Expression of Bisphosphoglycerate Mutase during Erythropoiesis

การแสดงออกของยีนบิสฟอสโฟกลีเซอเรตมิวเตสระหว่างกระบวนการสร้างเซลล์เม็ดเลือดแดง

Chanyanat Sukhuma (ชัญญานัฐ สุขุมะ)* Dr.Khanita Nuamsee (ดร.คณิตา น่วมศรี)**
Donny Nauphar (ดอนนี่ เนเปอร์)*** Dr.Phatchariya Phannasil (ดร.พัชริยา พรรณศิลป์)****
Dr.Saovaros Svasti (ดร.เสาวรส สวัสดิวัฒน์)*****

ABSTRACT

Releasing oxygen from hemoglobin is regulated by 2,3-bisphosphoglycerate (2,3-BPG) synthesized by bisphosphoglycerate mutase (BPGM). It is anticipated that the synthesis of hemoglobin and its allosteric modulator, 2,3-BPG, are synchronized. This study aims to examine BPGM expression during erythropoiesis. BPGM, α- and β-globin expression were measured by RT-qPCR. BPGM expression pattern during erythropoiesis was similar to α- and β-globin with the highest levels at basophilic and polychromatic erythroblast. The α- and β-globin showed a significant positive correlation. However, no correlation between BPGM and the globin genes was detected, suggesting different regulatory mechanisms of gene expression.

บทคัดย่อ

การปล่อยออกซิเจนของฮีโมโกลบินถูกควบคุมด้วย 2,3-bisphosphoglycerate (2,3-BPG) ซึ่งถูกสร้างด้วยเอ็นไซม์ bisphosphoglycerate mutase (BPGM) เนื่องจาก 2,3-BPG มีความสำคัญต่อการทำงานของฮีโมโกลบิน การแสดงออกของยีนที่สร้างฮีโมโกลบินและ 2,3-BPG จึงน่าจะสอดคล้องกัน การศึกษานี้มีวัตถุประสงค์เพื่อวิเคราะห์ระดับการแสดงออกของ BPGM ระหว่างกระบวนการสร้างเม็ดเลือดแดง โดยวิเคราะห์ระดับการแสดงออกของอัลฟ่าโกลบิน เบต้าโกลบิน และ BPGM ด้วยวิธี RT-qPCR พบว่ารูปแบบการแสดงออกของยีนทั้งสามมีความคล้ายคลึงกัน โดยมีการแสดงออกสูงสุดในเซลล์เม็ดเลือดแดงตัวอ่อนระยะ basophilic และ polychromatic erythroblast นอกจากนี้ยังพบความสัมพันธ์เชิงบวกระหว่างการแสดงออกของอัลฟ่าและเบต้าโกลบิน แต่ไม่พบความสัมพันธ์ระหว่าง BPGM และโกลบินทั้งสอง เนื่องจาก BPGM มีกลไกการควบคุมการแสดงออกที่ต่างกับอัลฟ่าและเบต้าโกลบิน

Keywords: Bisphosphoglycerate mutase, Hemoglobin, Erythropoiesis
คำสำคัญ: บิสฟอสโฟกลีเซอเรตมิวเตส ฮีโมโกลบิน กระบวนการสร้างเซลล์เม็ดเลือดแดง

*Student, Master of Science Program in Molecular Genetics and Genetic Engineering, Institute of Molecular Biosciences, Mahidol University

**Researcher, Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University

***Student, Doctor of Philosophy Program in Molecular Genetics and Genetic Engineering, Institute of Molecular Biosciences, Mahidol University

****Assistant Professor, Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University

*****Associate Professor, Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University

Introduction

Hemoglobin is a crucial protein in erythrocytes which function in oxygen transportation from lung to tissues and carry carbon dioxide from tissues back to lung (Ahmed et al., 2020). 2,3-bisphosphoglycerate (2,3-BPG), a major modulator of hemoglobin oxygen affinity, plays an important role in human erythrocytes by regulating their blood oxygen transport and delivery by facilitating the release of oxygen from hemoglobin. The normal erythrocyte concentration of 2,3-BPG can be altered in various disease states and environmental changes, including anemia, congenital heart disease and high altitude with higher concentrations of 2,3-BPG are evident. The intracellular concentration of 2,3-BPG is maintained by the erythroid-specific enzyme bisphosphoglycerate mutase (BPGM) that converts 1,3 bisphosphoglycerate (1,3-BPG), an intermediate metabolite in the glycolysis pathway, to 2,3-BPG (Chu et al., 2014).

During erythropoiesis, hematopoietic stem cells (HSCs) which were the origin of blood lineage were proceeded through various processes to develop into mature red blood cells. Besides, the morphology of cells and the expression of genes were continually changed during cell maturation (Dzierzak, Philipsen, 2013). During cell differentiation, genes involved with erythroid-specific functions such as heme metabolism, α-globin, and β-globin were highly expressed on proerythroblast (Shi et al., 2014). While expression pattern of α-globin and β-globin is well known, expression of BPGM during erythropoiesis is not well documented. Mello et al. (2019) studied gene expression profiling during normal erythropoiesis. They found that the BPGM expression was detected in erythroblast expressing CD71 or transferrin receptor. However, since CD71 can expose from burst-forming unit-erythroid (BFU-E) stage to orthochromatic erythroblast, the stage of cells that expressed BPGM was unclear (Moura et al., 2015).

Although it is known that BPGM expresses in erythroblast, its expression pattern during various erythroid differentiation states is less known. In addition, as 2,3-BPG is a hemoglobin allosteric modulator that decreases hemoglobin–oxygen binding affinity, it is interesting to examine the expression pattern of BPGM together with α-globin and β-globin. Thus, this study aimed to explore the expression level of the three genes during erythropoiesis. The results showed that the expression of the three genes was presented in the same trend.

Objectives of the study

This study aimed to analyze the gene expression level of BPGM, α-globin, and β-globin in erythroblast and the correlation among the expression of BPGM, α-globin, and β-globin genes during erythropoiesis of healthy donors.

Materials and methods

Subjects

This study was performed in accordance with the Helsinki declaration and was approved by Mahidol University Central Institutional Review Board (approval number MU-CIRB 2014/031.1703). Written informed consents were obtained from all individual participants in this study. Healthy individuals aged 18-45 years with normal erythrocyte indices and normal hemoglobin typing were recruited.

Erythroid progenitor isolation and culture

Fresh whole blood samples collected in CPDA-1 anticoagulant were collected. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Lymph prep, density 1.077 g/ml. The cells were then cultured in a two-phase liquid culture as previously described (Dai et al., 2016). The phase I (expansion phase) the PBMCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM), Penicillin-Streptomycin (P/S), 30% fetal bovine serum (FBS), 25 ng/ml human interleukin-3 (IL-3), 50 ng/ml human stem cell factor (SCF), and 0.1 U/ml erythropoietin (EPO) at 37 °C, 5% CO_2 for 7 days (E0-E7). After 7 days, erythroblast was collected and cultured in phase II (differentiation phase) media composed of IMDM, P/S, 30% FBS, 0.1 ng/ml human IL-3, 3 U/ml EPO at 37 °C, 5% CO_2 for 11 days (D0-D11). Finally, erythroid cells were collected on days 7, 9, 11 of phase II culture for analysis of erythroid cell differentiation by light microscopic analysis and flow cytometry, and gene expression by RT-qPCR.

Erythroid cell differentiation by light microscope and flow cytometry

Erythroid cell differentiation in erythroid progenitor cell culture from three healthy donors was examined by two methods, morphological examination by light microscopic analysis and erythroid differentiation marker by flow cytometry. For morphological analysis erythroid, $5x10^4$ cells were cytospun onto a glass slide. The cells were stained with Wright-Giemsa solution and counted 100 nucleated red blood cells under a light microscope. For flow cytometric analysis, $1x10^5$ cells were stained with three antibodies: fluorescein isothiocyanate (FITC) conjugated anti-human CD45 monoclonal antibody (BD Bioscience) (leukocyte marker), peridinin chlorophyll protein (PerCP)/Cy5.5 conjugated anti-human CD71 monoclonal antibody (BD Bioscience) (transferrin receptor marker) and phycoerythrin (PE) conjugated anti-human CD235a (Glycophorin A: GPA) monoclonal antibody (BD Bioscience) (erythroid cell lineage marker). The reaction was incubated for 30 minutes and measured by BD Accuri C6 Plus flow cytometer (BD Bioscience). Data from 20,000 events were acquired and analyzed using BD Accuri C6 plus software (BD Bioscience).

cDNA synthesis

Total RNA was isolated from $1x10^6$ erythroid progenitor cells by TRIzol reagent (Thermo Fisher Scientific). The procedure of RNA isolation was performed according to the manufacturer's instructions and RNA concentration was examined by NanoDrop spectrophotometer (Thermo Fisher Scientific). RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific) and oligo-dT primer were used to generate cDNA from 250 ng of total RNA.

RT-qPCR

cDNA was generated from erythroid progenitor cell culture obtained from seven healthy donors and used as a template for qPCR using iTaq universal SYBR green supermix (Bio-Rad) with 5 μM forward and reverse primers (Table 1). For PCR condition, annealing and extension temperature was set at 62 ℃ for amplification of BPGM and β-actin and 60 ℃, and 50 ℃ for amplification of α-globin and β-globin, respectively. The relative gene expression level was calculated using $2^{-\Delta Ct}$ method. The expression of β-actin gene was used for the normalization of gene expression.

Table 1 The sequences of PCR primers and expected product size.

Gene name	Primer sequences	Expected product size
Bisphosphoglycerate mutase (BPGM)	Forward primer: 5'GAGATCAGCCTGGACAACATAG 3' Reverse primer: 5'CGGCTTGGAACCAGATGAA 3'	214 bp
α-globin	Forward primer: 5'TGGACGACATGCCCAACGCGC 3' Reverse primer: 5'AGGAACTTGTCCAGGGAGGCG 3'	168 bp
β-globin	Forward primer: 5'CTCATGGCAAGAAAGTGCTCG 3' Reverse primer: 5'GTGATGGGCCAGCACACAGA 3'	171 bp
β-actin	Forward primer: 5'TCACCAACTGGGACGACATGG 3' Reverse primer: 5'GGTCTCAAACATGATCTGGGTC 3'	150 bp

Statistical analysis

Data were analyzed and presented as mean and standard error. Pearson correlation was used to analyze the statistical relationship among the three genes and paired sample t-test was used to analyze and compare the difference of expression level on days 7, 9, and 11 of each gene. These statistical analyses were performed by using SPSS Version 18.0 (IBM Collaboration, Armonk, NY).

Results

Erythroid progenitor differentiation in cell culture

The development of erythroid progenitor cell collected on days 7, 9, 11 of phase II culture was examined using a light microscopic analysis and flow cytometry. Microscopic analysis, on day 7 of phase II culture, most populations on this day were basophilic erythroblasts presented (mean ± standard error of the mean (SEM)) approximately 77±6.1%. Polychromatic erythroblasts and orthochromatic erythroblasts were 20.7±4.7% and 2.3±1.5%, respectively (Figure 1A). On day 9, basophilic erythroblasts were differentiated to polychromatic erythroblasts which were the main population. The proportion of basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts on day 9 were 34.3±8.4%, 52.3±3.7%, and 13.3±5%, respectively. On day 11 of culture, the main populations were

polychromatic normoblasts and orthochromatic normoblasts which had the proportion 55.3±7.2% and 37±8.1%, respectively. In addition, basophilic erythroblasts were 8.3±0.3%.

Flow cytometry analysis, the stage of erythroid differentiation was determined by gate $CD45^-$ erythroid progenitors and then determined stage of erythroblast by the expression of GPA and CD71. Since GPA was a surface marker for erythroid cell lineage and CD71 was decreased through the erythroid maturation. The population of cells in R1, R2, and R3 represented the cells at basophilic, polychromatic, and orthochromatic erythroblasts, respectively (Figure 1B). The main population on day 7 of culture was basophilic erythroblasts. Then, CD71 expression was reduced and the cells were differentiated to polychromatic erythroblasts which were the main population on day 9 of culture. Lastly, the main population on day 11 of culture was polychromatic and orthochromatic erythroblasts. The results from the two methods were consistent in both the main population and the proportion of cells on each day. This suggested that the two-phase liquid erythroid progenitor cell culture system could facilitate erythroid progenitor proliferation and differentiation and the culture system was suitable for the study of erythropoiesis.

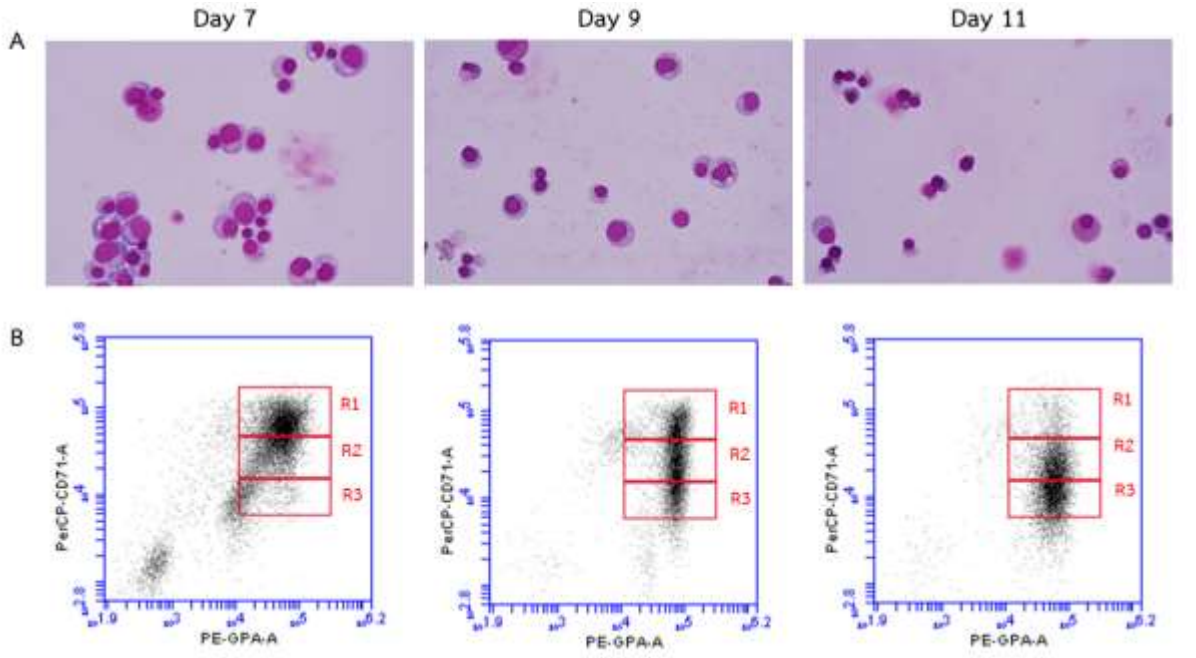

Figure 1 Differentiation of erythroid progenitor cells in culture system. Differentiation of erythroblasts on days 7, 9, and 11 of culture from three healthy donors was examined by light microscopic analysis and flow cytometry. (A) Morphological analysis of erythroid progenitor cells by stained with Wright-Giemsa and observed under the light microscope. (B) Surface expression of erythroid markers glycophorin A (GPA) and transferrin receptor (CD71) was measured by flow cytometry. R1, basophilic erythroblasts; R2, polychromatic erythroblasts; R3, orthochromatic erythroblasts.

Expression of BPGM during erythropoiesis

2,3-BPG is important for hemoglobin to efficiently deliver oxygen. The gene encoding for BPGM, a 2,3-BPG synthesis enzyme, and α-globin and β-globin, components of hemoglobin, may express simultaneously and tightly correlated. Herein, the expression pattern of genes encoding for BPGM, α-globin, and β-globin during erythropoiesis was examined by RT-qPCR. The results revealed that BPGM gene expression had a trend to be highest on days 7 and 9 of culture and it was decreased to the lowest level on day 11. However, a significant change of BPGM during erythropoiesis wasn't reached statistical significance (Figure 2A). While the expression of α- and β-globin genes was also highest on days 7 and 9 and significantly decreased on days 11 ($P < 0.05$) (Figure 2B and C). The level of α- and β-globin genes was higher compared to that of BPGM. It is noteworthy that the expression pattern was similar among the three genes. To determine whether the three genes had any synchronized expression, Pearson correlation test was performed.

The results showed the significance of the positive correlation between α-globin and β-globin ($r = 0.86$, $P < 0.0001$) indicated that the expression of the two genes was tightly simultaneous (Figure 3). However, expression of BPGM and α-globin or β-globin was not observed.

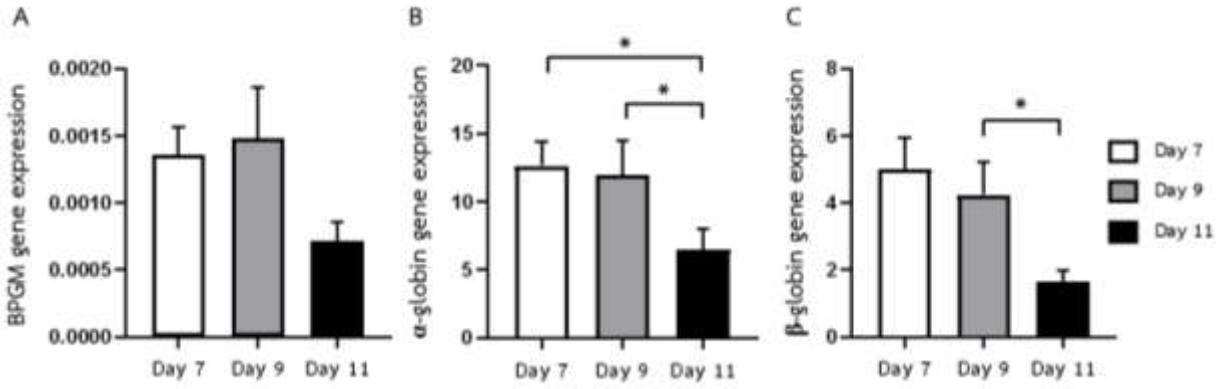

Figure 2 Expression of BPGM during erythropoiesis. The gene expression level of (A) BPGM, (B) α-globin, and (C) β-globin on days 7, 9, 11 of phase II culture from seven healthy donors was determined by RT-qPCR. The results were represented as mean with the standard error of the mean (SEM). * Statistical significance at $P < 0.05$

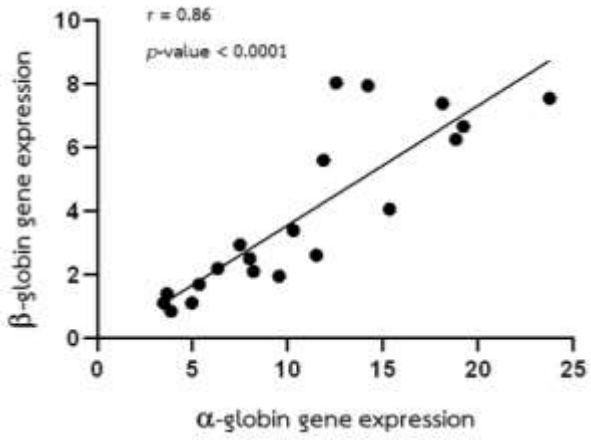

Figure 3 Correlation between α-globin and β-globin gene expression.

Discussion

2,3-BPG is required for efficiently deliver oxygen of Hemoglobin. The concentration of 2,3-BPG is modulated by changes in tissue oxygen demand in the body such as anemia, congenital heart disease, and high altitude. Synthesis of hemoglobin and its allosteric modulator, 2, 3-BPG, may need to be synchronized. This study showed the expression pattern of BPGM together with α-globin and β-globin was the same.

2,3-BPG is important in oxygen transport because it is a heterotrophic allosteric effector of oxygen binding by hemoglobin, by binding preferentially to the deoxygenated form of hemoglobin, it decreases the apparent affinity of hemoglobin for oxygen. The structure of hemoglobin is presented in two forms and the first one is a tense state (T state) which is deoxyhemoglobin and unstable form due to the positively charged pocket at the central cavity. Thus, the T state is likely to bind with oxygen to become a relaxed state. For the second form, the relaxed state (R state) is a fully oxygenated form (Berg et al., 2012). Hemoglobin can transport oxygen efficiently by cooperating with an anionic molecule called 2,3-BPG. Since pure hemoglobin binds tightly with oxygen and a small amount of oxygen is released in the tissues. In the presence of 2,3-BPG in the erythrocyte, this molecule binds to the center of hemoglobin and interacts with positively charged to stabilize the T state (Sahu et al., 2007). Therefore, the oxygen affinity of hemoglobin is reduced and oxygen is released more to the tissue compared to the absence of 2,3-BPG.

Hemoglobin is initially produced in the proerythroblastic stage. Then, the cells were differentiated to orthochromatic erythroblasts and the size of cells was decreased and showed a pyknotic nucleus which chromatin was condensed and prepared to extrude outside the erythroid cell with the gene expression levels was significantly reduced (Moras et al., 2017). In this study, the expression of α-globin and β-globin genes was highest on days 7 and 9 of culture which basophilic erythroblasts and polychromatic erythroblasts were the main populations, respectively. Then, the expression of both globin genes was decreased on day 11 that the main populations were polychromatic erythroblasts and orthochromatic erythroblasts. A recent study reported BPGM expression at immature erythroid cells

which presented CD71 or transferrin receptor (Mello et al., 2019). However, the study did not show BPGM expression level in detail at each state of erythroblast subpopulation. Here we showed the trend of BPGM expression was similar to α- and β-globin gene expression. The lowest BPGM expression was on day 11 of phase II culture. Nevertheless, no significant difference was presented on each day in BPGM and the statistical analysis showed the expression of α-globin and β-globin genes had a strong positive correlation indicating tightly synchronized expression of the two genes. The α-globin and β-globin genes were both regulated at the transcription level. To activate transcription and produce a high level of globin expression required enhancer elements, major regulatory element in α-globin cluster and locus control region in β-globin gene cluster, located upstream of the two globin gene clusters. These regions were characterized by DNaseI hypersensitive sites (DNaseI HS sites) (Cao, Moi, 2002; Philipsen, Hardison, 2018). Erythroid transcription factor GATA-1 binds to globin promoters and DNaseI HS sites played an important role in activating both α-globin and β-globin gene expression. (Anguita et al., 2004; Moriguchi, Yamamoto, 2014). Thus, the transcription of α-globin and β-globin genes was controlled and activated simultaneously due to the same set of transcription factors. On the other hand, there was no significant correlation between BPGM and the two globin gene expression. This may cause by different regulation of BPGM. The enzyme activity is mostly regulated at the protein level. BPGM is phosphorylated and activated by AMP-activated protein kinase (AMPK) and this protein plays a crucial role in the maintenance of energy homeostasis (Liu et al., 2016; Sayama et al., 2020). In addition, 2,3-BPG production is also metabolically influenced. BPGM is regulated by feedback inhibition with the 2,3-BPG product (Hsia 1998).

In summary, this study provided a basic understanding of the BPGM expression during erythropoiesis in relationship with α- and β-globin gene expression. As 2,3-BPG is important for hemoglobin function adaptive to oxygen level, these data may be used as fundamental for future study in other diseases such as hemoglobinopathy and hemolytic anemia.

Conclusion

2,3-BPG is essential for hemoglobin function. Here, we examined expression of genes responsible for hemoglobin and 2,3-BPG synthesis. The gene expression pattern of BPGM during erythropoiesis was similar trend as α-globin and β-globin with the highest expression at basophilic erythroblast and polychromatic erythroblast.

Acknowledgements

This work was supported by Office of the Permanent Secretary, Ministry of Higher Education, Science, Research and Innovation (RGNS 63-161), Mahidol University (MRC-MGR 01/2565) and Bilateral Programs, Japan Society for the Promotion of Science (JSPS) and National Research Council of Thailand (NRCT). DN was supported under Mahidol Postgraduate Scholarship, Faculty of Graduate Studies, Mahidol University.

References

Ahmed MH, Ghatge MS, Safo MK. Hemoglobin: structure, function and allostery. Subcell Biochem 2020; 94: 345-82.

Anguita E, Hughes J, Heyworth C, Blobel GA, Wood WG, Higgs DR. Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2. EMBO J 2004; 23(14): 2841-52.

Berg JM, Tymoczko JL, Stryer L. Biochemistry. New York: W. H. Freeman and Company; 2012.

Cao A, Moi P. Regulation of the globin genes. Pediatr Res 2002; 51(4): 415-21.

Chu WT, Zheng QC, Zhang HX. Insights into the phosphatase and the synthase activities of human bisphosphoglycerate mutase: a quantum mechanics/molecular mechanics simulation. Phys Chem Chem Phys 2014; 16(9): 3946-54.

Dai Y, Sangerman J, Luo HY, Fucharoen S, Chui DH, Faller DV, et al. Therapeutic fetal-globin inducers reduce transcriptional repression in hemoglobinopathy erythroid progenitors through distinct mechanisms. Blood Cells Mol Dis 2016; 56(1): 62-9.

Dzierzak E, Philipsen S. Erythropoiesis: development and differentiation. Cold Spring Harb Perspect Med 2013; 3(4): a011601.

Hsia CC. Respiratory function of hemoglobin. N Engl J Med 1998; 338(4): 239-47.

Liu H, Zhang Y, Wu H, D'Alessandro A, Yegutkin GG, Song A, et al. Beneficial role of erythrocyte adenosine A2B receptor-mediated AMP-activated protein kinase activation in high-altitude hypoxia. Circulation 2016; 134(5): 405-21.

Mello FV, Land MGP, Costa ES, Teodósio C, Sanchez ML, Bárcena P, et al. Maturation-associated gene expression profiles during normal human bone marrow erythropoiesis. Cell Death Discov 2019; 5: 69.

Moras M, Lefevre SD, Ostuni MA. From erythroblasts to mature red blood cells: organelle clearance in mammals. Front Physiol 2017; 8: 1076.

Moriguchi T, Yamamoto M. A regulatory network governing Gata1 and Gata2 gene transcription orchestrates erythroid lineage differentiation. Int J Hematol 2014; 100(5): 417-24.

Moura IC, Hermine O, Lacombe C, Mayeux P. Erythropoiesis and transferrin receptors. Curr Opin Hematol 2015; 22(3): 193-8.

Philipsen S, Hardison RC. Evolution of hemoglobin loci and their regulatory elements. Blood Cells Mol Dis 2018; 70: 2-12.

Sahu SC, Simplaceanu V, Gong Q, Ho NT, Tian F, Prestegard JH, et al. Insights into the solution structure of human deoxyhemoglobin in the absence and presence of an allosteric effector. Biochemistry 2007; 46(35): 9973-80.

Sayama S, Song A, Brown BC, Couturier J, Cai X, Xu P, et al. Maternal erythrocyte ENT1-mediated AMPK activation counteracts placental hypoxia and supports fetal growth. JCI Insight 2020; 5(10): e130205.

Shi L, Lin YH, Sierant MC, Zhu F, Cui S, Guan Y, et al. Developmental transcriptome analysis of human erythropoiesis. Hum Mol Genet 2014; 23(17): 4528-42.