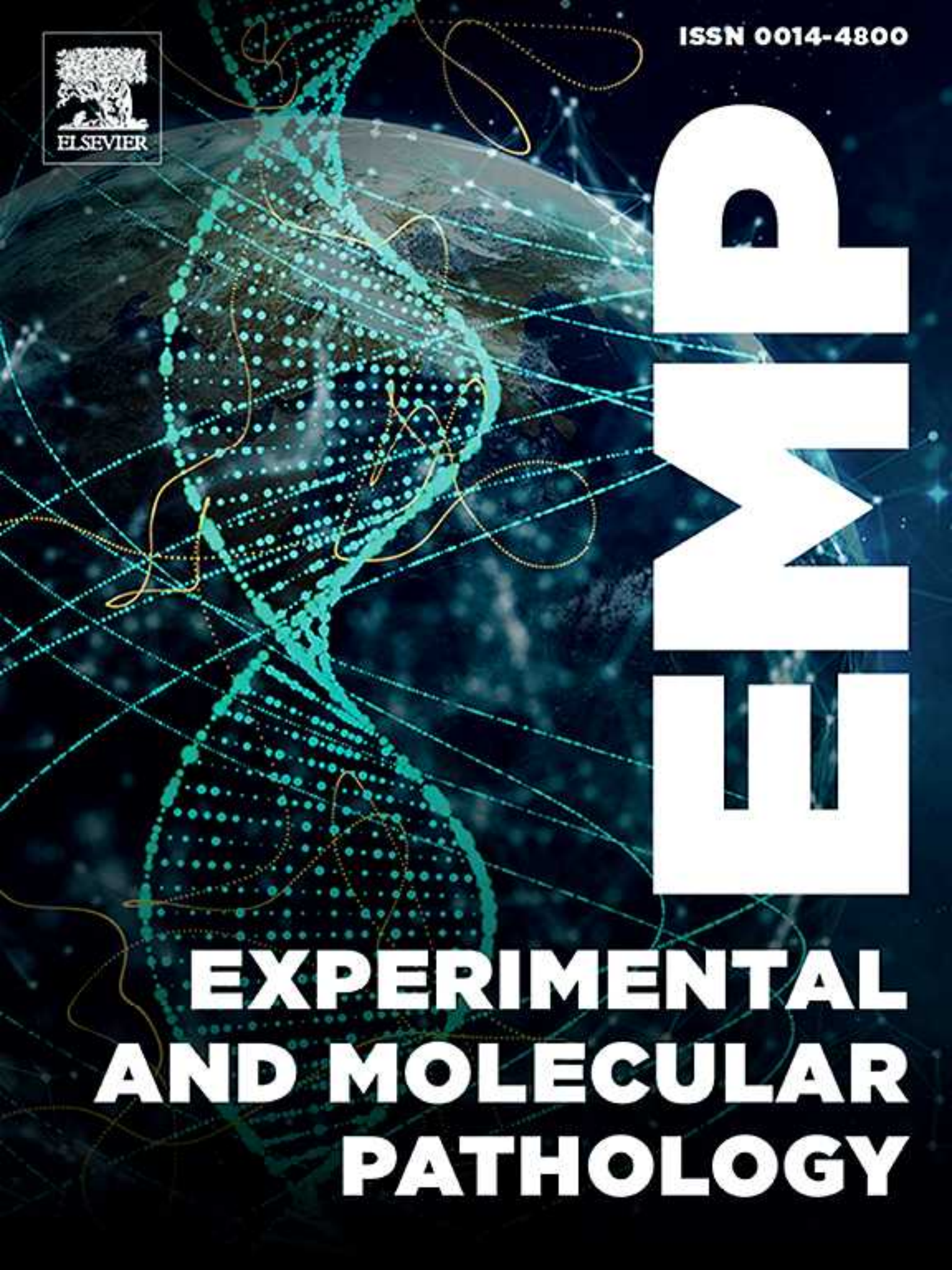

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Metabolic reprogramming during ineffective erythropoiesis in β-thalassemia/HbE disease

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ABSTRACT

Ineffective erythropoiesis, the main cause of anemia in β-thalassemia disease, is characterized by dramatic expansion of erythroblasts and increased erythroblast cell death. The absence or reduction of β-globin chains causes an accumulation of excess α-globin chains and generates cytotoxic reactive oxidant species, resulting in erythroblast cell death. Metabolism provides energy, building blocks for macromolecule synthesis, and cofactors for antioxidative defense systems. We hypothesized that β-thalassemia erythroblasts might alter their metabolism to cope with increased proliferation and cellular stress. Herein, transcriptomic analysis of basophilic and polychromatic erythroblasts isolated from bone marrow obtained from β-thalassemia/HbE patients showed the global up-regulation of metabolic genes in glycolysis, TCA cycle, pentose phosphate pathway, ATP, and fatty acid synthesis pathway. The expression of metabolic genes during terminal erythropoiesis was further determined by PCR array and RT-qPCR in erythroblast culture obtained from β-thalassemia/HbE patients with mild and severe symptoms. The increased expression of enolase1, isocitrate dehydrogenase 1, and bisphosphoglycerate mutase was observed in mild cases compared to severe patients, suggesting that mild patients might modulate metabolic flux for cellular stress defense mechanisms, reducing disease severity. Moreover, the role of BPGM in regulating erythroid differentiation was demonstrated in K562 cells. Inhibition of BPGM promotes cell differentiation in K562 cells. Understanding metabolic reprogramming in thalassemia erythropoiesis opens new therapeutic approaches for β-thalassemia/HbE treatment. Further research is needed to explore how metabolism affects ineffective erythropoiesis and supports thalassemic erythroblasts' high proliferation and oxidative stress defense.

1. Introduction

β-Thalassemia/HbE disease, an inherited red blood cells disorder, is caused by mutations at the β-globin gene and results in the reduction or absence of the β-globin chain synthesis. Co-inheritance of β-thalassemia with hemoglobin (Hb) E results in β-thalassemia/HbE the most

Abbreviations: ACO1, Aconitase 1; *ALDOA*, Aldolase A; *ALDOC*, Aldolase C, fructose-bisphosphate; *BPGM*, Bisphosphoglycerate mutase; *ENO1*, Enolase 1; *ENO2*, Enolase 2 (gamma, neuronal); *FH*, Fumarate hydratase; *HK1*, Hexokinase 1; *IDH1*, Isocitrate dehydrogenase 1; *PDK4*, Pyruvate dehydrogenase kinase, isozyme 4; *PFKM*, Phosphofructokinase, muscle; *PGAM2*, Phosphoglycerate mutase 2 (muscle); *PHKG1*, Phosphorylase kinase, gamma 1 (muscle); *PKLR*, Pyruvate kinase L/R; *PRPS1L1*, Phosphoribosyl pyrophosphate synthetase 1-like 1; *PYGM*, Phosphorylase, glycogen, muscle; *SDHD*, Succinate dehydrogenase subunit D; *TALDO1*, Transaldolase 1.

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prevalent β-thalassemia disease in Southeast Asia and comprises about 50 % of β-thalassemia disease globally (Fucharoen and Weatherall, 2012). The clinical symptoms of β-thalassemia/HbE were extremely varied from asymptomatic to requiring regular blood transfusion, with Hb levels ranging from 3 to 13 g/dL (Fucharoen and Weatherall, 2012; Taher et al., 2021). The absence or reduction of β-globin chains in erythroblasts causes accumulation of excess α-globin, which forms cytotoxic intracellular precipitates that lead to oxidative stress, ultimately leading to cell death and ineffective erythropoiesis, a major determinant of anemia. The hallmark of ineffective erythropoiesis in β-thalassemia is a massive increase in erythroblast proliferation, together with increased erythroblast cell death *via* apoptosis and autophagy (Chaichompoo et al., 2022a; Chaichompoo et al., 2022b; Lithanatudom et al., 2010; Lithanatudom et al., 2011; Rivella, 2009). The bone marrow of β-thalassemia patients has increased numbers of erythroblasts, especially basophilic erythroblasts and polychromatic erythroblasts, with a decreased level of later-stage erythroblasts, such as orthochromatic erythroblasts (Chaichompoo et al., 2022a; Mathias et al., 2000). Furthermore, *in vitro* studies showed that the expansion fold of erythroblasts in β-thalassemia/HbE patients was higher than in normal controls (Phannasil et al., 2023; Wannatung et al., 2009). However, the detailed mechanism of ineffective erythropoiesis is still unknown.

Metabolism is required for cell proliferation as it generates ATP and *de novo* synthesis of nucleotides, lipids, and proteins. Metabolic reprogramming is associated with tumorigenesis to support the high demand for cancer cell rapid proliferation (Smith et al., 2016). Erythropoiesis involves a series of differentiation steps beginning with hematopoietic stem cells (HSCs) and erythroid progenitor (erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) differentiation, terminal erythropoiesis, and reticulocyte maturation (Koury and Haase, 2015). During terminal erythroid differentiation, proerythroblasts undergo differentiation into orthochromatic erythroblasts. Metabolic switching occurs in HSCs and differentiated cells. The major metabolic pathway of HSCs was glycolysis before switching to mitochondrial oxidative phosphorylation when cells differentiated (Ito and Ito, 2018). Recently, dynamic changes of protein machinery for metabolic pathways during erythroid progenitor differentiation and terminal erythropoiesis were reported (Gautier et al., 2016; Papoin et al., 2023). However, the metabolic change during thalassemic erythroblast differentiation is not elucidated.

Increased of several proteins involved with glucose metabolism including glycolysis and tricarboxylic acid cycle (TCA) cycle in β-thalassemic erythroblast has been reported (Leecharoenkiat et al., 2011). In addition, fifty mitochondrial proteins were significantly increased in β-thalassemia/HbE erythroblasts compared to normal controls suggesting the mitochondrial change in β^0-thalassemia/HbE disease (Khungwanmaythawee et al., 2016). Moreover, the NADH/NAD^+ ratio in β-thalassemia/HbE erythroblasts was lower than that of normal erythroblasts indicating the increase in the metabolic state of β-thalassemic erythroblasts (Leecharoenkiat et al., 2011). The up-regulation of several metabolic enzymes suggested that metabolic reprogramming may contribute to ineffective erythropoiesis in β-thalassemia/HbE by increase the energy production and macromolecule synthesis required for rapid proliferation.

In this study, the metabolic reprogramming during erythropoiesis in β-thalassemia/HbE disease was determined. The transcriptomics analysis was performed in bone marrow samples obtained from β-thalassemia/HbE patients. PCR array focusing on the glucose metabolic pathway and RT-qPCR were performed in erythroblast culture obtained from β-thalassemia/HbE patients with different clinical severities to determine the metabolic gene expression. Herein, we demonstrated the differential expression of metabolic genes in several pathways, including glycolysis, TCA cycle, Luebering-Rapoport shunt, ATP, and fatty acid synthesis, suggesting the metabolic reprogramming during ineffective erythropoiesis in β-thalassemia/HbE disease.

2. Materials and methods

2.1. Human bone marrow collection and transcriptomics analysis

This study was performed following the Helsinki Declaration and was approved by the Mahidol University Central Institutional Review Board (MU-CIRB 2014/031.1703). Written informed consent was obtained from all individual participants in this study. Hematological data analysis was performed using an automated cell counter (ADVIA 2120, Bayer, Tarrytown, NY). Hemoglobin analysis and quantification were determined using automated hemoglobin cation exchange high-performance liquid chromatography (Bio-Rad Variant II, Bio-Rad Hercules, CA). The β-thalassemia mutations were characterized using reverse dot blot hybridization (Winichagoon et al., 1999). The α-thalassemia deletional mutations (α-thalassemia 2, $-^{3.7}$, and $-^{4.2}$ kb deletion, and α-thalassemia 1, $--^{SEA}$, and $-^{THAI}$ deletion) were genotyped by multiplex GAP-PCR (Chong et al., 2000).

Patients under treatment with hydroxyurea, aspirin, antibiotics, antidepressants, beta-blockers, and anti-platelets were excluded, and no participant had been hospitalized or blood transfused within 4 weeks before sample collection. Human bone marrow samples were collected from 3 β-thalassemia/HbE patients and 3 control subjects into citrate phosphate dextrose adenine-1 solution (CPDA-1) anticoagulant by using the bone marrow aspiration technique. Samples were processed within an hour after aspiration. $CD45^-$erythroblasts from bone marrow samples were isolated using a MACS cell separation system (Miltenyi Biotec, Bergen Gladbach, Germany) following the manufacturer's instructions. The $CD45^-$erythroblasts were stained with fluorochrome-conjugated monoclonal antibodies specific to glycophorin A (GPA, RBC marker), CD45 (leukocyte marker) and CD71 (transferrin receptor as blast cell marker) for sorting the basophilic and polychromatic erythroblasts ($CD71^{bright}GPA^{bright}CD45^-$ erythroblasts) using flow cytometer FACSAria III sorter (Becton Dickison, BD Biosciences, San Jose, CA). All antibodies were purchased from BD Biosciences (Supplementary Table S1). Isolated human bone marrow erythroblasts were stored at −80 °C until transfer to Beijing Genomics Institute (BGI, Shenzhen, China) for RNA extraction, sample quality control test, and transcriptomics analysis by RNA sequencing. After the total RNA extraction and DNase I treatment, magnetic beads with Oligo (dT) were used to isolate mRNA. The mRNA was fragmented into short fragments. Then, cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and resolved with EB buffer for end reparation and the addition of single nucleotide A (adenine). After that, the short fragments were connected with adapters. After agarose gel electrophoresis, the suitable fragments are selected as templates for the PCR amplification. During the QC steps, the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System are used to quantify and qualify the sample library. At last, the library could be sequenced using Illumina HiSeqTM 2000. Bioinformatic analyses were conducted using R program. Principal component analysis (PCA) was performed with the prcomp function (stats package) and visualized using ggplot2. Differential expression and volcano plots were generated using the DESeq2 package (Love et al., 2014). FPKM values were z-transformed, and heatmaps were created with the heatmap.2 function (gplots package). Gene set enrichment analysis (GSEA) was conducted using clusterProfiler (v4.10.1) (Wu et al., 2021), with gene annotation based on the org.Hs.eg.db database.

2.2. Human blood sample collection

To validate the results from bone marrow transcriptomics, the whole blood obtained from healthy and β-thalassemia/HbE patients was collected for PCR array and RT-qPCR. The study design is shown in supplementary Fig. S1. The written informed consent was obtained from all participants recruited in this study. This study was approved by Mahidol University Central Institutional Review Broad (MU-CIRB 2014/

031.1703) and Mahidol University Multi-Faculty Cooperative IRB review (MU-MOU 2022/129.0812). This study recruited ten β-thalassemia/HbE patients (5 mild and 5 severe clinical symptoms) and 5 healthy subjects. The disease severity of β-thalassemia/HbE patients was determined by the scoring system of 6 parameters as described in the previous study, hemoglobin at steady state; age at receiving first blood transfusion; requirement for blood transfusion; size of spleen; age at thalassemia presentation; and growth and development (Sripichai et al., 2008).

2.3. Erythroid cell culture

50 mL of whole blood from normal subjects and 30 mL from mild and severe patients were collected in CPDA-1 anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Lymphoprep solution, density 1.077 g/mL (Axis-Shield, Oslo, Norway). The erythroid progenitor cells were cultured using a two-phase culture system consisting of an expansion phase (Phase I), and a differentiation phase (Phase II), as described in a previous study (Phannasil et al., 2023) and supplementary Fig. S2A. Briefly, the erythroid progenitor cells were expanded in the expansion phase (phase I) by cultured with Iscove's modified Dulbecco's medium (IMDM; Gibco, Carlsbad, CA) supplemented with 30 % fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 50 ng/ml stem cell factor (SCF; Cell Signaling Technology, Danvers, MA), 25 ng/mL interleukin-3 (IL-3; Cell Signaling Technology) and 0.1 U/mL erythropoietin (EPO; Janssen-Cilag, New Brunswick, NJ) at 37 °C with 5 % CO_2 for 7 days. Then, the media was changed to a differentiation phase (phase II) containing IMDM supplemented with 30 % FBS, 0.1 ng/mL IL-3, and 3 U/mL EPO at 37 °C with 5 % CO_2 for 11 days. Erythroblasts in the differentiation phase were collected on days 7, 9, and 11 to determine cell proliferation, differentiation, and gene expression.

2.4. Erythroid cell proliferation analysis

The erythroblasts from β-thalassemia/HbE patients and healthy controls were counted at days 0, 7, 9, and 11 of the differentiation phase culture by using 0.4 % trypan blue (Gibco) to determine the number and cell viability under a light microscope at 100× magnification.

2.5. Erythroid cell differentiation analysis

Stages of erythroid cells at days 0, 7, 9, and 11 of differentiation phase culture were determined by 2 methods; morphological analysis and flow cytometric analysis. In the morphological analysis, 5×10^4 cells were collected and cytospun onto the glass slide, then, stained with Wright-Giemsa solution (BioTechnical, Bangkok, Thailand). One hundred erythroblasts were counted, and the cell stages were identified under a light microscope at 1000× total magnification. Flow cytometric analysis, 1×10^5 erythroid cells were harvested and stained using monoclonal antibodies specific to glycophorin A (GPA, RBC marker), CD45 (leukocyte marker), and CD71 (transferrin receptor as blast cell marker) (Supplementary Table S1) for erythroid classification into 3 stages including basophilic erythroblasts ($CD71^{bright}GPA^{bright}CD45^{-}$ erythroblasts), polychromatic erythroblasts ($CD71^{dim}GPA^{bright}CD45^{-}$ erythroblasts) and orthochromatic erythroblasts ($CD71^{-}GPA^{bright}CD45^{-}$ erythroblasts). The stained erythroblasts were acquired and analyzed by using a BD Accuri C6 plus flow cytometer (BD Bioscience). The diagram of erythroid differentiation gating is shown in Supplementary Fig. S2B.

2.6. RNA extraction

Erythroblasts at the differentiation phase on days 7, 9, and 11 were harvested and RNA extraction was performed using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. RNA concentration was measured by NanoDrop 2000/2000c Spectrophotometers (Thermo Fisher Scientific, Waltham, MA).

2.7. Gene expression profiles using PCR array

The total RNA was extracted from erythroblasts on day 11 at the differentiation phase from 6 β-thalassemia/HbE patients (3 mild and 3 severe cases) and 3 normal controls. The total RNA was converted to cDNA using RT^2 First Strand Kit (Qiagen, cat. No. 330401). The cDNA was mixed with RT^2 SYBR Green qPCR Mastermix (QIAGEN, cat. No. 330500). The metabolic gene expression profiles were determined using an RT^2 Profiler PCR array with a 96-well plate format coated with specific primers and probes of 84 glucose metabolic genes (Qiagen). The PCR array plate was subjected to a real-time PCR machine (Biorad, CFX 96). The expression of metabolic genes was determined as a relative fold change and calculated by the $2^{-\Delta\Delta CT}$ method.

2.8. Gene expression analysis using reverse transcription-quantitative PCR (RT-qPCR)

The total RNA was extracted from erythroblasts at the differentiation phase on days 7, 9, and 11 from 10 β-thalassemia/HbE patients (5 mild and 5 severe cases) and 5 normal controls. Total RNA was synthesized to cDNA by oligo $(dT)_{18}$ primer using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific), and the procedure was performed according to the manufacturer's protocol. In brief, 250 ng of total RNA was mixed with oligo $(dT)_{18}$ primer and incubated at 65 °C for 5 min. Then, the master mix (5× reaction buffer, 10 mM dNTP mix, 20 U/μL Ribolock RNase inhibitor, and 200 U/μL RevertAid M-MuL V RT) was added to the reaction at 42 °C for 60 min, followed by 70 °C for 5 min.

The cDNA samples were mixed with 2× iTaq universal SYBR green supermix (Bio-Rad), forward, and reverse primers. The primer sequences and concentration used in qPCR are shown in Supplementary Table S2. PCR was performed at 95 °C for 2 min for the enzyme activation and 40 cycles of denaturation at 95 °C for 15 s; the annealing and extension temperature according to Supplementary Table S2 for 1 min. The candidate gene expression was normalized using the β-actin gene, and relative expression was calculated by the $2^{-\Delta CT}$ method.

2.9. Western blot analysis

Protein was extracted from erythroblasts by resuspending cell pellet in 100 μL of RIPA buffer (0.1 % SDS, 0.5 % sodium deoxycholate, 1 % (*v*/v) NP-40 and 1× protease inhibitor cocktail (Sigma-Aldrich, MA, USA). The sample was centrifuged at 14,000 x*g* for 10 min at 4 °C. 40 μg of protein was loaded onto an SDS-PAGE gel. The protein was then transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were blocked overnight at 4 °C with 5 % skim milk in 1×-TBST (Tris-buffered saline with Tween 20). For BPGM detection, the membranes were incubated with a 1:100 dilution of mouse anti-BPGM monoclonal IgG antibody (Santa Cruz, CA, USA) overnight at 4 °C and subsequently incubated with a 1:10,000 dilution of horseradish peroxidase (HRP) conjugated mouse IgG Fc binding protein antibody (Santa Cruz, CA, USA) for 90 min at room temperature. For β-actin detection, the membranes were incubated with 1:250,000 diluted HRP conjugated mouse anti-β-actin monoclonal IgG antibody (Abcam, Cambridge, UK) for 60 min at room temperature. Then, the enhanced chemiluminescence substrate solution (ECL) (Cytiva, MA, USA) was used to detect the band, and the chemiluminescent signal was imaged using X-ray film.

2.10. Virtual screening and functional analysis of BPGM inhibitor in the K562 cell line

Virtual screening was conducted using AutoDock Vina to identify compounds potentially binding to the BPGM enzyme (PDB ID: 2HHJ)

from the FDA-approved drug library available in the MTiOpenScreen database. Among the compounds screened, dihydroergocristine, which exhibited high docking scores (low ΔG values), was chosen as a potential BPGM inhibitor. To evaluate the role of BPGM, K562 cells (3.4×10^5 cells/ml) were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum and treated with 25 μM dihydroergocristine mesylate (Merck, NJ, USA) for 24 h. Cell proliferation was assessed using 0.04 % trypan blue staining. For cell differentiation analysis, 1×10^5 cells were resuspended in 50 μl of 1×-DPBS and stained with anti-human CD71 antibody (BioLegend, CA, USA) and anti-human CD235a (Glycophorin A: GPA) antibody (BD Biosciences, NJ, USA) (Supplementary Table 1). The differentiation of K562 cells was categorized based on CD71 and GPA expression into intermediate-stage erythroid cells ($CD71^+GPA^+$) and late-stage erythroid cells ($CD71^-GPA^+$) (Klaihmon et al., 2024). Apoptosis was analyzed using the PE Annexin V Apoptosis Detection Kit I (cat. No. 559763, BD Biosciences, NJ, USA), following the manufacturer's instructions. To identify early and late apoptotic cells, 7-Amino-Actinomycin (7-AAD) was used in parallel with PE Annexin V. In this assay, viable cells are negative for both PE Annexin V and 7-AAD, early apoptotic cells are PE Annexin V positive and 7-AAD negative, while late apoptotic cells are positive for both PE Annexin V and 7-AAD.

2.11. Statistical analysis

All data were shown as mean with standard error of the mean (SEM), and statistical analysis was performed using SPSS program version 18.0. Mann-Whitney *U* test was used to compare the difference in the number of erythroid cells and gene expression among groups, and the Wilcoxon signed-rank test was used to compare the different expressions among days 7, 9, and 11 in each group. The statistical significance of all comparisons was determined at a *P*-value <0.05.

3. Results

3.1. Transcriptomics reveal the increased metabolism of β-thalassemia/HbE erythroblasts in bone marrow

To investigate the mechanism of ineffective erythropoiesis in β-thalassemia/HbE disease, erythroblasts from bone marrow were isolated, and transcriptomics was analyzed by using RNA sequencing. β-Thalassemia/HbE patients ($n = 3$) had microcytic hypochromic anemia with 51 ± 11 % HbE and 41 ± 19 % HbF while control subjects (n = 3) had normochromic normocytic without anemia, normal Hb typing (A_2A, 87 ± 1 % HbA, and 3 ± 0.2 % HbA_2), negative for α-thalassemia (Supplementary Table S3). Transcriptomics demonstrated global differential expression genes in β-thalassemia/HbE patients compared to normal controls. The volcano plot showed 4363 up-regulated genes and 4633 down-regulated genes in patients compared to normal controls (Fig. 1A). The gene set enrichment analysis (GSEA) using the R library revealed the significant enrichment of genes associated with glycolysis/gluconeogenesis (*p*-value = 0.0004) and fatty acid metabolism (p-value = 0.0023) pathways (Fig. 1B and C). Focusing on the metabolic pathways, the 40 genes of those involved in glycolysis, pyruvate to acetyl CoA, ATP synthesis, TCA cycle, pentose phosphate pathway, fatty acid synthesis, gluconeogenesis, and pyruvate dehydrogenase complex regulation were up-regulated in β-thalassemia/HbE patients compared to normal controls (Fig. 1D and Supplementary Table S4). The PCA plot showed that the β-thalassemia/HbE patients and normal subjects could be differentiated by 40 metabolic genes (Fig. 1E). The change in metabolic gene expression may be involved in ineffective erythropoiesis in β-thalassemia/HbE. Erythropoiesis is a dynamic process of erythroblast differentiation. Thus, it is interesting to examine the change in metabolic gene expression during terminal erythropoiesis.

3.2. Abnormal metabolic gene expression in polychromatic and orthochromatic erythroblasts from β-thalassemia/HbE patients

To investigate the dynamic of metabolic gene expression during erythropoiesis in β-thalassemia/HbE patients, we performed a two-phase culture system, which was an expansion phase and a differentiation phase to differentiate stem cells into erythroid lineage.

Erythroblast proliferation and differentiation of erythroblasts on days 0, 7, 9, and 11 of the differentiation phase culture in β-thalassemia/HbE patients who had either mild or severe clinical symptoms and healthy subjects were determined. The pattern of cell proliferation was similar between patients and healthysubjects, which increased the growth rate from day 0 to day 7, while it was constant from day 7 to day 11 (Fig. 2A). Interestingly, the proliferation rate in mild and severe β-thalassemic erythroblasts was 2–4 fold higher than that of normal erythroblasts ($P < 0.05$). The results showed a mixed population of erythroblasts during differentiation. In healthysubjects, the main population in the culture system at day 7 was basophilic erythroblasts approximately 73.8 ± 5.1 % (mean ± standard error of the mean (SEM)); at day 9 was polychromatic erythroblast presented at 51.4 ± 2 %; and at day 11 was polychromatic (52.2 ± 4.6 %), and orthochromatic erythroblasts (39.4 ± 5.3 %) (Fig. 2B). In β-thalassemia/HbE patients with mild clinical symptoms, the main population in the culture system at day 7 was basophilic erythroblasts (64 ± 14.6 %); at day 9 was polychromatic erythroblasts (58 ± 11.7 %); and at day 11 was polychromatic erythroblasts (67.4 ± 10.2 %) (Fig. 2C). In β-thalassemia/HbE patients with severe clinical symptoms, the main populations in the culture system at day 7 were basophilic erythroblasts (71.6 ± 5.4 %); at day 9 was polychromatic erythroblasts (51 ± 11 %); and at day 11 was polychromatic erythroblasts (67.2 ± 10.7 %) (Fig. 2D). Therefore, the main population in all groups on day 7 was basophilic erythroblasts; on day 9 was basophilic and polychromatic erythroblasts; on day 11 was polychromatic erythroblasts and orthochromatic erythroblasts (Fig. 2B-D). The two-phase culture system could efficiently support the proliferation and differentiation of erythroblasts. Besides, the number of cells from mild and severe cases was significantly increased compared to the healthy subjects, indicating a high proliferation rate in β-thalassemia/HbE patients, representing a distinctive characteristic of ineffective erythropoiesis. Therefore, the culture system can be used in further experiments to determine metabolic gene expression during terminal erythropoiesis in β-thalassemia.

The clinical symptoms of β-thalassemia/HbE were varied. As increased Hb synthesis and accumulation during erythroblast differentiation, the effect of excess α-globin is more pronounced in polychromatic erythroblasts, an erythroblast stage that has increased cell death in β-thalassemia. The metabolic gene expression of day 11 erythroblast cultures from mild and severe patients was examined by PCR array to understand the metabolic gene expression during erythropoiesis in different disease severities. β-thalassemia/HbE patients who had either mild or severe clinical symptoms and healthy subjects were recruited. The characteristics of the subjects are shown in Supplementary Table S5. Both mild and severe patients had microcytic hypochromic anemia, but the different disease severity was categorized based on the scoring system (Sripichai et al., 2008).

The RT^2 Profiler PCR array was used to determine the metabolic gene expression profiles. Erythroblasts on day 11 obtained from 3 healthy subjects, 3 mild, and 3 severe patients were used in this experiment. Among 84 metabolic genes, there are 5 up-regulated genes and 11 down-regulated genes in mild patients compared to healthy subjects (Fig. 3A and Table 1), and 5 up-regulated genes and 17 down-regulated genes were investigated in severe patients compared to healthy subjects (Fig. 3B and Table 2). The metabolic genes in glycolysis, such as aldolase A (*ALDOA*), were significantly decreased in severe patients, and enolase 2 (*ENO2*) was significantly reduced in both mild and severe patients at the late stages of erythroblasts compared to healthy subjects ($P < 0.05$). Moreover, PCR array analysis showed that the phosphorylase kinase

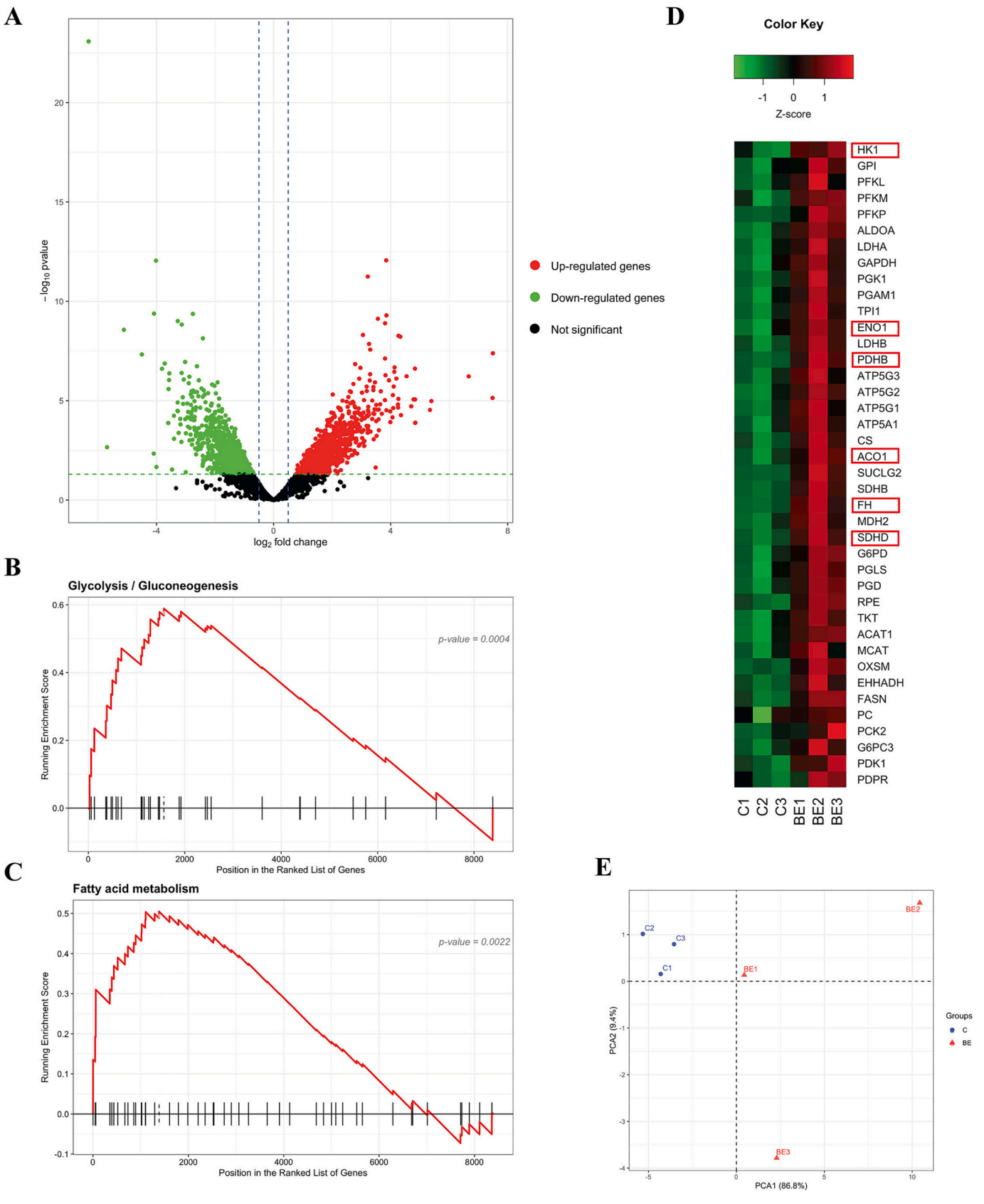

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Fig. 1. Transcriptomics analysis of bone marrow samples obtained from 3 control subjects (C) and 3 β-thalassemia/HbE patients (BE). (A) The volcano plot shows the global differential gene expression. The red, green, and black dots represent up-regulated, down-regulated, and not significant genes, respectively. (B, C) Gene set enrichment analysis (GSEA) shows a significant enrichment of genes associated with metabolic pathways. The red lines indicate running enrichment scores across the fold change-ranked genes and the black vertical tick marks below the curve indicate the location of individual genes within the fold change-ranked gene list. (D) The heat map shows the up-regulation of metabolic genes in β-thalassemia/HbE patients. Each column represents the gene expression of the individual subject. The log2 fold change of the relative gene expression scale is depicted on the top. The red and green colors show up-regulation and down-regulation of the individual gene in comparison between β-thalassemia/HbE patients and control subjects. The red box represents the genes that had a similar trend of gene expression in the PCR array and/or RT-qPCR. (E) The principal component analysis (PCA) plot shows the cluster of control subjects and β-thalassemia/HbE patients. The transcriptomes of control subjects and β-thalassemia/HbE patients were shown in blue and red dots, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gamma 1 (*PHKG1*), which participated in the glycogen storage and breakdown pathway, was significantly down-regulated in mild patients. Interestingly, succinate dehydrogenase subunit D (*SDHD*) was increased in mild patients compared to healthy subjects ($P < 0.05$) (Tables 1, 2), similar to transcriptomic analysis in human bone marrow (Supplementary Table S4). Therefore, it might be compensation or abnormal metabolic changes in β-thalassemia/HbE patients with mild clinical symptoms compared to severe patients and healthy subjects.

3.3. Metabolic changes in β-thalassemia/HbE patients with mild and severe clinical symptoms

To reveal the dynamic metabolic changes in β-thalassemia/HbE patients with mild and severe clinical symptom compared to healthy subjects, the candidate metabolic genes involving in glycolysis such as hexokinase 1 (*HK1*), phosphofructokinase, muscle (*PFKM*), *ALDOA*, enolase 1 (*ENO1*), and pyruvate kinase (*PKLR*); TCA cycle such as aconitase 1 (*ACO1*), isocitrate dehydrogenase 1 (*IDH1*) and succinate dehydrogenase subunit D (*SDHD*); pentose phosphate pathway such as transaldolase 1 (*TALDO1*); and Luebering-Rapoport shunt such as bisphosphoglycerate mutase (*BPGM*) were determined in the erythroid culture at days 7, 9 and 11 of differentiation phase by using RT-qPCR. The candidate genes were selected based on differential expression in transcriptomics and PCR array and their key roles in metabolic pathways, particularly as rate-limiting enzymes. These candidate genes can help us investigate various cellular activities, such as ATP production, synthesis of biomolecules, and the antioxidant defense system.

In healthy subjects, basophilic erythroblasts as the major population on day 7 had shown high expression of *HK1, PFKM, ALDOA*, and *PKLR* genes involved in the glycolysis pathway (Fig. 4) and *ACO1*, and *IDH1* genes involved in the TCA cycle (Fig. 5) suggesting the increased energy for cell proliferation and macromolecule production such as hemoglobin. Normal erythroid differentiation from day 7 to 11 represented a reduction of *HK1, PKLR, IDH1, TALDO1*, and *BPGM* as decreased metabolism to preparation of terminal erythroid differentiation.

Unlike healthy subjects, β-thalassemia/HbE patients had defects in β-globin production resulting in high levels of heme and cellular stress. Severe patients had increased erythroblast death in bone marrow due to ineffective erythropoiesis, leading to severe anemia compared to mild patients. The pattern of *ALDOA*, *IDH1*, and *SDHD* from day 7 to day 11 of erythroblasts from severe patients differed from healthy subjects. Defects on *IDH1* expression in severe patients since day 7 might affect ATP and macromolecule production in the TCA pathway (Fig. 5B).

Importantly, mild patients had a special pattern of erythroid metabolism. *PKLR* expression on day 7 in mild patients was significantly decreased when compared to normal erythroblasts at day 7 ($P < 0.05$) (Fig. 4E), but not other genes in the glycolysis pathway. In the TCA cycle, mild patients showed increased expression of *ACO1* and *IDH1* on day 9, followed by a decrease on day 11. In contrast, the expression of these genes remained unchanged from day 7 to day 11 in severe patients (Fig. 5A-B). Moreover, *ENO1* expression in mild patients was continuously increased from day 7 to 11. It needs to be noted that *ENO1* expression in mild patients was higher than in healthy subjects on day 9, while its expression was higher than in severe patients on day 11 (Fig. 4D). Additionally, erythroblasts of mild patients from day 7 to day 11 had maintenance levels of *BPGM* (Fig. 5E). It could be mentioned that *ENO1* plays a role in the glycolysis pathway to produce acetyl-CoA and viceversa; it can also catalyze phosphoenolpyruvate to 2-phosphoglycerate. These results suggested the metabolic reprogramming in thalassemic erythroblast. The diagram showing the up-regulation and down-regulation of metabolic genes is summarized in Fig. S3. Moreover, the BPGM protein expression was determined using Western blot analysis. The erythroblasts of healthy subjects, mild, and severe patients had increased levels of BPGM protein from day 7 to day 11. Overall, Western blot analysis suggests a trend of higher BPGM protein levels in β-thalassemia patients compared to normal controls, especially on day 11, but the difference is not statistically significant (Fig. 6). This may be caused by the small number of samples used in this experiment.

This finding suggests the dynamic of erythroblast metabolic change in mild clinical symptoms of β-thalassemia/HbE disease. The purpose of metabolic change could be to increase the ATP production and/or macromolecule synthesis such as amino acids, fatty acids, and nucleotides required for high proliferation of erythroblasts and/or anti-oxidant synthesis to eliminate reactive oxygen species (ROS). However, further study is needed to validate this hypothesis.

3.4. BPGM inhibitor induces cell differentiation in K562 cells

According to gene expression data from the PCR array and RT-qPCR, BPGM stood out due to its significantly higher expression in mild patients compared to healthy subjects. BPGM produces 2,3-BPG in the Luebering-Rapoport shunt, influencing ATP production and potentially affecting erythroid cell lifespan. Since this pathway is active mainly in erythroid cells, BPGM represents a promising and accessible target for therapeutic intervention. Therefore, BPGM was selected as the initial target for further investigation. The virtual screening of the FDA-approved drug library was conducted to identify potential BPGM inhibitors, resulting in the discovery of over a hundred compounds. Among these, dihydroergocristine (DHEC) emerged as a noteworthy candidate, demonstrating a strong affinity for BPGM with a ΔG score of −10.1. To investigate the role of BPGM in cell differentiation, K562 cells were treated with DHEC, the BPGM inhibitor, and their differentiation was subsequently assessed. After incubating K562 cells with 25 μM of DHEC for 24 h, the expression of the erythroid differentiation markers CD71 and GPA was analyzed using flow cytometry. The results indicated a significant decrease in CD71 expression in treated cells, which suggests differentiation to late-stage erythroid cells (CD71−/GPA+ cells) when compared to untreated cells (Fig. 7A). The percentage of intermediate-stage erythroid cells ($CD71^{+}/GPA^{+}$) significantly decreased to 51.10 % in K562 cells treated with DHEC, compared to 80.73 % in the untreated control. Specifically, the percentage of late-stage erythroid cells ($CD71^{-}/GPA^{+}$) significantly increased to 33.33 % in the DHEC-treated cells, whereas the untreated cells had only 10.87 %, indicating that DHEC induces cell differentiation to late-stage erythroid cells (Fig. 7B). Furthermore, cell proliferation assessed by trypan blue exclusion showed a slight decrease in cell viability between untreated (6.1×10^5 cells/ml) and treated cells (4.7×10^5 cells/ml), though this difference was not statistically significant. However, the apoptosis assay revealed a significant reduction in viable cells, with a slight increase in early and late apoptotic cells in the treated group, although this difference was not

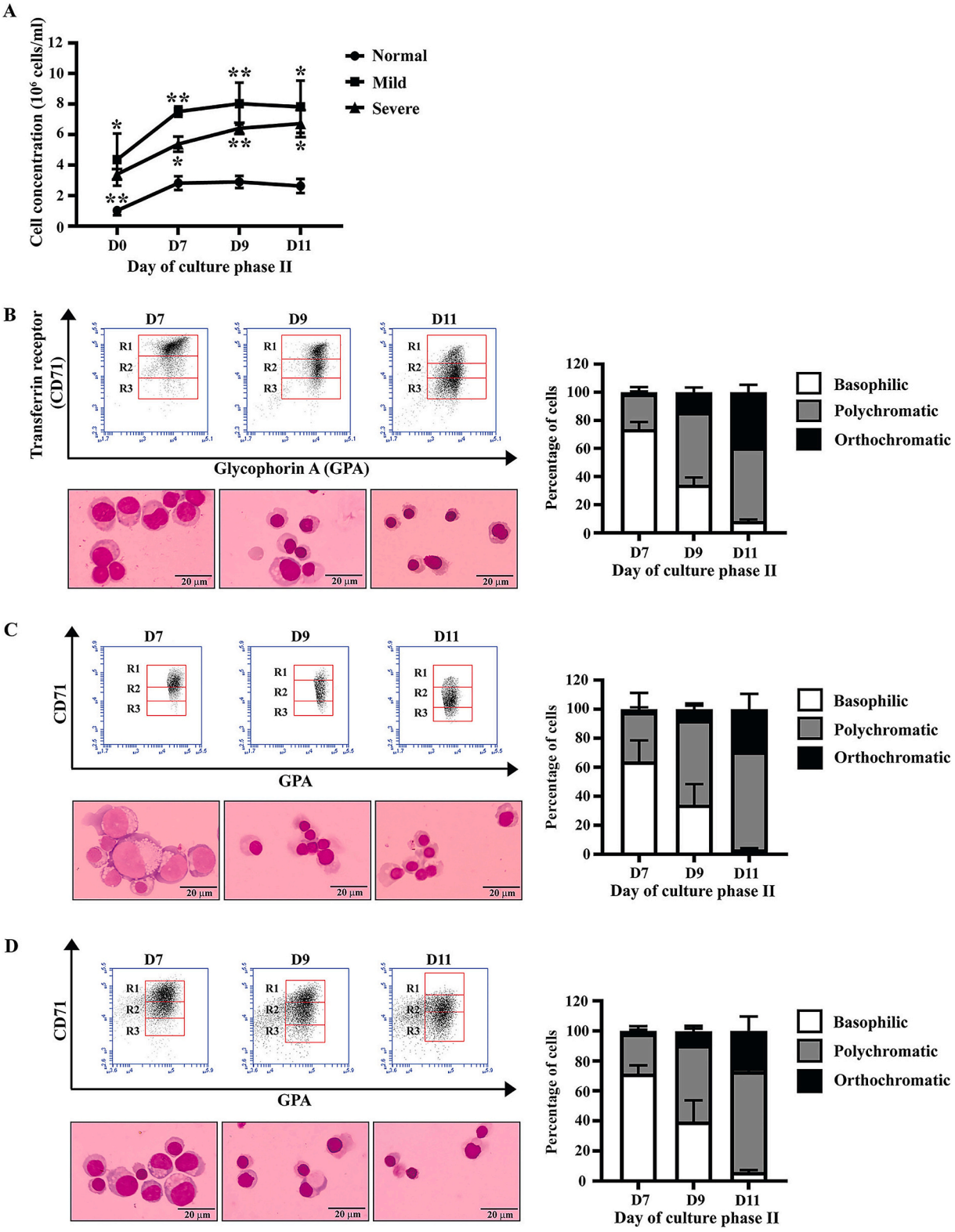

Fig. 2. Erythroid proliferation and differentiation in the culture system. Erythroid cells were harvested from the differentiation phase at day 0, 7, 9, and 11 from healthy subjects ($n = 5$) and β-thalassemia/HbE patients (5 mild and 5 severe clinical symptoms) to determine (A) cell proliferation using trypan blue staining and erythroid differentiation of (B) healthy subjects, (C) mild, and (D) severe β-thalassemia/HbE. Erythroid differentiation was determined by using flow cytometric analysis (top-left; R1, basophilic erythroblasts; R2, polychromatic erythroblasts; R3, orthochromatic erythroblasts) and Wright-Giemsa staining (bottom-left; scale bar is 20 µm). The percentages of stages of erythroid cells at days 7, 9, and 11 from differentiation phase culture were calculated from Wright-Giemsa staining. Data represents mean ± standard error of the mean (SEM). *, ** indicated *P-value* < 0.05 and < 0.01, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

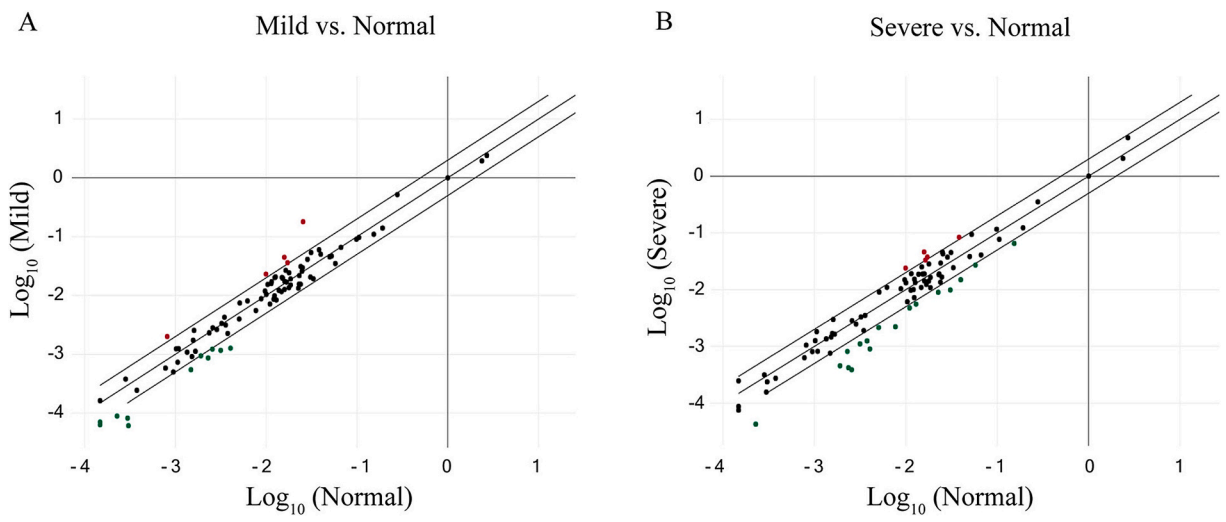

Fig. 3. The PCR array analysis of glucose metabolic gene expression was conducted in erythroblasts obtained from 3 healthy subjects and 6 β-thalassemia/HbE patients (3 with mild and 3 with severe clinical symptoms) on day 11 of the differentiation phase culture. Scatter plot showing dysregulated genes between β-thalassemia/HbE patients and healthy subjects. Differential expression of metabolic genes in (A) β-thalassemia/HbE patients who had mild clinical symptoms and (B) β-thalassemia/HbE patients who had severe clinical symptoms compared to healthy subjects. The black, red, and green dots represented unchanged genes, up-regulated genes, and down-regulated genes in β-thalassemia/HbE patients compared to healthy subjects, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
PCR array analysis of glucose metabolic genes in β-thalassemia/HbE patients who had mild clinical symptoms compared to healthy subjects.

No.	Gene Symbol	Gene Name	Fold change	*P*-value
Upregulated gene				
1.	*BPGM*	2,3-bisphosphoglycerate mutase [a]	7.05	0.16
2.	*PDK4*	Pyruvate dehydrogenase kinase, isozyme 4	2.81	0.29
3.	*ACO1*	Aconitase 1, soluble [a,b]	2.47	0.23
4.	*SDHD*	Succinate dehydrogenase complex, subunit D, integral membrane protein [b]	2.32	0.04[c]
5.	*FH*	Fumarate hydratase [b]	2.10	0.32
Downregulated gene				
1.	*GCK*	Glucokinase (hexokinase 4)	−4.99	0.09
2.	*MDH1B*	Malate dehydrogenase 1B, NAD (soluble)	−3.63	0.48
3.	*ENO2*	Enolase 2 (gamma, neuronal)	−3.19	0.02[c]
4.	*G6PC*	Glucose-6-phosphatase, catalytic subunit	−2.71	0.31
5.	*PGAM2*	Phosphoglycerate mutase 2 (muscle)	−2.71	0.22
6.	*ALDOC*	Aldolase C, fructose-bisphosphate	−2.65	0.07
7.	*PRPS1L1*	Phosphoribosyl pyrophosphate synthetase 1-like 1	−2.57	0.49
8.	*PCK1*	Phosphoenolpyruvate carboxykinase 1 (soluble)	−2.34	0.76
9.	*FBP2*	Fructose-1,6-bisphosphatase 2	−2.11	1.00
10.	*PYGM*	Phosphorylase, glycogen, muscle	−2.09	0.26
11.	*PHKG1*	Phosphorylase kinase, gamma 1 (muscle)	−2.03	0.04[c]

[a] Genes exhibiting similar expression trends as observed in transcriptomics.
[b] Genes exhibiting similar expression trends as observed in RT-qPCR.
[c] Significant difference between groups at $P < 0.05$.

Table 2
PCR array analysis of glucose metabolic genes in β-thalassemia/HbE patients who had severe clinical symptoms compared to healthy subjects.

No.	Gene Symbol	Gene Name	Fold change	*P*-value
Upregulated gene				
1.	*PDK4*	Pyruvate dehydrogenase kinase, isozyme 4	2.91	0.30
2.	*SDHD*	Succinate dehydrogenase complex, subunit D, integral membrane protein [a]	2.41	0.18
3.	*FH*	Fumarate hydratase [a]	2.19	0.17
4.	*UGP2*	UDP-glucose pyrophosphorylase 2	2.19	0.16
5.	*PDHB*	Pyruvate dehydrogenase (lipoamide) beta [a]	2.02	0.24
Downregulated gene				
1.	*PYGM*	Phosphorylase, glycogen, muscle	−6.59	0.15
2.	*PKLR*	Pyruvate kinase, liver and RBC [b]	−5.60	0.06
3.	*PRPS1L1*	Phosphoribosyl pyrophosphate synthetase 1-like 1	−5.35	0.52
4.	*ENO2*	Enolase 2 (gamma, neuronal)	−4.49	0.02[c]
5.	*PHKG1*	Phosphorylase kinase, gamma 1 (muscle)	−4.19	0.63
6.	*PCK2*	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	−3.47	0.09
7.	*GSK3A*	Glycogen synthase kinase 3 alpha	−3.11	0.14
8.	*PHKG2*	Phosphorylase kinase, gamma 2 (testis)	−3.02	0.15
9.	*PGAM2*	Phosphoglycerate mutase 2 (muscle)	−2.85	0.45
10.	*ALDOC*	Aldolase C, fructose-bisphosphate	−2.82	0.93
11.	*PDK1*	Pyruvate dehydrogenase kinase, isozyme 1	−2.66	0.56
12.	*IDH3G*	Isocitrate dehydrogenase 3 (NAD+) gamma	−2.52	0.09
13.	*ALDOA*	Aldolase A, fructose-bisphosphate [b]	−2.35	0.02[c]
14.	*PDPR*	Pyruvate dehydrogenase phosphatase regulatory subunit	−2.35	0.46
15.	*ACO2*	Aconitase 2, mitochondrial	−2.33	0.08
16.	*PFKL*	Phosphofructokinase, liver	−2.31	0.11
17.	*TPI1*	Triosephosphate isomerase 1	−2.14	0.20

[a] Genes exhibiting similar expression trends as observed in transcriptomics.
[b] Genes exhibiting similar expression trends as observed in RT-qPCR.
[c] Significant difference between groups at $P < 0.05$.

statistically significant (Fig. 7C-D). Overall, these findings suggest that BPGM inhibition effectively facilitates differentiation in K562 cells, underscoring the role of BPGM in cell differentiation.

4. Discussion

Ineffective erythropoiesis is a hallmark of β-thalassemia, and its characteristics are the high proliferation of erythroblasts and increased cell death at late-stage erythroblasts, especially polychromatic erythroblasts in bone marrow, leading to a decreased amount of mature red blood cells and anemia (Mathias et al., 2000). Khungwanmaythawee

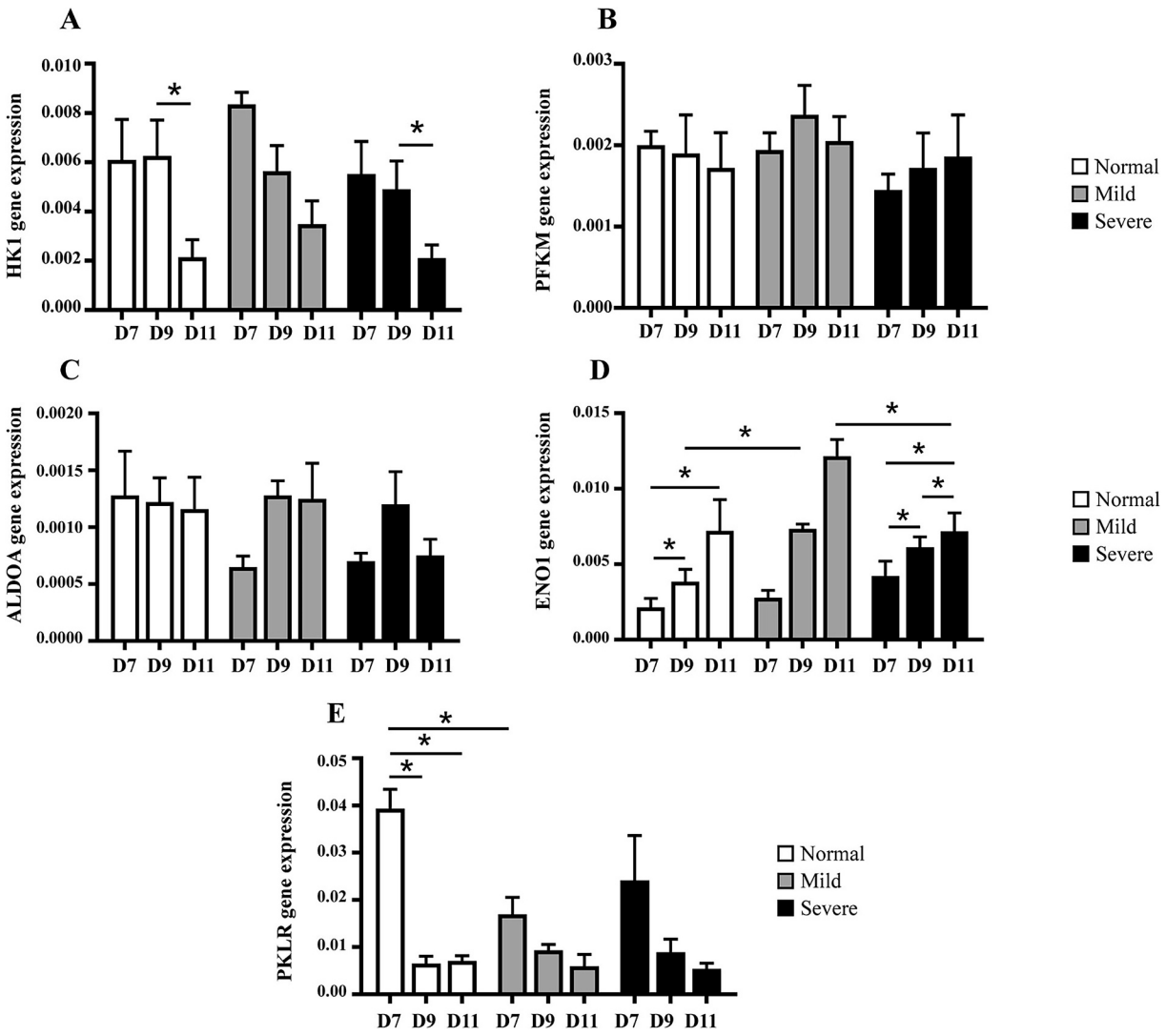

Fig. 4. The expression of metabolic genes in the glycolysis pathway. The gene expression was analyzed in erythroblasts obtained from 5 normal subjects, and 10 β-thalassemia/HbE patients (5 mild and 5 severe clinical symptoms) on days 7, 9, and 11 of differentiation phase culture. The expression levels of (A) hexokinase 1 (*HK1*) (B) phosphofructokinase, muscle (*PFKM*) (C) aldolase A (*ALDOA*) (D) enolase 1 (*ENO1*), and (E) pyruvate kinase L/R (*PKLR*) were determined using RT-qPCR. * Significant difference at *P-value* < 0.05.

et al. (2016) observed mitochondrial content during erythroblast differentiation using Mitotracker staining and reported higher staining intensity in β°-thalassemia/HbE erythroblasts compared to healthy controls. Mitochondrial levels were further quantified by quantitative PCR, revealing elevated mitochondrial content in cells from both mild and severe β°-thalassemia/HbE patients on day 10 of culture (Khungwanmaythawee et al., 2016). Thalassemic erythroblasts might require enhanced energy production, macromolecule synthesis, and cofactors for antioxidant compounds to sustain a high proliferation rate and cope with increased oxidative stress due to excess α-globin. For this reason, the expression of metabolic genes in late-stage erythroblasts was examined. Transcriptomic analysis of bone marrow basophilic erythroblasts and polychromatic erythroblasts, together with PCR array and RT-qPCR analysis of erythroblasts culture, revealed the metabolic reprogramming that is associated with ineffective erythropoiesis and disease severity in β-thalassemia/HbE disease.

The transcriptomics analysis revealed that β-thalassemia/HbE patients have global up-regulation of several metabolic pathways, including glycolysis, ATP synthesis, TCA cycle, pentose phosphate pathway, and fatty acid synthesis. Glucose metabolic genes examined by PCR array analysis of day 11 erythroblast culture showed that some metabolic genes were up-regulated in mild and severe patients, which correlated with transcriptomics results. Since this experiment was focused on mRNA expression levels, it might be a limitation in interpreting which metabolic pathway changes in β-thalassemia/HbE erythroblasts contribute to ineffective erythropoiesis. Decreased correlation between mRNA and proteins in the late stages of erythroblast differentiation was reported (Gautier et al., 2016). Although, there was some discrepancy between transcriptomic and PCR array results, which may be caused by the difference between bone marrow cells and erythroblast culture. The increased expression of metabolic genes is consistent with the proteomics study, in which β-thalassemia/HbE patients have increased several metabolic proteins, including phosphoglycerate mutase 1, enolase 1, triosephosphate isomerase 1, aldolase A, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase A. Furthermore, the NADH/NAD^+ ratio was reduced, indicating increased metabolism in patients (Leecharoenkiat et al., 2011). Metabolic genes are found to be up-regulated in several types of cancer to increase the biosynthesis of nucleotides and fatty acids, enhance ATP production, and support cell proliferation (Higashimura et al., 2011; Nong et al., 2023; Phannasil et al., 2015). Change in the expression of these metabolic genes in β-thalassemia/HbE erythroblasts might support

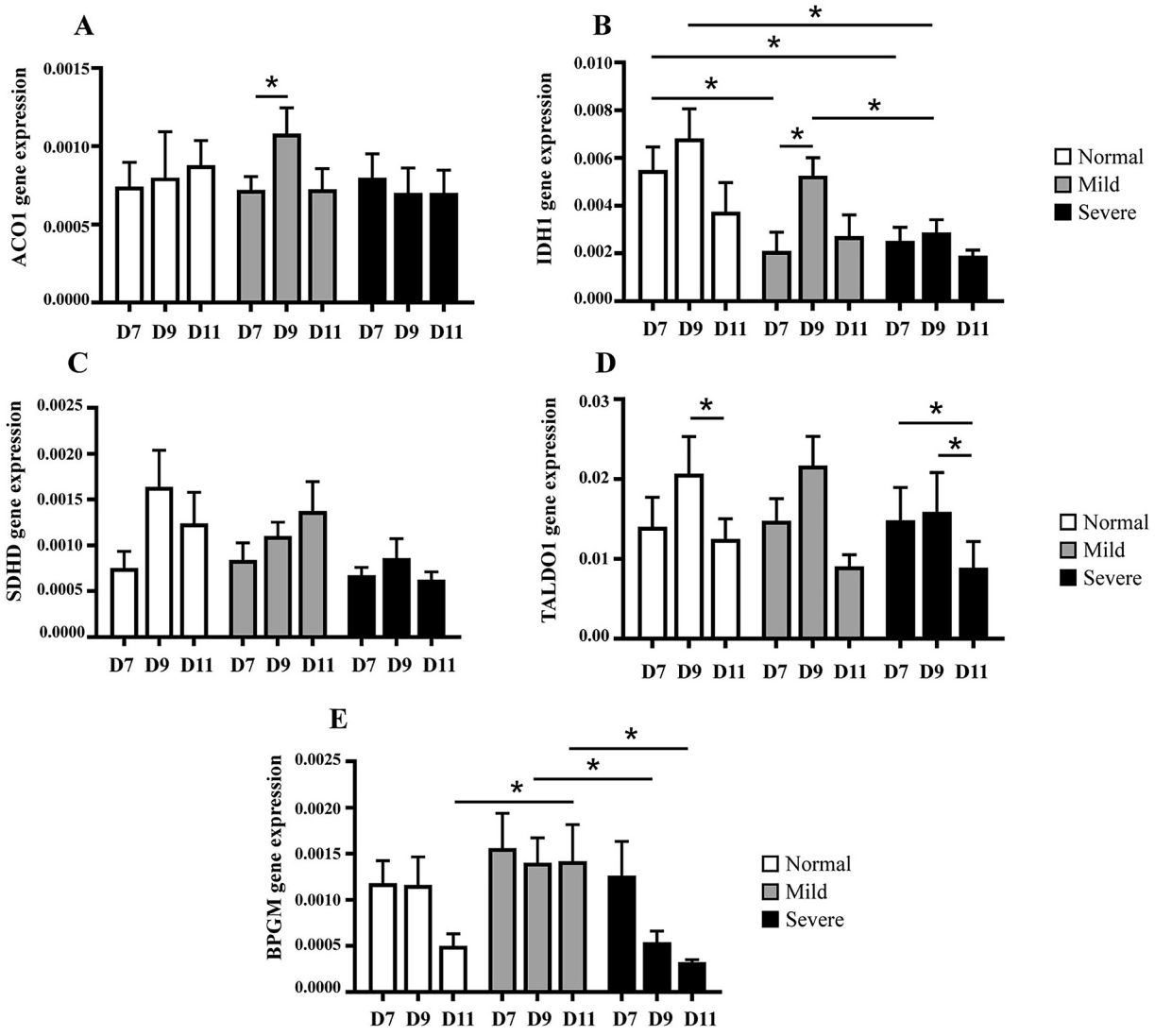

Fig. 5. The expression of metabolic genes in the TCA cycle, pentose phosphate pathway and Luebering-Rapoport shunt. The gene expression was analyzed in erythroblasts obtained from 5 normal subjects, and 10 β-thalassemia/HbE patients (5 mild and 5 severe clinical symptoms) on days 7, 9, and 11 of differentiation phase culture. The expression levels of (A) aconitase 1 (*ACO1*) (B) isocitrate dehydrogenase 1 (*IDH1*), (C) succinate dehydrogenase subunit D (*SDHD*), (D) transaldolase 1 (*TALDO1*), and (E) bisphosphoglycerate mutase (*BPGM*) were determined using RT-qPCR. * Significant difference at *P-value* < 0.05.

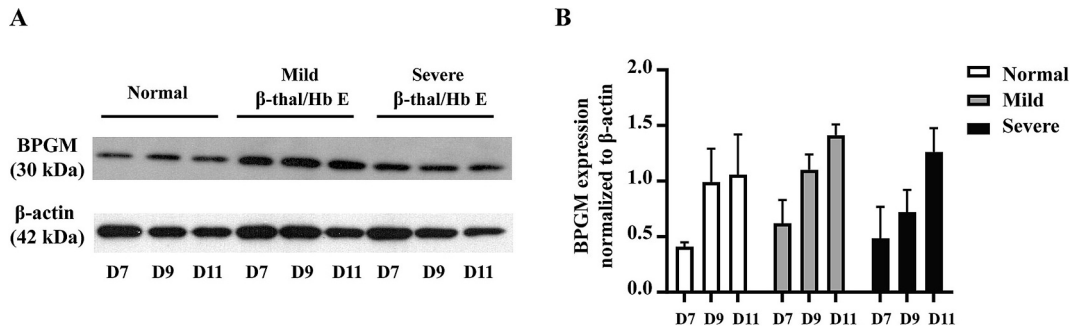

Fig. 6. The expression of BPGM protein. The protein expression was determined in erythroblasts obtained from 2 normal subjects, and 4 β-thalassemia/HbE patients (2 mild and 2 severe patients) on days 7, 9, and 11 of the differentiation phase (phase II) culture. (A) Western blot analysis of BPGM protein. β-actin was used as a loading control. (B) The immunoreactive band intensity of BPGM in A was quantitated and normalized with that of β-actin and shown as the relative BPGM expression.

the high expansion of erythroblasts and response to oxidative stress.

In the glycolysis pathway, several metabolic genes were significantly changed in β-thalassemia/HbE erythroblasts. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was up-regulated, especially in β-thalassemia/HbE patients analyzed from the transcriptomics study. The RT-qPCR analysis showed higher *ENO1* expression, while reduced

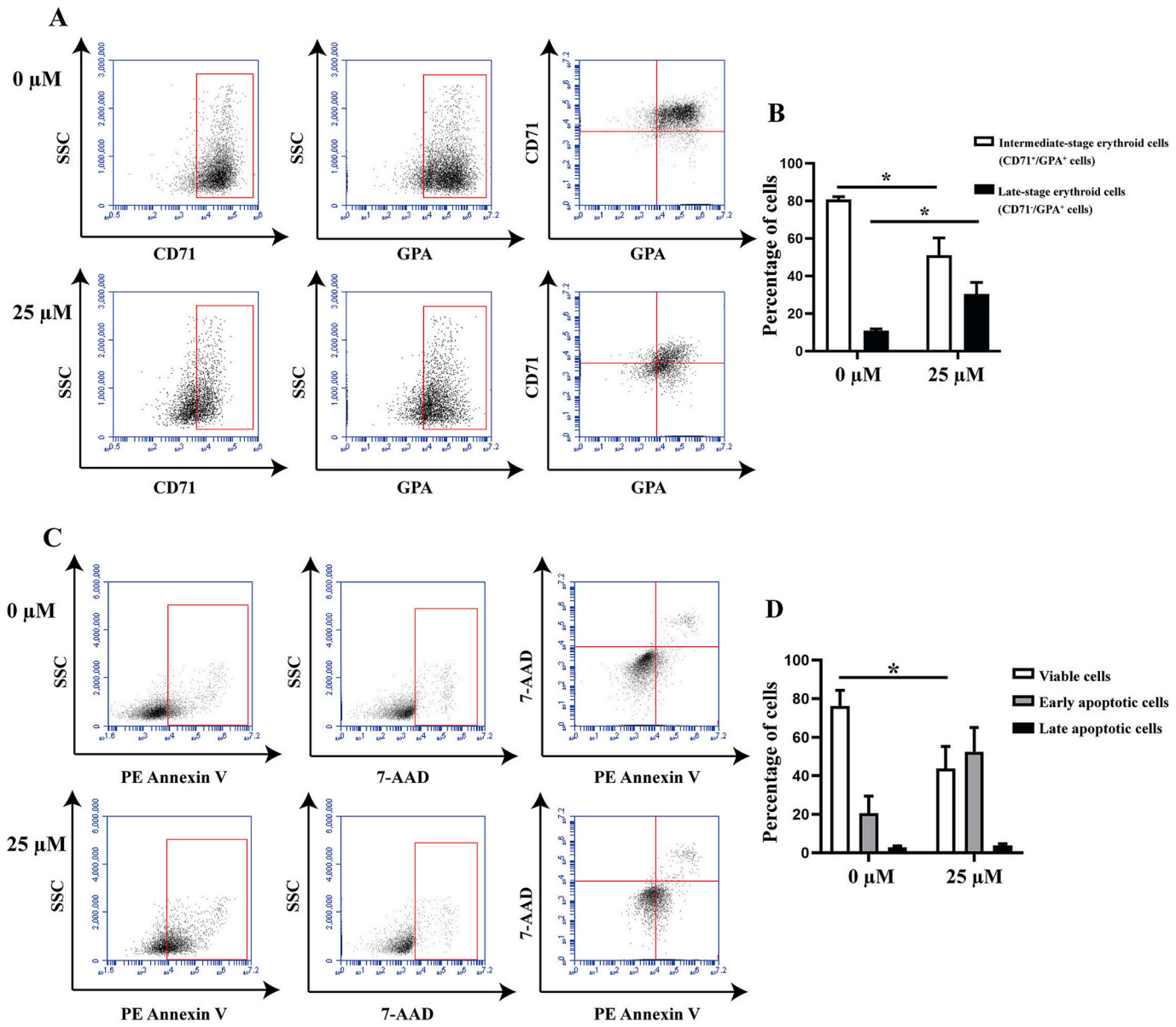

Fig. 7. BPGM inhibitor treatment in K562 cells. (A) Flow cytometry analysis was conducted using CD71 and GPA erythroid markers to assess the differentiation of erythroid cells treated with 25 µM DHEC compared to untreated cells (top-right; intermediate-stage erythroid cells; bottom-right; late-stage erythroid cells). (B) The percentage of intermediate-stage erythroid cells (CD71+ GPA+) and late-stage erythroid cells (CD71- GPA+) was analyzed. (C) The apoptosis assay was performed using PE Annexin V and 7-AAD to compare 25 µM DHEC treated cells with untreated cells. (D) The percentage of viable cells (PE Annexin V^- and 7-AAD^-), as well as early (PE Annexin V^+ and 7-AAD^-) and late apoptotic cells (PE Annexin V^+ and 7-AAD^+), was quantified in the bar graph. * Significant difference at P-value <0.05.

PKLR expression in mild patients. Overexpression of GAPDH and ENO1 was detected in several cancer types (Almaguel et al., 2020; Higashimura et al., 2011; Tang et al., 2012; Yang et al., 2020). Therefore, the up-regulated *GAPDH* and *ENO1* in β-thalassemia/HbE erythroblasts might occur in promoting cell proliferation and response to oxidative stress during ineffective erythropoiesis.

After the glycolysis pathway, pyruvate is converted to acetyl-CoA, and then enters the TCA cycle by condensing with oxaloacetate. First, pyruvate dehydrogenase (PDH) converts pyruvate to acetyl-CoA, which fuels the TCA cycle to generate electron carriers NADH and $FADH_2$. Most ATP is then produced through oxidative phosphorylation (Judge and Dodd, 2020). According to our results, the *PDH* gene expression analyzed from the PCR array was increased in β-thalassemia/HbE erythroblasts, suggesting an increase in energy production. Moreover, the up-regulation of *PDH* may lead to an increase in TCA cycle intermediates used for the production of macromolecules to support the high proliferation of erythroblasts. Second, pyruvate can be converted to oxaloacetate by pyruvate carboxylase (PC), which was significantly increased in β-thalassemia/HbE erythroblasts. The up-regulation of PC was found in several cancers such as lung cancer, gallbladder cancer, thyroid cancer, and breast cancer (Kiesel et al., 2021; Phannasil et al., 2015). In breast cancer, PC is essential to support cancer cell proliferation since the role of PC is to provide the TCA cycle intermediates to support mitochondrial biosynthesis of precursors of cellular components (Phannasil et al., 2017; Phannasil et al., 2015). Besides, there was a tendency that PC had the potential to protect the cells from oxidative stress because PC-depleted cancer cells were more sensitive to oxidants than the cells where PC was still functioning (Kiesel et al., 2021). Therefore, up-regulated PC in β-thalassemia/HbE erythroblasts might be responsible for promoting cell proliferation and protecting cells from oxidative stress.

In the TCA cycle, several metabolic genes, including *IDH1*, *SDHD*,

and *MDH2* were significantly changed in β-thalassemia/HbE erythroblasts. IDH is a rate-limiting enzyme of the TCA cycle. The expression of *IDH1* analyzed from RT-qPCR was significantly lower in both mild and severe patients. IDH knockdown caused an increase in ROS, which affects cellular enucleation and cell maturation in erythroid cells (Gonzalez-Menendez et al., 2021). Moreover, IDH mutation caused a reduction in heme production, leading to ROS accumulation, cell death, and anemia in IDH-mutated mice (Gu et al., 2021). The lower expression of *IDH* in our study may contribute to high ROS and might be responsible for the impairment of cell development and maturation in β-thalassemia/HbE patients. *SDHD* analyzed from the transcriptomics and PCR array was significantly up-regulated in β-thalassemia/HbE erythroblasts. The function of SDHD is to convert succinate to fumarate in the TCA cycle. In addition, SDHD is an enzyme complex II, which is one of the members of the electron transport chain. Its function is to generate an electrochemical proton gradient across the inner mitochondrial membrane to produce ATP (Rustin et al., 2002). Therefore, the up-regulation of *SDHD* might support macromolecules and indirectly support ATP synthesis in β-thalassemia/HbE erythroblasts.

In addition, fatty acid synthase (FASN) is an enzyme used to synthesize fatty acid, and the expression of *FASN* was significantly increased in β-thalassemia/HbE erythroblasts. The overexpression of FASN was identified in many cancer types, leading to the promotion of several characteristics of cancer, such as resisting cell death and supporting cell proliferation (Vanauberg et al., 2023). Thus, the up-regulation of the *FASN* gene might promote cell proliferation in β-thalassemia/HbE erythroblasts.

Thalassemic erythroblasts and RBCs have elevated oxidative stress from heme released by excess denatured α-globin chains, promoting ROS formation. ROS levels are higher in RBCs from β-thalassemia/HbE patients with severe symptoms compared to those with mild symptoms (Chaichompoo et al., 2015). Glucose metabolism is crucial for the RBC redox system, which is the only metabolic pathway able to supply ATP and cofactors for antioxidative defense systems. The interplay between glycolysis and the pentose phosphate pathway is important for cellular metabolism. Increased *ENO1* on day 11 of mild patients might influence the metabolic flux and provide substrates for the production of NADPH from the pentose phosphate pathway, which is essential for oxidative stress defense mechanisms. This might result in a reduced degree of disease severity.

Heme is essential for hemoglobin synthesis, with erythroid-specific 5-aminolevulinate synthase (ALAS2) catalyzing the first step by converting succinyl-CoA and glycine into 5-aminolevulinic acid (ALA). Aconitase 1 (ACO1), also known as IRP1, can regulate iron metabolism depending on its form. In its apo form, it binds to the 5′ IRE of ALAS2 mRNA and inhibits translation. (Ye et al., 2010). In β-thalassemia/HbE, reduced β-globin synthesis may cause excess heme, leading to oxidative stress and cell death. In mild cases, increased ACO1 expression may serve as a protective mechanism by limiting heme production through ALAS2 suppression, helping to reduce heme toxicity and disease severity.

Hemoglobin oxygen affinity is regulated by 2,3-bisphosphoglycerate (2,3-BPG). When the oxygen supply is insufficient, BPGM in the Luebering-Rapoport shunt is activated to synthesize 2,3 BPG, which binds to hemoglobin and releases oxygen into tissues (Liu et al., 2016a). On day 11, *BPGM* gene expression appeared downregulated in healthy controls and severe β-thalassemia/HbE patients compared to days 7 and 9. Interestingly, *BPGM* expression remained elevated in mild patients on day 11, with significantly higher levels compared to both healthy controls and severe patients. Regarding protein levels, BPGM showed a lower expression trend on day 7, followed by sustained higher levels on days 9 and 11 across all groups. Although BPGM protein levels were higher in mild patients compared to normal controls and severe patients, the differences were not statistically significant. The observed discrepancy between mRNA and protein levels may be attributed to post-transcriptional regulation, variability in translation efficiency, and differences in protein stability or degradation, which are known to contribute to the uncoupling of mRNA and protein expression (Liu et al., 2016b; Vogel and Marcotte, 2012). To date, no studies have specifically investigated BPGM regulation in thalassemia, highlighting the need for further research in this area. Nevertheless, the elevated expression of BPGM may contribute to improved oxygen delivery and reduced tissue hypoxia, potentially explaining the milder clinical phenotype observed in this group.

Ineffective erythropoiesis is the main cause of β-thalassemia pathology, and several targeted therapies are currently in development (Chaichompoo et al., 2022b). RBCs rely on ATP from glycolysis due to a lack of mitochondria. Mitapivat (AG-348), an allosteric activator of pyruvate kinase, enhanced ATP production and reduced ROS. In β-thalassemic mice, oral administration of mitapivat ameliorated anemia and ineffective erythropoiesis (Matte et al., 2021). In a phase 2 multicenter clinical trial (NCT03692052), mitapivat was orally administered to 20 non–transfusion-dependent thalassemia patients (5 with α-thalassemia and 15 with β-thalassemia). Most patients showed an increase in hemoglobin levels from baseline (Kuo et al., 2022). Phase 3 studies were recruited to evaluate the efficacy and safety of mitapivat in β-thalassemia (NCT04770753). However, some patients did not respond to this drug, and most patients had treatment-emergent adverse events. Therefore, other novel targets are still needed to further investigate to improve drug efficacy and to cover different severities of β-thalassemia. Here, we demonstrate the role of BPGM in cell differentiation in K562 cells. These findings highlight the critical role of BPGM in regulating erythroid differentiation. Inhibition of BPGM by DHEC promotes differentiation of K562 cells into late-stage erythroid cells, suggesting that BPGM may be a promising therapeutic target for modulating erythroid maturation. Further studies are needed to investigate its role in human erythroid progenitor cells, to better understand the underlying mechanisms, and to explore the broader therapeutic potential of BPGM inhibition in different severities of β-thalassemia/HbE.

In conclusion, transcriptomic analysis of bone marrow samples revealed the global up-regulation of metabolic genes in glycolysis, TCA cycle, pentose phosphate pathway, ATP, and fatty acid synthesis pathway in β-thalassemia/HbE compared to normal erythroblasts. Expression of metabolic genes during terminal erythropoiesis was further determined by PCR array and RT-qPCR, demonstrating the increased expression of *ENO1*, *IDH1*, and *BPGM* in mild compared to severe patients. Inhibition of BPGM enhances differentiation of K562 into late-stage erythroid cells, suggesting that BPGM may be a promising therapeutic target for modulating erythroid maturation. This study provides the basic knowledge of metabolic reprogramming during ineffective erythropoiesis in β-thalassemia/HbE, leading to novel approaches for β-thalassemia to ameliorate ineffective erythropoiesis and improve anemia in patients.

4.1. Limitations

The small sample size of bone marrow used in the initial transcriptomic analysis limits our ability to clearly distinguish biological differences, as bone marrow aspiration was performed only in participants who voluntarily consented due to the lack of a clinical indication. Another limitation is that transcriptomics was performed after erythroblast cell sorting. Cell sorting or FACS has been reported to increase oxidative stress and alter the metabolic state of cells. The sorted cells showed an alteration of some metabolite levels compared to unsorted cells in metabolomics analysis (Llufrio et al., 2018). Thus, this point should be a concern for further experiments related to metabolic activity and metabolite level analysis. The small number of samples was used to validate BPGM protein expression. We attempted this validation to support the interpretation at the gene expression level, aiming to strengthen the overall conclusions. However, more work is needed to provide more comprehensive evidence to understand how this metabolic reprogramming is functionally involved in the ineffective

erythropoiesis and disease severities in β-thalassemia disease. The validation of metabolic enzyme expression, metabolomics, and functional analysis will be performed to prove this hypothesis.

CRediT authorship contribution statement

Chanyanat Sukhuma: Writing – original draft, Methodology, Investigation, Formal analysis. **Donny Nauphar:** Investigation. **Khanita Nuamsee:** Investigation. **Wasinee Kheansaard:** Resources, Formal analysis. **Kittiphong Paiboonsukwong:** Resources. **Alisa Wilantho:** Formal analysis, Data curation. **Chumpol Ngamphiw:** Formal analysis, Data curation. **Pornthip Chaichompoo:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization. **Sissades Tongsima:** Formal analysis, Data curation, Conceptualization. **Saovaros Svasti:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Phatchariya Phannasil:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethics approval

This study was approved by Mahidol University Central Institutional Review Broad (MU-CIRB 2014/031.1703) and Mahidol University Multi-Faculty Cooperative IRB review (MU-MOU 2022/129.0812). The written informed consent was obtained from each participant.

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Declaration of competing interest

The authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2025.104980.

Data availability

All data related to this study can be obtained on request, while all the analyzed data were included in this published article and its supplementary information files.

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