รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาบทบาทของไมโตฟาจีในกระบวนการสร้างเม็ดเลือดแดง

โดย นางสาวอรพรรณ ศรีพิชัย และคณะ

1 กรกฎาคม 2557

สัญญาเลขที่ MRG5580049

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Abstract

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Project Title : Role of mitophagy in erythropoiesis

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Anemia in β-thalassemia/HbE patients is due to an overall reduction in hemoglobin production, ineffective erythropoiesis and hemolysis of mature erythrocytes. Immature reticulocyte is an indicator of stress erythropoiesis and is markedly increased in these patients. Significant increases in reticulocyte percentage and immature reticulocyte fraction were observed in severe and moderate cases compared to mild cases, suggesting a higher degree of bone marrow stimulation in the former groups. In addition, an *ex vivo* reticulocyte culture exhibited delay mitochondrial clearance in β-thalassemia/HbE reticulocytes compared to normal reticulocytes. Mitochondria in erythroblast are primarily responsible for ATP and heme synthesis, and undergo elimination during terminal differentiation. Removal of unwanted and damaged mitochondria, before it causes activation of cell death, occurs by mitochondrial autophagy (mitophagy). In order to study the mitochondria dynamic during erythropoiesis, an *ex vivo* erythroblast culture were performed. Inductions of autophagy in early stage of differentiation were observed in thalassemic erythroblast. The stimulation of autophagy was concomitant with the transcriptional upregulation of several mitophagy-related genes, in which modulation of its levels affects number of mitochondria and the amount of cell death. Together, these results suggested that the dysregulation of mitochondrial clearance in β-thalassemia/HbE may contribute to the pathophysilogy of patients.

Keywords : erythropoiesis, reticulocyte, thalassemia, mitophagy

บทคัดย่อ

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ภาวะโลหิตจางในผู้ป่วยโรคเบต้า-ธาลัสซีเมียเกิดจากการสร้างฮีโมโกลบินในเซลล์เม็ดเลือดแดงลดลง ร่วมกับมีกระบวนการสร้างเม็ดเลือดแดงที่ไม่มีประสิทธิภาพ และเกิดการแตกของเม็ดเลือดแดงตัวเต็มวัย ผู้ป่วยเบต้า-ธาลัสซีเมียมีจำนวนเรคติคคิวโลไซต์ระยะตัวอ่อนสูงขึ้นซึ่งเป็นตัวบ่งชี้ว่ามีการสร้างเม็ดเลือดแดงภายใต้ภาวะกดดัน โดยผู้ป่วยที่มีอาการมากและอาการปานกลางพบว่ามีปริมาณเรคติค-คิวโลไซต์และสัดส่วนของเรคติคคิวโลไซต์ระยะตัวอ่อนที่สูงกว่าในผู้ป่วยที่มีอาการน้อยซึ่งแสดงให้เห็นว่ามีระดับการกระตุ้นไขกระดูกให้สร้างเม็ดเลือดแดงที่สูงกว่า นอกจากนี้จากการเพาะเลี้ยงเรคติคคิวโลไซต์ในหลอดทดลองพบว่าเรคติคคิวโลไซต์โรคเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีมีการกำจัดไมโตคอนเดรียช้ากว่าเรคติคคิวโลไซต์ปกติ ไมโตคอนเดรียเป็นแหล่งผลิตพลังงานและเป็นที่ผลิตฮีมสำหรับสร้างฮีโมโกลบินในเม็ดเลือดแดงตัวอ่อน และจะถูกกำจัดเมื่อเซลล์เม็ดเลือดแดงตัวอ่อนเจริญถึงระยะสุดท้ายของการเจริญเติบโต ไมโตคอนเดรียที่เซลล์ไม่ต้องการแล้วหรือไมโตคอนเดรียที่เสียสภาพจะถูกกำจัดด้วยกระบวนการออโตฟาจีของไมโตคอนเดรีย (ไมโตฟาจี) ซึ่งจะเกิดขึ้นก่อนที่ไมโตคอนเดรียเหล่านี้จะกลายเป็นสาเหตุของการกระตุ้นให้เกิดการตายของเซลล์ จากการเพาะเลี้ยงเม็ดเลือดแดงตัวอ่อนในหลอดทดลองเพื่อศึกษาการเปลี่ยนแปลงไมโตคอนเดรียในระหว่างกระบวนการสร้างเม็ดเลือดแดง พบว่าเซลล์เม็ดเลือดแดงตัวอ่อนโรคธาลัสซีเมียมีการเพิ่มขึ้นของออโตฟาจีในระยะเริ่มต้นของการเจริญเติบโตของเซลล์ การกระตุ้นออโตฟาจีนี้พบว่าเกิดขึ้นพร้อมกับการเพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการไมโตฟาจีหลายตัว ซึ่งการเปลี่ยนแปลงปริมาณของยีนเหล่านี้จะส่งผลต่อจำนวนไมโตคอน-เดรียในเซลล์และการตายของเซลล์ ผลจากการศึกษาวิจัยเหล่านี้แสดงให้เห็นว่าการควบคุมกระบวนการกำจัดไมโตคอนเดรียที่ผิดปกติในเม็ดเลือดแดงโรคเบต้า-ธาลัสซีเมีย/ฮีโมโกลบินอีอาจส่งผลกระทบต่อพยาธิสภาพของโรคในผู้ป่วย

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INTRODUCTION

Cell growth and differentiation requires a well-controlled balance of organelle biosynthesis versus turnover. Autophagy is the major pathway for degradation of cellular constituents, such as the cytosol, organelles and protein aggregates, which is particularly important during development and under certain stress conditions.[1] Macroautophagy is the major inducible pathway for the cytoplasmic components turnover in all nucleated cell types. The process involved in the sequestered of the portions of cytoplasm with double-membrane vesicles, known as autophagosomes in yeast or autophagic vacuoles in mammalian cells, which transport cytoplasmic cargo to the lysosome for degradation.[2,3]

Mitochondria perform essential functions for the cell, such as ATP production via oxidative phosphorylation, heme biosynthesis, and calcium homeostasis. Degradation of mitochondria (mitophagy) is a major mechanism of mitochondrial turnover. If the energetic demand is low, excess mitochondria are unnecessary and may generate excessive reactive oxygen species (ROS), therefore mitophagy is an efficient cytoprotective response.[4] Some damage to mitochondria can lead to the depolarization of the inner mitochondrial membrane, thereby sensitizing impaired mitochondria for selective elimination by mitophagy. Furthermore, damaged mitochondria may leads to release of ROS, cytochrome c and apoptosis-inducing factor (AIF), triggering caspase activation and apoptosis.[5] Hence, clearance of unstable and potentially dangerous mitochondria will maintain cellular homeostasis. In addition to a role in mitochondrial quality control and preventing cell death, mitophagy has been shown to have a role in differentiation of T lymphocyte and reticulocytes.[6,7]

Erythroid differentiation involves progression through morphologically distinct nucleated precursor stages, from proerythroblast to orthochromatic erythroblasts, prior to enucleation and maturation of the nascent reticulocyte. Reticulocytes undergo extensive membrane remodeling, volume changes, and elimination of all intracellular organelles, to ensure that critical function, such as hemoglobin production, oxygen transportation, and deformability are optimized in mature red blood cells. This transformative process results in a successive reduction in cell volume, massive increase in hemoglobin production and conversion to a purely glycolytic

pathway for ATP production. Since approximately 30% of red cell hemoglobin is produced in reticulocytes, and heme is synthesized in the mitochondria these organelles are among the last to be eliminated.[8] Recent studies in erythroid cells have shown that autophagy is an important process for the physiological clearance of mitochondria during terminal differentiation.[7,9] However, the molecular pathways involved in mitophagy during erythropoiesis, especially in reticulocytes are not fully understood, although several important insights have been made into this process.

Molecular genetic studies in yeast and mammalian cells have characterized many of genes involved in all forms of autophagy. The four major factors of autophagy are Beclin1, Atg5, Atg7 and Atg8.[10] Similar to autophagy of other cellular organelles, mitophagy is sequestration by autophagosomes and followed by delivery to the lysosomal compartment for degradation. Although the molecular machinery of mitophagy has not been fully uncovered, Atg1/Ulk1, Bnip3L, Perkin and Pink1 have been identified as mitophagy-related genes.

a.) Beclin 1 (BECN1), an orthologue of yeast Atg6, is a critical effecter of autophagy.[11] Interaction of Beclin 1 and its cofactors promote the Beclin-1 core complex, which is involved in the initiation of autophagosome formation. In contrast, the BH3 domain of Beclin 1 is interacted with Bcl-2 and Bcl-xL to inhibit autophagy.[12,13] Caspase-mediated cleavage of Beclin1 reduces the level of autophagy and promotes crosstalk between apoptosis and autophagy.[14,15]

b.) ATG5 is an autophagic regulator required at the stage of autophagosome-precursor synthesis.[16] Atg5 knockout mice appear normal at birth, but exhibit amino acid and energy insufficient, and die within 10 h after birth[17]. ATG5 is found to be cleaved during apoptosis and the cleavage product appears to promote mitochondria-mediated apoptosis.[18]

c.) ATG7 encodes the E1-like enzyme and is an autophagy protein which required for the elongation phase of autophagosome formation.[19] ATG7 plays a key role in mitochondrial autophagy in mammalian hematopoietic cells. A mouse knocked out for Atg7 in the hematopoietic system develop severe anemia.[20] The absence of ATG7 in erythroid cells leads to an accumulation of damaged mitochondria with altered membrane potential, resulting in cell death.[7] Increased death in both nucleated and enucleated erythroid cells was observed in Atg7 knockout erythroid cells, suggesting that mitophagy starts in nucleated erythroblasts and is ongoing until the mature erythrocyte stage is reached.

d.) Atg8, a ubiquitin-like protein, is central coordinators of autophagosome assembly, maturation and lysosomal fusion.[2] LC3, an autophagosomal ortholog of yeast Atg8, is subjected to lipidation processing during formation of autophagosomes and incorporated into the

autophagosomal membrane where it promotes recruitment of cargo.[21] A lipidated form of LC3, LC3-II, has been shown to be an autophagosomal marker.

e.) ULK1, a serine/threonine kinase homologs of yeast Atg1, is a key initiator of autophagy and played important role in clearance of mitochondria and RNA-containing ribosomes in reticulocytes.[22] Ulk1-deficient mice exhibit a mild anemia and defect in reticulocyte maturation, which impairs the mitophagy and clearance of ribosomes.[23]

f.) BNIP3L, a BH3-only member of Bcl-2 family also called NIX, was shown to be essential in the normal maturation of erythrocytes and its absence in mice caused a mild, nonlethal anemia.[24] BNIP3L, which is up-regulated during terminal erythroid differentiation, functions as an autophagy receptor to mediates mitochondria clearance after mitochondrial damage and during erythroid differentiation by target mitochondria to autophagosome-like structures.[25,26]

g.) Parkin (PARK2), an E3 ligase located in the cytosol and originally discovered as mutated in Parkinson's disease which showed abnormalities in mitochondrial respiration and increased oxidative stress, that also exhibit increased mitochondrial autophagy.[27] Parkin was found to specifically recruit from the cytosol to damaged mitochondria to induce their mitophagy.[28]

h.) PINK1 (PTEN-induced kinase 1) is a cytosolic and mitochondrially localized 581-amino acid serine/threonine kinase that harbors N-terminal mitochondrial targeting sequence.[29] PINK1 in mitochondria is regulated by voltage-dependent proteolysis to maintain low levels on healthy and polarized mitochondria, while facilitating the rapid accumulation of PINK1 on damage mitochondria. Recent studies indicate that PINK1 and Parkin interact genetically to prevent oxdative stress and PINK1 accumulation in mitochondria is necessary and sufficient for Parkin recruitment and subsequent autophagy of depolarized mitochondria.[30] In HeLa cells, PINK1 knockdown results in significant alterations of mitochondrial morphology and a loss of mitochondrial membrane potential.[31] PINK1 is upregulated during terminal erythroid differentiation,[32] however the role of PINK1 in erythropoiesis, specifically erythroid mitophagy was not studied. Interestingly, microarray study showed differentially express of PINK1 in β-thalassemia erythroblast compared to that of normal control, suggesting its role in stress erythropoiesis (Fucharoen S *et al.*, unpublished data).

Thalassemia is a form of chronic anemia due to an inherited deficiency in the production of the globin chain and hemoglobin production; mostly are α-thalassemia and β-thalassemia. Individuals with α-thalassemia have deficient production of α-globin chain whereas those with β-thalassemia have deficient β-globin chain production.[33,34] Defective synthesis of one of the globin chain leads to the imbalance of α/non-α chains production and consequently

precipitation of the excessive unmatched normal synthesized globin chains in erythroblasts (causing ineffective erythropoiesis) and mature erythrocytes (causing hemolysis).[35] The excess globin chains precipitate in the bone marrow, lead to red blood cell membrane rigidity and ineffective erythropoiesis, and premature red blood cell destruction results in anemia.

Abnormal production of α-globin chains results in a relative excess of γ-globin chains in fetuses and newborns and of β-globin chains in children and adults in α-thalassemia. The β-globin chains are capable of forming soluble tetramers (β4, HbH) that is unstable and tends to precipitate within the cells, forming insoluble inclusions that damage the red cell membrane. HbH containing red blood cells are sensitive to oxidative stress, thus more susceptible to hemolysis when oxidants are administered. Aging erythrocytes contain more precipitated HbH than younger erythrocytes, consequently they are removed from the circulation prematurely. However, HbH inclusions are rare in α-thalassemia bone marrow and erythropoiesis is apparently effective. Therefore, anemia in α-thalassemia is primarily causing from reduced hemoglobinization of individual red blood cells and hemolysis.

In β-thalassemia, the degraded products of unbound α-globin such as heme, hemichrome and free iron cause oxidative damage of erythroid cells which leads to ineffective erythropoiesis and shortened red-cell survival, consequently severe anemia. β-Thalassemia bone marrow shows marked erythroid hyperplasia and accelerated erythroid differentiation. However, despite the apparent increased erythroid commitment, ineffective erythropoiesis occurs due to apoptosis at the polychromatophilic erythroblasts.[36,37] The tight correlations between the extent of apoptosis and the erythroid expansion as measured either by the absolute number of marrow erythroid precursors or by serum soluble transferrin receptor levels suggesting that the extreme rates of erythroid proliferation lend themselves to cellular errors that turn on apoptotic programs.[38] Hence, the most deleterious effects on erythropoiesis in β-thalassemia patients occur as a result of ineffective erythropoiesis and hemolysis of mature red cells. A significant higher degree of autophagy induction was seen in β-thalassemic erythroblasts as compared to that of normal control.[39] Based on the dual functions of autophagy, the induction of autophagy in β-thalassemia may either contribute to the increased levels of apoptosis that leads to ineffective erythropoiesis or being the response mechanism for cell survival under the stress erythropoiesis. To better understand the role of autophagy in erythropoiesis and thalassemia, therefore, the further explore in molecular mechanism and the comparative study among healthy normal, β-thalassemia and α-thalassemia will be employed in the present study.

MATERIALS AND METHODS

Study Samples and Ethical Consideration

Potential participants received full explanations of the nature of the disease and the procedures of examinations from both physician and participant project information sheet (appendix-1-II and -III). Each subject was involved only in a physical examination for disease diagnosis, a one-time donation of a blood sample, and the completion of a patient informed consent form (appendix-1-iV). Informed consent, which was approved by the Institutional Review Boards of Mahidol University (Bangkok, Thailand), as shown in appendix-1, was obtained from each subject before the collection of blood samples. Clinical data was obtained from the patients' interviews, medical records and physical examination by a physician. All patients' clinical and hematologic data were recorded in individual patient record forms and in an electronic file (Microsoft Office Excel 2007, Microsoft Corporation). To ensure confidentiality and protect the privacy of the subjects in processes, they were identified by an in house code instead of by individual name. Only authorized personal were able to access confidential data of the patients.

Mitochondria clearance analysis in reticulocyte

1. Reticulocyte enrichment and *ex vivo* culture

Venous blood was collected into EDTA tubes. Complete blood counts including reticulocyte count were determined using an automated blood cell analyzer (ADVIA 120, Bayer, NY, USA). Enriched reticulocytes were isolated from 3 mL EDTA blood samples by using gradient density 70% Percoll and centrifugation. First, the EDTA blood was transferred to 15 mL centrifuge tube then centrifuged at 3000 rpm for 5 minutes. Supernatants were discarded and resuspended packed cell to 20% hematocrit with RPMI incomplete medium. Four milliliters of cell suspensions were overlayed on 4 ml of 70% Percoll. Cells were centrifuged at 1200 g for 30 minutes without break. After centrifugation at room temperature for 30 minutes, the samples were separated to four phases; plasma phase, reticulocyte phase, percoll phase and pack red cell phase, respectively. The reticulocyte phase was collected into new 15 mL centrifuge tubes and washed enrich reticulocytes 3 times with 10 mL of RPMI 1640. Enrich reticulocytes were cultured in RPMI supplemented with 20% FBS (Sigma) and antibiotics (penicillin, streptomycin) for 4 days.

2. MitoTracker and Thiazol-orange staining

A total of $2x10^6$ red blood cells, approximately 2 uL of blood was diluted with 500 uL of PBS buffer. To stain mitochondria, 500 uL of the dilution were incubated for 30 minutes at 37°C with 200nM MitoTracker deep red (Molecular Probes). The cells were subsequently washed

with cold PBS and incubated with thiazole-orange (Sigma) for 40 minutes at room temperature, after that washed twice with cold PBS. Subsequently MitoTracker and thiazole-orange cells were co-stained with appropriate concentrations of FITC-anti-CD71 (BD Biosciences). Samples were analyzed on a BD FACSCanto-II using FACSDiva Software ver 6.1.3. RBC-gate was set from forward scatter (FSC)/side scatter (SSC). Unstained cells were used as negative controls.

Mitophagy analysis during erythropoiesis

1. Human hematopoietic CD34+ progenitor cells separation

Blood samples were taken from five healthy normal and five β-thalassemia/HbE patient volunteers who are aged at least 18 year-old. The healthy subjects must not carry any one of the α-thalassemia, β-thalassemia, and Hb variants. The β-thalassemia/HbE patients do not received blood transfusions at least 1 month. Primary progenitor cells (CD34+) were obtained from peripheral blood samples of healthy volunteers or thalassemia patients. The erythroid separation was carried out by density gradient centrifugation using Ficoll Hypaque following standard Ficoll-seperation technique. In brief, 20 mL of whole blood were collected in 50 mL centrifuge tube with anticoagulant citrate dextrose (ACD) after informed consent was obtained. The samples were centrifuged at 600 g for 10 minutes, and then discarded plasma. The samples were diluted to 20% hematocrit by using PBS containing 2mM EDTA and mixed by pipette. The samples were overlayed on Ficoll (Lymphoprep, Norway) then centrifuged at 600 g for 20 minutes. Mononuclear cells (MNCs) were collected into a new 50 mL centrifuge tube and washed twice with PBS containing 2mM EDTA. Then the supernatants were decanted and 10 mL of PBS-EDTA containing 0.5% HSA were added into the pellets. The cells were resuspended and counted by using hemocytometer chamber. Isolation of erythroid precursors were accomplished by the CD34 MicroBead Kit (Miltenyibiotec) according to the manufacturer's protocol.

2. Primary erythroblast cultures

Primary $CD34^+$ cells were cultured using two-phase culture system. Phase I culture media for day 0 to day 4 contains Iscove's modified Dulbecco's medium (IMDM; Gibco) adding 20% FBS (Gibco), 100 U/ml penicillin-steptomycin (Gibco), 0.3 mg/mL human holo-transferrin (Sigma), 50 ng/mL stem cell factor (SCF; R&D systems), 10 ng/mL interleukin-3 (IL3; Cell Signaling) and 2 U/mL erythropoietin (EPO; Cilag-Janssen), while phase II culture media for day 4 to day 14 contains 5 U/mL EPO without SCF and IL3. Cellular growth and differentiation were confirmed by flow cytometry and cytospin. The cultures were kept for up to 14 days at 37°C and 5% CO2 in a fully humidified incubator. Cell growth was monitored and cultures were replenished with fresh medium in order to maintain the cells at a concentration to $<1x10^6$ /mL

3. Cell staining, flow cytometry and microscopy

An approximately 1×10^5 cell cultures on day 8, 10, 12 and 14 were collected and stained by phycoerythrin (PE) mouse monoclonal anti-human CD71 (transferrin receptor) and allophycocyanin (APC) conjugated mouse monoclonal anti-human CD235a (glycophorin A) (BioLegend). One microliter of each 1:20 diluted antibody was added to cultured cells after washing once with 1 mL PBS. The cultured cells were stained for 15 minutes at room temperature in the dark. After staining, cells were suspended in PBS prior to analysis. All flow cytometric analyses were performed on a BD FACSCalibur flow cytometer. Fifty thousand cultured cells were prepared for cytospins (StatSpin) and air dried before staining with modified Giemsa stain (Sigma-Aldrich). Microscopy and photograph were done with an Olympus instrument. Apoptosis detection was carried out by AnnexinV-FITC/PI staining. Briefly, cells were harvested and washed with phosphate-buffered saline (PBS), then resuspended with AnnexinV-binding buffer and stained with Annexin V-FITC for 15 min at room temperature in the dark. Then, PI was added to the cells and further incubated for 5 min at room temperature. Cells were analyzed immediately using flow cytometer.

4. Mitochondrial damage and potential.

Cells were washed once in PBS and stained with 50 nM MitoTracker Green, 50 nM MitoTracker Deep Red and 20 nM TMRM. Cells were incubated in the dark for 30 min at 37°C. Cells were centrifuged at 500 × g for 5 min and suspended in RPMI without phenol red with 10% FBS. Cells were kept in the dark until measurement on FACS CANTO-II. Analysis was done with FACS Diva software.

5. RNA isolation and quantification

In order to investigate the transcripts levels, total RNA was isolated from $2\ x10^6$ cells of cultured erythroblasts using TRIzol reagent (Invitrogen) according to manufacturer's instructions. The reverse transcription (RT) reaction was carried out using the iScript cDNA™ synthesis kit (Bio-Rad) according to the manufacturer's instructions. TaqMan® Gene Expression Assays containing FAM-labeled primer/probe sets specific for gene-of-interest were obtained from Applied Biosystems (Table 1.). The real-time PCR reactions were performed in a 20 µl reaction volume containing 900 nM of each primer, 250 nM TaqMan probe, and 10 µl TaqMan Universal PCR Master Mix (ABI, Germany) on CFX96™ Real-Time System (Bio-Rad) according to the manufacturer's instructions. The PCR thermal cycle progress consisted of one cycle of denaturation for 5 min at 95°C; 40 cycles of denaturation for 15 sec at 95°C, annealing and extension for 1 min at 60°C. The relative fold changes will be calculated based on the ΔΔCt method by normalizing against glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

expression. This ΔCt value obtained with an individual sample was then subtracted from the ΔCt value obtained from the reference sample, giving a ΔΔCt value. Comparative gene expressions were analyzed further by comparison to a reference sample with the following formula based on the assumption that the amplification efficiencies of target and endogenous control genes are equal to 1: Relative expression $= 2^{-\Delta\Delta_{Ct}}$.

The mean values were normalized to the internal GAPDH control and were calculated from at least three independent experiments. Data were reported as the fold of the reference sample.

Table 1. TaqMan® gene expression assays used for real-time quantitative RT-PCR.

Gene Name	**TaqMan® Gene Expression Assays** *(Applied Biosystems)*
BECN1; Beclin1	Hs00186838_m1
ATG5; autophagy related 5	Hs00169468_m1
ATG7; autophagy related 7	Hs00197348_m1
ATG8/GABARAPL2; GABA(A) receptor-associated protein-like 2	Hs00371854_m1
ULK1; unc-51 like autophagy activating kinase 1	Hs00177504_m1
NIX/BNIP3L; BCL2/adenovirus E1B 19kDa interacting protein 3-like	Hs01087963_m1
PARK2; parkin RBR E3 ubiquitin protein ligase	Hs01038325_m1
PINK1; PTEN induced putative kinase 1	Hs00260868_m1

NIX and PINK1 shRNA Knockdown

1. Targeted shRNA expression constructs

Three different sequences of human NIX shRNA, PINK shRNA and one non-targeted shRNA were chosen randomly from human shRNA library provided by the Sigma Life Science (Sigma-Aldrich) and The RNAi Consortium (TRC) and then the double stranded shRNA was generated by annealing with its complementary strand. Details of selected shRNA and its complementary were shown in Table 2. In order to generate shRNA, several oligonucleotides were properly added; RNA Pol III transcription start site, hairpin loop sequence, RNA Pol III terminator sequence and 5' end *Xho*I overhang (Figure 1A). The annealed oligonucleotides were introduced into lentiviral expression vector pLL3.7-Puro which was digested with *Hpa*I and *Xho*I restriction enzymes. The map of pLL3.7-Puro is shown in Figure 1B. The ligation reaction of the fragment and vector were transformed into *Escherichia coli* strain DH5α. The transformant clones were analyzed by restriction analysis and the selected clones were verified by DNA sequencing.

Table 2. Human NIX shRNA, PINK1 shRNA, and non-targeted shRNA sequences and its complementary oligonucleotides (underlined).

NIX sh1	Sense: 5'-TGCCCTAAACGTTCTGTGTCTTTTTCAAGAGAAAAAGACACAGAACGTTTAGGGCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGCCCTAAACGTTCTGTGTCTTTTCTCTCTTGAAAAAGACACAGAACGTTTAGGGCA-3'
NIX sh2	Sense: 5'-TGGCTAGGCATCTATATTGGAAATTCAAGAGATTTCCAATATAGATGCCTAGCCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGGCTAGGCATCTATATTGGAAATCTCTTGAATTTCCAATATAGATGCCTAGCCA-3'
NIX sh3	Sense: 5'- TGCAGTCAGAAGAAGAAGTTGTATTCAAGAGATACAACTTCTTCTTCTGACTGCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGCAGTCAGAAGAAGAAGTTGTATCTCTTGAATACAACTTCTTCTTCTGACTGCA-3'
PINK1 sh1	Sense: 5'-TGCGGCTGGAGGAGTATCTGATATTCAAGAGATATCAGATACTCCTCCAGCCGCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGCGGCTGGAGTATCTGATATCTCTTGAATATCAGATACTCCTCCAGCCGCA-3'
PINK1 sh2	Sense: 5'-TGCCTAACCGTCTCCGCTTCTTCTTCAAGAGA GAAGAAGCGGAGACGGTTAGGCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGCCTAACCGTCTCCGCTTCTTCTCTCTTGAAGAAGAAGCGGAGACGGTTAGGCA-3'
PINK1 sh3	Sense: 5'-TGGAAGCCACCATGCCTACATTGTTCAAGAGA CAATGTAGGCATGGTGGCTTCCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGGAAGCCACCATGCCTACATTGTCTCTTGAACAATGTAGGCATGGTGGCTTCCA-3'
Non-targeted shRNA	Sense: 5'-TGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGAGATATCGCGCCTTTTTTC-3'
	Antisense: 5'-TCGAGAAAAAAGGCGCGATATCTCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCCA-3'

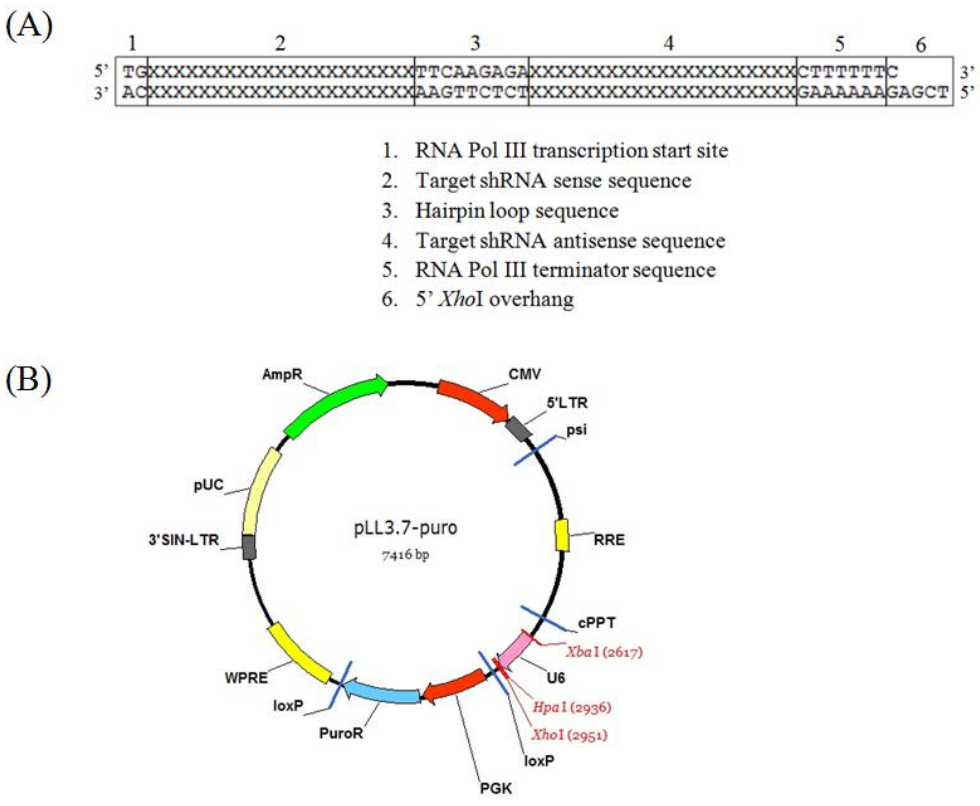

Figure 1. shRNA Expression Constructs. (A) Several oligonucleotides were properly added in order to generate shRNA of interest. (B) The physical map of third generation lentiviral expression vector pLL3.7-Puro. Not all the restriction sites are shown.

RESULTS

1. Mitochondria Clearance in Reticulocytes

Normal erythroid mitophagy begins during enucleation of the orthochromatophilic erythroblasts and continues in reticulocytes. Following enucleation of human orthochromatophilic erythroblasts, nascent reticulocytes mature over the course of 48-72 hours.

Heterogeneity of reticulocyte parameters in β-thalassemia/HbE. To study the reticulocyte maturation in stressed-erythroid terminal differentiation, the reticulocyte parameters were analyzed in the β-thalassemia/HbE cohort which included patients with different degrees of anemia from both non-spleenectomized and spleenectomized, but exclude patients who regularly (every 3 – 8 weeks) or recently (within 2 months) receive blood transfusion.[40,41] β-thalassemia/HbE patients were divided into 6 groups according to disease severity and genetic backgrounds (Table 3).

Table 3. Distribution of reticulocyte parameters in different β-thalassemia/HbE disease severity groups.

CHARACTERISTICS	Mild β^+/β^E	Mild β^0/β^E α-thalassemia	Mild β^0/β^E αα/αα	Moderate β^0/β^E αα/αα	Severe β^0/β^E αα/αα	Severe β^0/β^E Splenectomized	Total
No. (%)	15 (3.5)	46 (10.7)	128 (29.8)	86 (20.0)	36 (8.4)	119 (27.7)	430
Gender, female (%)	11 (73.3)	19 (41.3)	76 (59.4)	46 (53.5)	13 (36.1)	62 (52.1)	227 (52.8)
Age (year)	23.9 ± 23.4 (4 - 87)	16.8 ± 13.3 (3 - 57)	18.6 ± 13.9 (2 - 76)	18.2 ± 13.3 (2 - 49)	11.3 ± 7.1 (3 - 35)	18.3 ± 10.6 (5 - 53)	17.8 ± 13.0 (2 - 87)
Reticulocyte							
Retic (x10e9/L)	132.5 ± 42.3 (64 - 208)	160.6 ± 42.2 (72 - 250)	183.0 ± 49.3 (80 - 350)	198.5 ± 117.4 (51 - 863)	188.4 ± 45.6 (101 - 306)	574.8 ± 165.9 (233 - 1115)	290.5 ± 206.5 (51 - 1115)
Retic (%)	2.7 ± 1.0 (1.1 - 4.9)	3.4 ± 1.0 (1.6 - 5.1)	4.4 ± 1.2 (1.7 - 7.8)	5.7 ± 4.3 (1.1 - 37.2)	5.9 ± 1.8 (3.5 - 12.8)	18.6 ± 5.4 (7.4 - 38.1)	8.6 ± 7.2 (1.1 - 38.1)
MCVr (fL)	72.9 ± 6.8 (63.2 - 82.7)	70.9 ± 4.7 (59.8 - 79.3)	77.9 ± 6.0 (65.5 - 96.6)	77.4 ± 6.2 (65.1 - 89.8)	78.9 ± 5.4 (70.4 - 92.6)	81.2 ± 6.3 (68.2 - 101.0)	77.9 ± 6.7 (59.8 - 101.0)
CHr (pg)	20.5 ± 1.5 (18.2 - 23.3)	19.2 ± 1.4 (16.7 - 23.6)	20.8 ± 1.7 (16.8 - 27.9)	20.2 ± 1.6 (17.3 - 24.6)	20.0 ± 1.5 (17.7 - 24.4)	22.5 ± 1.9 (18.8 - 28.8)	20.9 ± 2.0 (16.7 - 28.8)
CHCMr (g/dL)	28.5 ± 1.3 (26.4 - 30.5)	27.4 ± 1.3 (24.8 - 30.8)	27.0 ± 1.3 (23.8 - 30.6)	26.6 ± 1.1 (24.1 - 29.2)	25.8 ± 0.8 (24.2 - 27.3)	28.1 ± 0.9 (25.7 - 30.3)	27.2 ± 1.3 (23.8 - 30.8)
L-Retic (%)	84.6 ± 9.1	77.4 ± 7.7	74.9 ± 7.4	70.0 ± 9.9	65.2 ± 10.6	46.5 ± 11.5	65.8 ± 15.7
M-Retic (%)	11.1 ± 5.1	15.6 ± 3.7	16.1 ± 3.4	17.3 ± 3.7	17.7 ± 4.0	21.6 ± 2.6	17.7 ± 4.3
H-Retic (%)	4.4 ± 4.5	7.1 ± 4.6	9.1 ± 4.8	12.7 ± 6.9	17.1 ± 7.4	31.9 ± 12.4	16.4 ± 12.9

Hematological analyses of β-thalassemia/HbE patients was performed using a Bayer Advia 120 hematology analyzer. Retic, reticulocyte; MCVr, reticulocyte mean corpuscular volume; CHr, reticulocyte hemoglobin content; CHCMr, reticulocyte mean corpuscular hemoglobin concentration; L-Retic, low fluorescence ratio reticulocyte; M-retic, medium fluorescence ratio reticulocyte; H-Retic, high fluorescence reticulocyte. Data are presented as mean±s.d. and range.

Ineffective erythropoiesis were presented in all patients as stated by extremely low reticulocyte hemoglobin content (CHr mean = 20.3 ± 1.6; range 16.7 – 27.9 pg). However the mean in the mild group showed no statistical difference compared to other groups. Significant increases

(P<0.001) in reticulocyte percentage and immature reticulocyte fraction (IRF) were observed in severe and moderate cases compared to mild cases. The reticulocyte counts were increased (5.9 ± 1.8%) with a relative increase in the most immature fractions (H-Retic: 17.1 ± 7.4%) in severe group and as expected the highest number was found in the patients with splenectomized (H-Retic: 31.9 ± 12.4%). Among the mild group, lower absolute reticulocyte counts, reticulocyte percentage and immature reticulocyte fraction were found in β^{+}-thalassemia/HbE and β^{0}-thalassemia/HbE coinheritance with α-thalassemia (P<0.01).

Detection of mitophagy in reticulocytes. In order to assess mitophagy in reticulocytes, mitochondria in cells were monitored by MitoTracker which accumulate in mitochondria independent of mitochondrial membrane potential and by tetramethyl rhodamine methy ester (TMRM) which accumulates only in polarized mitochondria. A slightly increase in the level of MitoTracker and TMRM were detected in alpha-thalassemia reticulocytes compared to normal control (Figure 2A and 2B, middle panel vs. upper panel). A largely increase in the level of MitoTracker and TMRM were detected in beta-thalassemia reticulocytes compared to normal control (Figure 2A and 2B, bottom panel vs. upper panel). However, more samples are required to confirm these finding and for further statistic analysis.

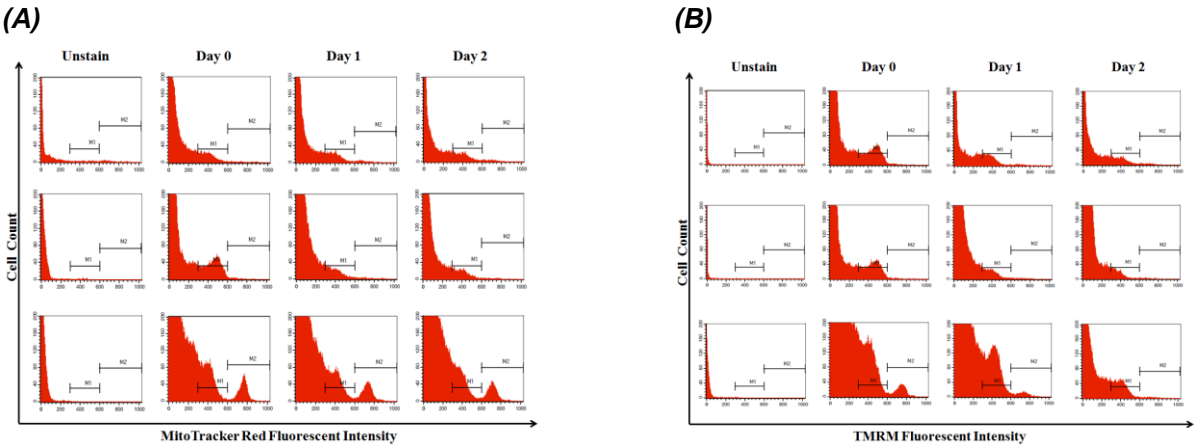

Figure 2. Flow cytometry analysis of (upper panel) normal, (middle panel) alpha-thalassemia, and (lower panel) beta-thalassemia reticulocytes stained with (A) MitoTracker Red and (B) TMRM at baseline (D0) or after 1 and 2 days in ex vivo culture.

Enrichment of reticulocyte from peripheral blood. As show in Table 3 that the very low number of reticulocytes is expressed in the peripheral blood, this was affected the detection of mitochondria in the mitochondria clearance study ; low positive cell population in normal control.

To overcome this problem, the enrichment of reticulocyte using density gradient centrifugation was performed in peripheral blood. The result show successfully enrichment of reticulocyte up to 30% when compared to 1% in un-enriched blood (Figure 3).

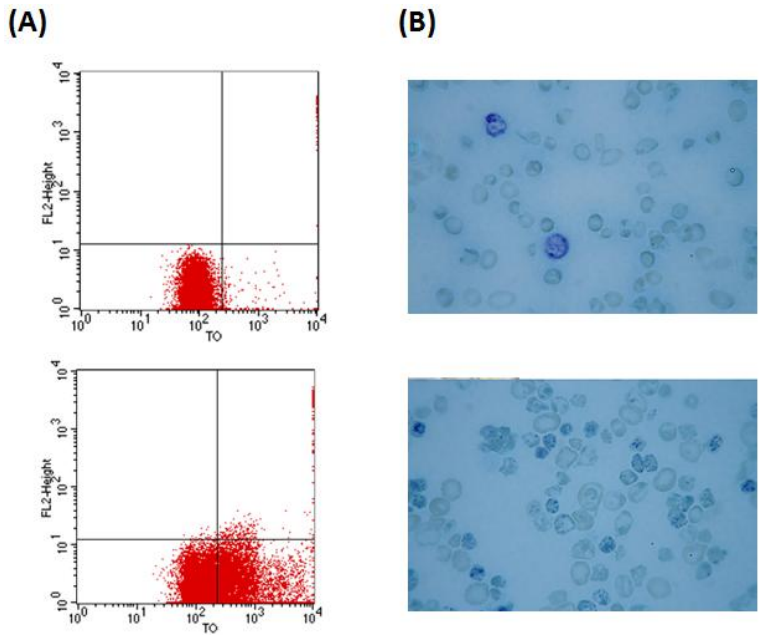

Figure 3. Analysis of enriched-reticulocyte blood. (A) Histogram plot of Thiazole orange (TO) positive cells in (upper panel) peripheral blood and (lower panel) enriched-reticulocyte blood. (B) Methyleneblue staining of (upper panel) peripheral blood and (lower panel) enriched-reticulocyte blood.

Immature reticulocyte fraction in β-thalassemia/HbE. Analysis of β-thalassemia/HbE erythrocytes by thiazol orange staining and flow cytometry confirmed the presence of marked reticulocytosis with elevated numbers of IRF. As representatively shown in Figure 4, second panel, RNA content levels in β-thalassemia/HbE erythrocytes were higher than those of α-thalassemia (HbH disease) and normal control. In addition to RNA content levels, the intensity of MitoTracker was also shown to be enhanced in thalassemia patients (Figure 4, third panel). Transferrin receptor positive reticulocytes are the youngest red blood cell cohort in blood.[42] Immature and transferrin receptor positive reticulocytes were observed in all cases (Figure 4, right panel). In these analyses, transferrin receptor positive cells were always thiazol-orange and MitoTracker positive.

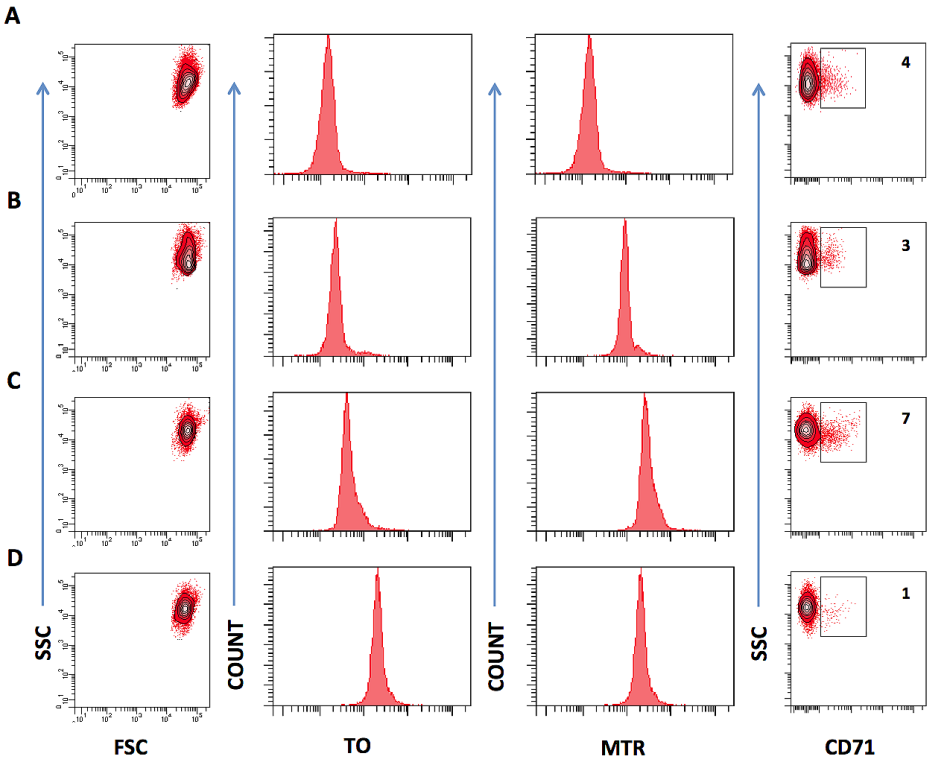

Figure 4. Flow cytometry analysis of erythroid cells from the peripheral blood. (A) Normal control, (B) α-thalassemia (HbH disease), (C) β-thalassemia/HbE with high reticulocyte count, and (D) β-thalassemia/HbE with low reticulocyte count. Left panel: contour plots of FSC versus SSC dot plot showing the erythrocyte population. Second panel: histogram plot showing thiazol-orange (TO) stained erythrocytes. Third panel: histogram plots showing MitoTracker (MTR) stained erythrocytes. Right panel: contour plots of CD71 versus SSC showing the percentages of CD71 erythrocytes positive population.

Delay mitochondrial clearance in β-thalassemia/HbE reticulocytes. In general, the amount of RNA in reticulocytes decreases as cells mature, and in older reticulocytes the various organelles decrease in number, usually mitochondria will be disappeared first and ribosomal last. Flow cytometry were used to detect mitochondria and monitor the mitochondrial clearance over three days in *ex vivo* culture. The expression of surface CD71 and the presence of mitochondria and RNA were used to distinguish between reticulocytes and mature red blood cells. CD71 is found on the surface of erythroblasts and reticulocytes, but is eliminated from red cell membranes. Enumerating reticulocyte were performed using thiazole orange for RNA staining. Mitochondria were detected by MitoTracker. The higher MitoTracker intensity in thalassemia erythrocytes (Figure 5, third panel) leading to further examination whether β-thalassemia/HbE reticulocytes might be defective in eliminating the mitochondria. Notably, less RNA and mitochondria contents were observed in freshly isolated β-thalassemia/HbE

reticulocytes (Figure 5, left panel). In contrast, fewer normal reticulocytes were negative for mitochondrial staining (Figure 5A, left panel). After culture for in vitro maturation for three days, RNA and mitochondria in normal reticulocytes were completely removed (Figure 5A, right panel). While a slightly decline of MitoTracker and thiazol-orange double positive reticulocytes was observed in β-thalassemia/HbE reticulocytes (Figure 5B and C).

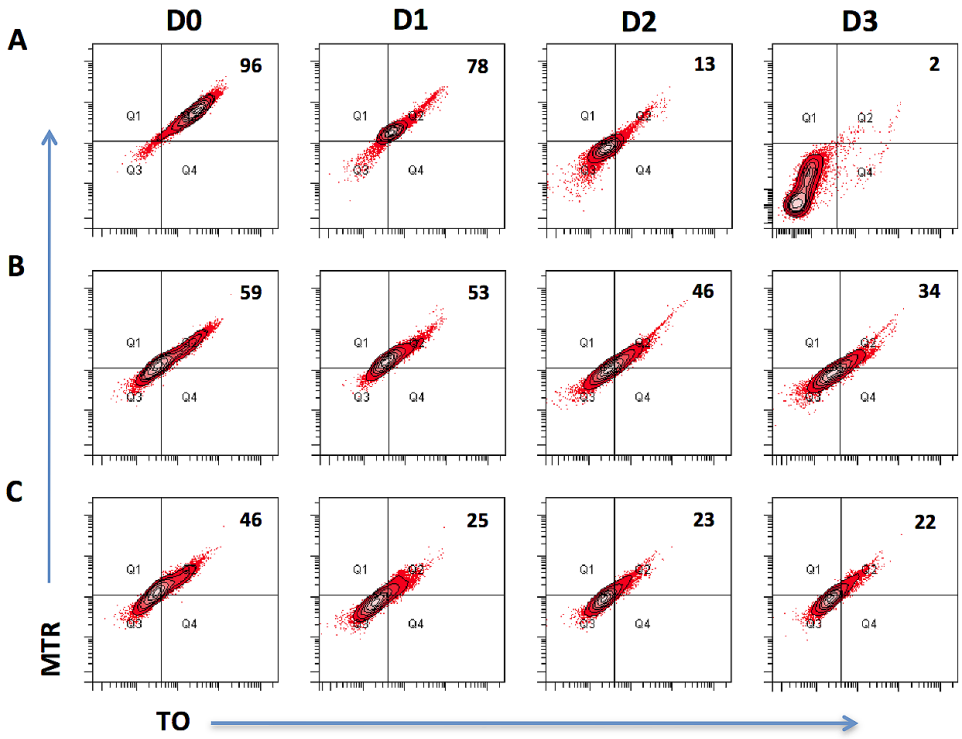

Figure 5. Flow cytometry analysis of the RNA and mitochondria contents in reticulocytes. Thiazol-orange (TO) and MitoTracker (MTR) contour plots of (A) non-thalassemic control, (B) β-thalassemia/HbE with high reticulocyte count, and (C) β-thalassemia/HbE with low reticulocytes count at in vitro cultured days 0, 1, 2, and 3.

2. Mitophagy during erythroblast differentiation.

Ex vivo primary CD34+ hematopoietic stem cells culture condition. To study mitophagy during erythropoiesis, a human *ex vivo* erythropoiesis culture system have been chosen and adapted from a previously developed protocol in which cell differentiate towards the erythroid lineage. This erythroblast culture system showed that it recapitulates all stages of erythroid differentiation as determined by flow cytometry using the fluorescence labeled antibody against erythroid surface markers, transferrin receptor (CD71) and glycophorin A (Figure 6).

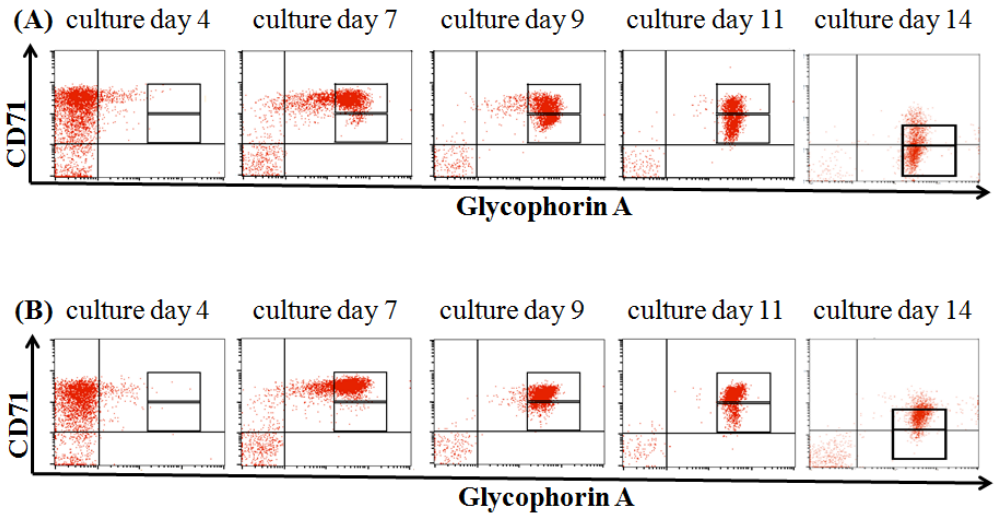

Figure 6. *Ex vivo* differentiation of erythroblast derived from (A) normal and (B) beta-thalassemia CD34+ progenitor cells study by flow cytometry using abtibody against erythroid markers: transferrin receptor (CD71) and Glycophorin A.

While the *ex vivo* vs. *in vivo* timing of differentiation might slightly differ, the demonstration that this system can obtain complete enucleation of erythroid cells for both normal and beta-thalassemia (Figure 7). Of note, percentage of enucleation in beta-thalassemia samples is lower than normal samples suggesting defect in the erythroid termination processes. These data validate the current *ex vivo* culture system is suitable for further study the mitophagy during erythropoiesis using progenitor cells of different origins.

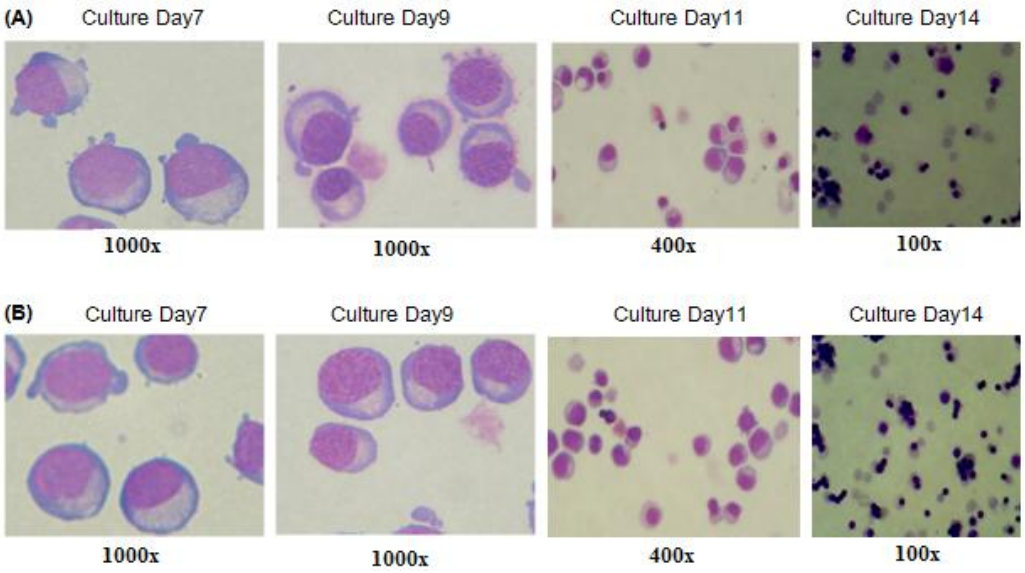

Figure 7. *Ex vivo* differentiation of erythroblast derived from (A) normal and (B) beta-thalassemia CD34+ progenitor cells study by Wright-Giemsa cytospin preparation and observed under light microscope.

By Mitotracker Red staining and flow cytometry, majority of day 7 cultured erythrocytes contained mitochondria, while β-thalassemia/HbE day 10 erythroblasts contain less mitochondria than normal erythroblast (Figure 8C). As expected, percentage of apoptosis cells is higher in β-thalassemia/HbE compared to normal erythroblast (Figure 8D).

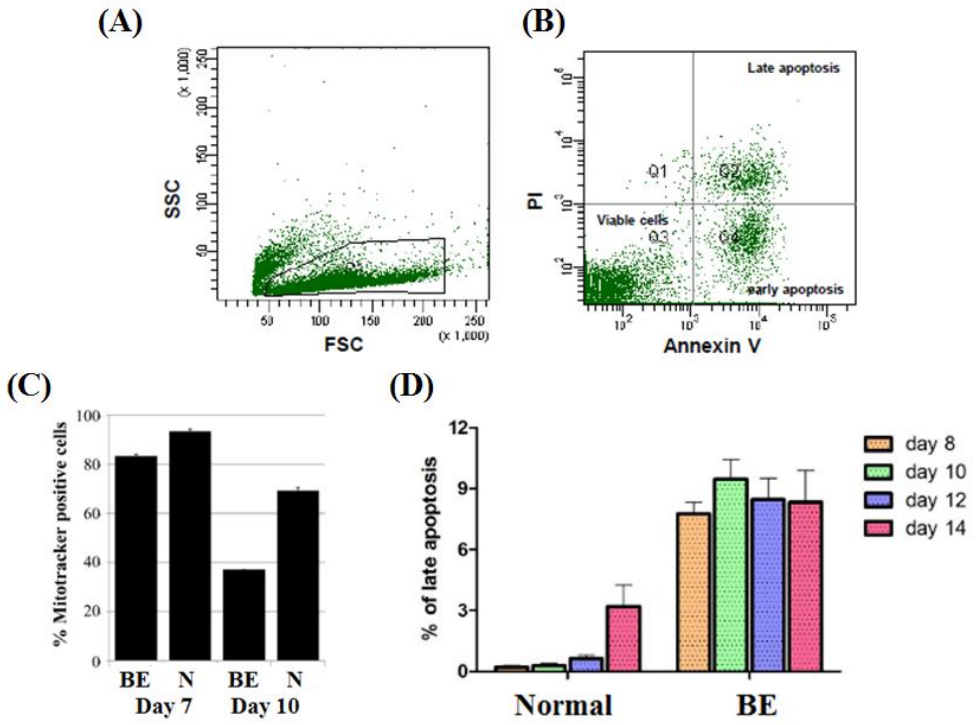

Figure 8. Flow cytometry analysis of cultured erythroblast. (A) Selected gate for analysis and (B) gating of apoptosis positive cells. (C) Mitochondrial content of cultured erythroblasts day 7 and day 10 was assessed by Mitotracker staining. (D) Percentage of late apoptosis in cultured erythroblasts day 8, 10, 12, and 14. BE; β-thalassemia/HbE and N; normal erythroblasts.

3. Molecular mechanism underlying mitophagy in thalassemic erythropoiesis.

To further extend the autophagy in thalassemia at the molecular level and identify the mechanisms that involve in stressed-erythroid mitophagy. This aim is designed to determine whether enhanced thalassemic erythroid autophagy/mitophagy is due to the activation/repression of certain autophagy- and mitophagy-related genes.

Differentially expression of autophagy-related genes in β-thalassemia/HbE. The primary progenitor cells (CD34+) culture was carried out using the protocol described above. Cultured erythroblasts were determined for stage of differentiation and sorted by flow cytometry into four subpopulations depending on the specific staining intensity: $CD71^{high}/GPA^{negative}$ corresponded

to proerythroblasts, $CD71^{high}/GPA^{positive}$ corresponded to basophilic and early polychromatophilic erythroblasts, $CD71^{medium}/GPA^{positive}$ corresponded to late polychromatophilic erythroblasts, and $CD71^{low}/GPA^{positive}$ corresponded to orthochromatophilic erythroblasts and reticulocytes (Figure 9).

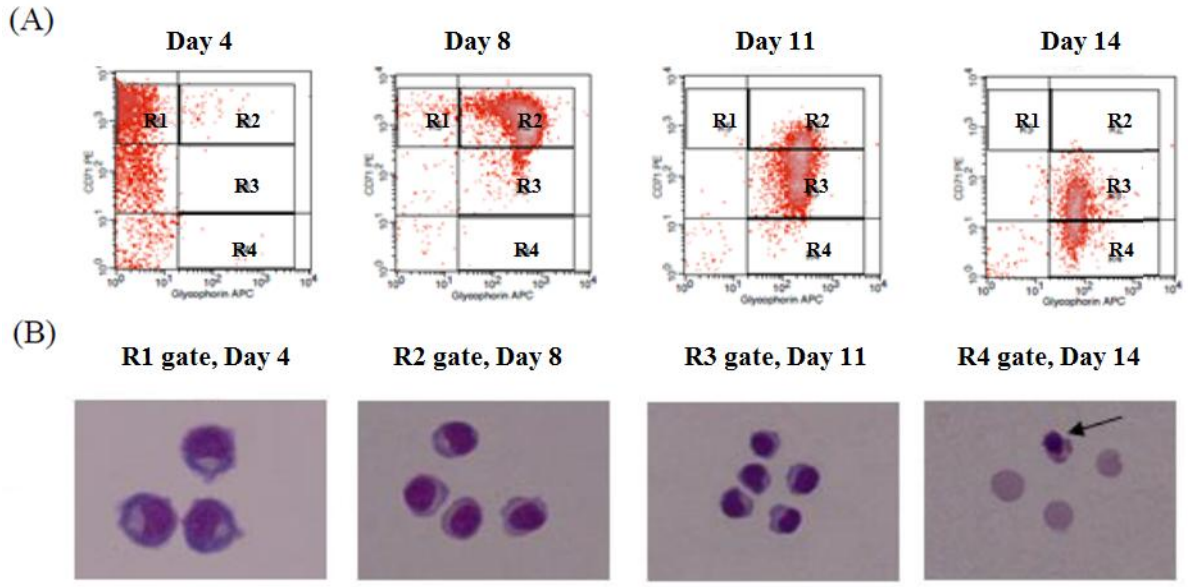

Figure 9. *Ex vivo* primary erythroblast differentiation and maturation. (A) Flow cytometric results show decrease of transferrin receptor (CD71; y-axis) while glycophorin A (CD235a; x-axis) tends to increase throughout cell differentiation. (B) Microscopic results show normal erythroid cell morphology during cell proliferation and maturation. Cell stage from left to right: R1; pronormoblast, R2; basophilic normoblast, R3; polychromatic normoblast and orthochromatic normoblast, and R4; erythrocyte (mature red blood cell) with enucleated cell (arrow).

RNA was extracted from $CD71^{high}/GPA^{positive}$ population-sorted day 8 cultured erythroblasts. BECN1, ATG5, ATG7, ATG8, ULK1, BNIP3L/NIX, PARK2, and PINK1 were selected to determine the expression level in thalassemic erythroblasts, due to its function and expression during normal erythropoiesis and potential associate with apoptosis, using quantitative real-time RT-PCR method (Table 4). Up-regulation of NIX and PINK1 were observed in β-thalassemia/HbE erythroblasts compared to normal control (fold changed = 37.9 $\pm$ 15.2 and 18.4 $\pm$ 7.8, respectively). There is no significant changed of BECN1, ATG5, ATG7, ATG8, ULK1, and PARK2 were found in this study.

Table 4. Quantitative RT-PCR expression of autophagy-related genes in erythroblasts. The experiments was carried out in triplicate in three independent subjects of β-thalassemia/HbE and healthy normal.

Gene Name	Fold change expression β-thalassemia/HbE vs. normal
BECN1; Beclin1	< 2-fold changed
ATG5; autophagy related 5	< 2-fold changed
ATG7; autophagy related 7	< 2-fold changed
ATG8/GABARAPL2; GABA(A) receptor-associated protein-like 2	< 2-fold changed
ULK1; unc-51 like autophagy activating kinase 1	< 2-fold changed
NIX/BNIP3L; BCL2/adenovirus E1B 19kDa interacting protein 3-like	unregulated 37.9±15.2-fold
PARK2; parkin RBR E3 ubiquitin protein ligase	< 2-fold changed
PINK1; PTEN induced putative kinase 1	unregulated 18.4±7.8-fold

Expression of NIX and PINK1 during erythroblast differentiation.

To study the dynamic expression of NIX and PINK1 in β-thalassemia/HbE and normal erythroblast during late stage of differentiation, quantitative RT-PCR was performed in cDNA from cultured day 4, 6, 8, 10, 12, and 14 erythroblasts. Nix and PINK1 genes expressions were upregulated at the transcriptional level during human erythroid differentiation (Figure 10). Both NIX and PINK1 start to transcribe on cultured erythroblast day 4. The highest expression level in normal is on day 14 cultured erythroblast whereas in β-thalassemia/HbE is highly transcribed on cultured erythroblast day 12.

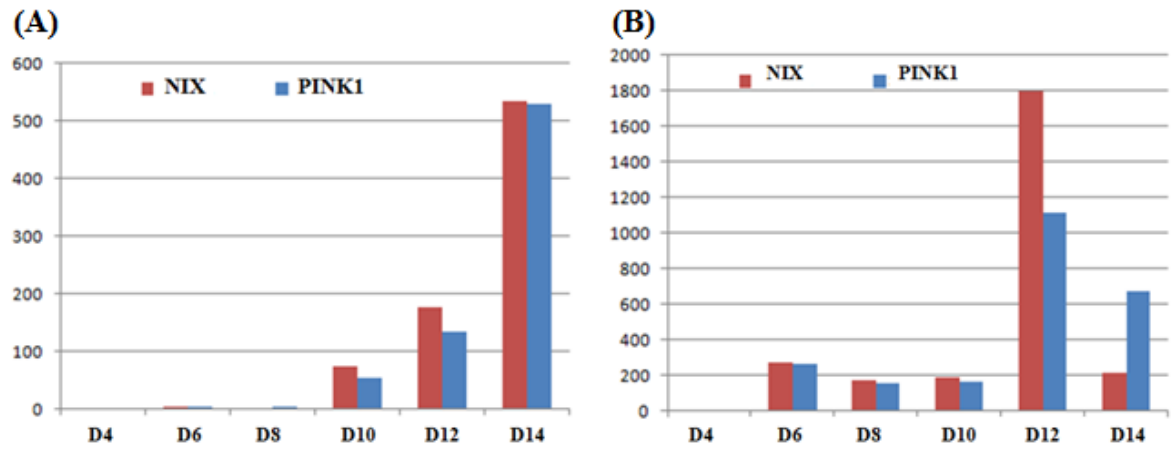

Figure 10. NIX and PINK1 transcript levels in (A) normal and (B) β-thalassemia/HbE cultured erythroblasts. The relative fold changes were calculated based on the ΔΔCt method. X-axis is day of culture and Y-axis is fold-changed compare to day 4.

4. Role of NIX and PINK1 in thalassemic erythropoiesis.

Generation of NIX shRNA and PINK1 shRNA lentiviral vector.

The shRNA knockdown of targeted gene is used to reveal whether the expression of NIX and/or PINK1 is required for mitophagy in during erythropoiesis. A third generation self-inactivation (SIN) LentiLox 3.7 (pLL 3.7) lentiviral vector system was used to evaluate the effects of shRNA-mediated knockdown of PINK1 in erythroids. This lentiviral vector system is very well characterized and has previously been used to efficiently silence proteins in hematopoietic stem cells and their progeny. NIX shRNA, or PINK1 shRNA, or non-targeted shRNA sequences were inserted into pLL 3.7 vector under the control of the human U6 Pol III promoter. Construction of lentiviral expression vector containing shRNA of interest transformation into Escherichia coli strain DH5α and ampicillin drug resistant selection were performed. Suspected recombinant clones were picked up for screening by restriction analysis using *Xba*I and *Xho*I as diagnostic restriction sites (Figure 1B). The positive clones give rise to a bigger product size than negative due to the insertion (Figure 10).

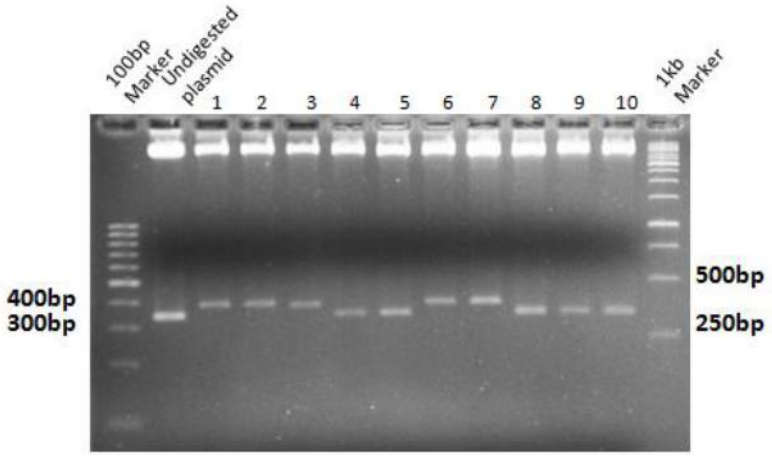

Figure 10. Screening the recombinant plasmids by restriction analysis using the diagnostic restriction site. 1-10 are shRNA of interest. Five positive (1, 2, 3, 6 and 7) clones give rise to a bigger product size (~395 bp) than negative clones (~335 bp) due to insertion.

After screening, those selected clones were confirmed the identity by sequencing and aligned with the reference sequence. Only correct clones showed 100% identity with reference sequence which mean these clones are lentiviral expression vectors containing shRNA of interest (Figure 11). Seven lentiviral expression vectors carrying shRNA of interest were constructed.

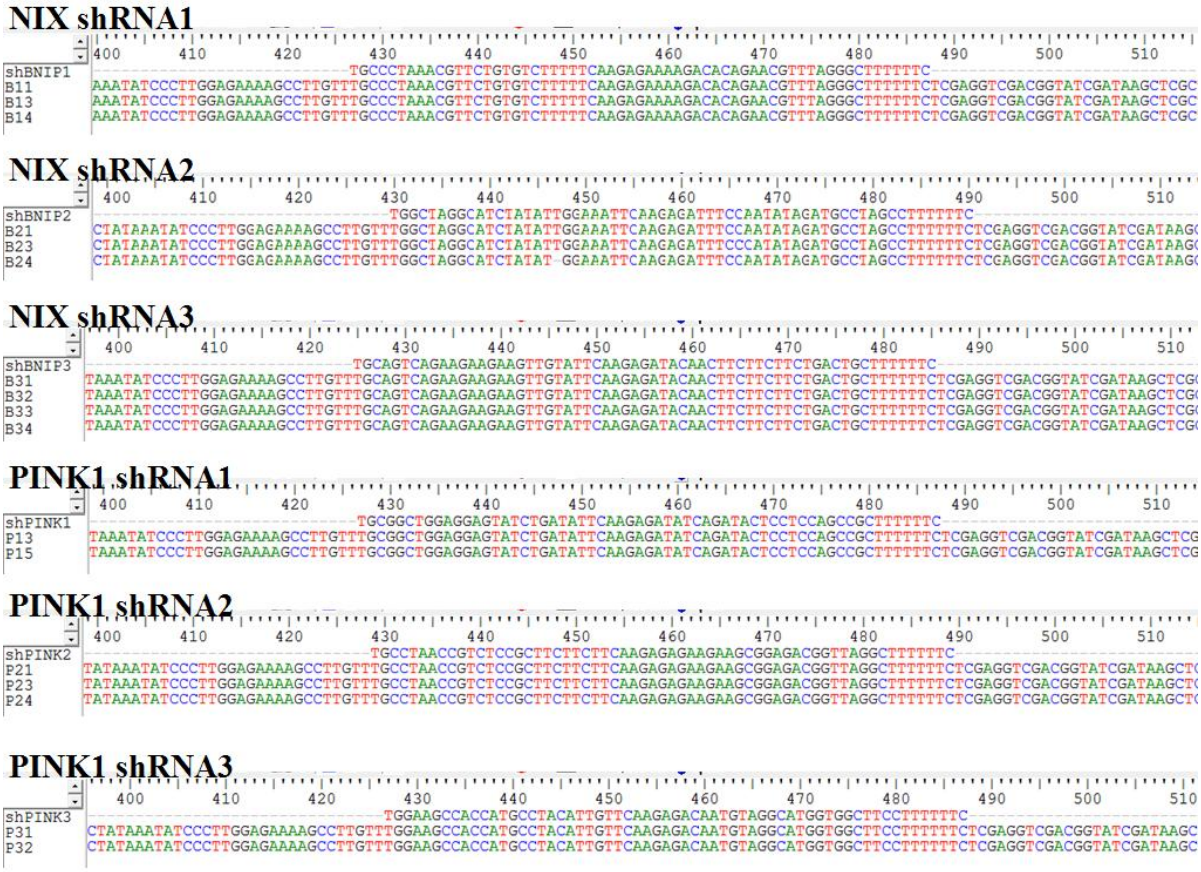

Figure 11. Confirmation of positive clones by DNA sequencing and alignment with reference sequence for (A) NIX shRNA, (B) PINK1 shRNA, and (C) non-targeted shRNA.

Transduction of non-targeted shRNA lentiviral in erythroblasts. The lentiviral particle containing non-targeted shRNA was generated by transfection of HEK 293T with pLL3.7-puro shRNA lentiviral plasmid and packaging plasmids. The supernatant containing lentiviral particles was collected at 48 and 72 hours after transfection. The viral titer was determined by transduction of HEK 293T and counting the colony after drug selection. The viral titer was 10^8 to 10^9 virus particles/mL. For transduction, CD34+ cells grown for 4 days were transferred to 24-well plates at a concentration of 2-4 x10^5 cells/mL and incubated overnight with vector particles (multiplicity of infection [MOI], 20-50). The next day, cells were returned to the expansion medium. Drug selection was initiated at 48 hours after infection, followed by erythroid differentiation for 5–7 days. Cell morphology was assessed by Wright-Giemsa staining of cytocentrifuge preparations. Dead cells were showed in non-target shRNA lentivirus transduced cells (Figure 12A, middle and lower panel) compared to healthy cells in untransduced control

(Figure 12A, upper panel). Puromycin at concentration of 1 ug/mL was kill all untransduced cells (Figure 9C, upper panel) compared to 70% live cell in untreated cells (Figure 12B). The number of live cells increased with the higher amount of viral vector transduction; MOI 20 (Figure 12C, middle panel) versus MOI 50 (Figure 12C, lower panel). Erythroblast differentiation was not different between cells were untransduced and transduced with non-target shRNA lentivirus (Figure 12D).

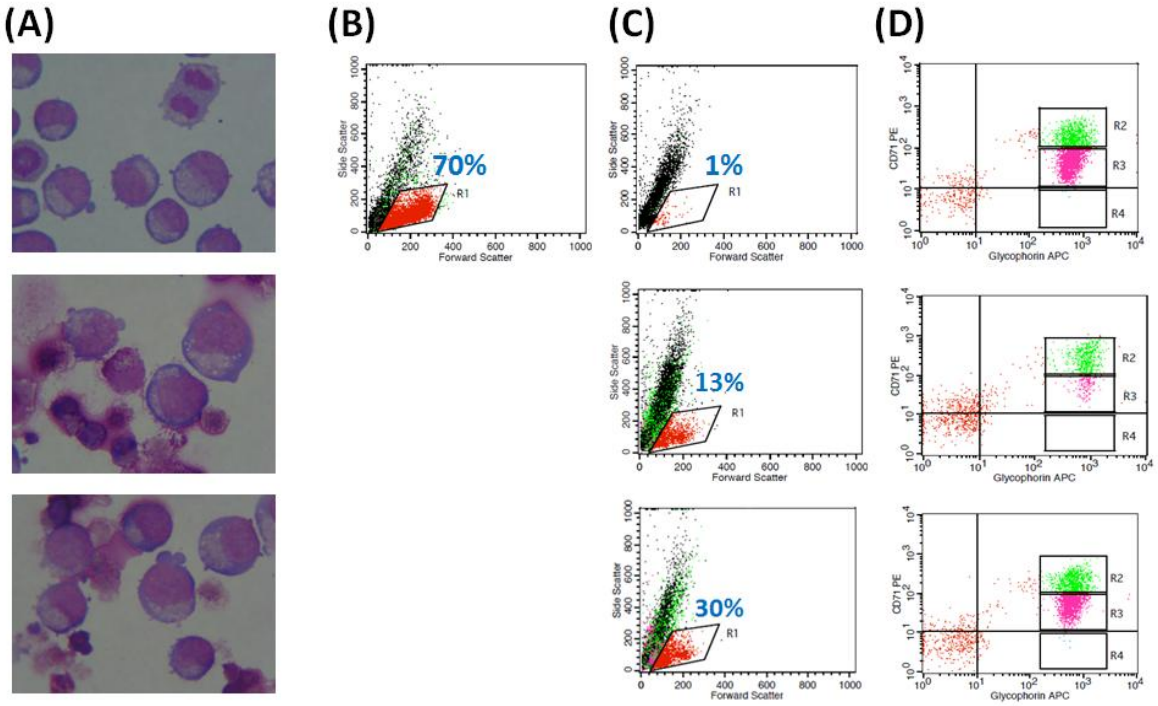

Figure 12. (A) Cytospin of human erythroid progenitors of day 8 untransduced erythroblasts without drug selection (upper panel) compared to MOI 20 (middle panel) and MOI 50 (lower panel) lentiviral shRNA transduced cells after drug selection for 48 hours. Flow cytometry analysis of live cell gate (red dot) of day 14 untransduced erythroblasts (B) without drug selection and (C, upper panel) with drug selection. Higher percentage of live cells in day 14 MOI 50 (lower panel) lentiviral shRNA transduced cells compare to MOI 20 (middle panel). (D) Differentiation of day 14 erythroblast study by flow cytometry using abtibody against erythroid markers: x-axis; Glyphophorin A and y-axis; transferring receptor (CD71), (upper panel) untransduced erythroblasts without drug selection and MOI 20 (middle panel) and MOI 50 (lower panel) lentiviral shRNA transduced cells with drug selection.

DISCUSSION

Mitochondria Clearance in Reticulocytes

With the life span of reticulocytes in the circulation being only 24-48 hours, reticulocyte cellular characteristics provide useful information of marrow erythropoietic activity. Patients clearly reveal the significant heterogeneity in the reticulocyte population. An increase in the percentage of reticulocyte was related to degree of anemia in the patients, as significantly higher in the severe compared to mild cases ($P<0.001$, Table 2). Accelerated erythropoiesis may result in arrival of immature stress reticulocytes in peripheral blood.[43] In severely affected patients, absolute reticulocyte counts showed no increase despite the rise in immature reticulocytes. This pattern of reticulocyte counts and shift in reticulocyte fractions may reflect ineffective erythropoiesis in this group of patients. The apparent discrepancy between reticulocyte counts and IMF can likewisely be explained by neocytolysis and apoptosis of immature reticulocytes.[44] In order to circulate through capillaries and respond to a range of shear stresses, mature RBCs must have the capacity to undergo marked membrane deformation and shape changes. During terminal erythroid maturation, changes in both protein content and membrane organization occur. Specifically, it is known that membrane vesiculation leads to approximately 20% loss of surface area[45] and that membranes of young reticulocytes are mechanically much less stable than those of mature cells.[46] As globular shapes confer a lesser deformability than biconcave shapes, the present of the biconcave-like geometries first detected in the low expression of CD71 reticulocytes population contribute to its increased flexibility.[47] In addition to shape effect, later stages of reticulocyte development also shows significant increases to deformability and mechanical stability.[48] Under basal erythropoiesis, reticulocytes have a lifespan in the systemic circulation of approximately 24 hours before developing into mature RBCs, however, the reticulocyte lifespan in the circulation increases to an estimated 2-3 days during stress erythropoiesis.[49] An early release of less flexibility and deformability immature reticulocyte from bone marrow into the blood circulation may lead to hemolysis of these cells.

In mammalian erythroids, the expulsion of the nucleus followed by the removal of other organelles is necessary for maturation. Mitochondria are the most abundant organelles to be cleared for the completion of erythropoiesis. Since approximately 30% of hemoglobin is produced in reticulocytes, and heme is synthesized in the mitochondria these organelles are among the last to be eliminated.[8] The loading and unloading of oxygen by hemoglobin can induce oxidant stress in RBCs.[50] The mitochondrion is a major site for the production of reactive oxygen species (ROS) and can function as an apoptotic machinery.[51] Therefore, timely

elimination of mitochondria is essential for erythropoiesis and survival of RBCs. Within 2 to 3 days of enucleation, the mitochondria are eliminated through an autophagy-related process. After culture for in vitro maturation, β-thalassemia/HbE reticulocytes showed significant delays in the removal of the mitochondria compared to normal controls, clearly showing that mitochondrial clearance is defective. Recent studies in erythroid cells have shown that autophagy is an important process for the physiological clearance of mitochondria during terminal erythroid differentiation.[7,9] Defective autophagic removal of mitochondria during maturation of erythroid cells have been linked to anemia.[52] Therefore, understanding the mechanisms for mitochondrial autophagy in erythroid cells should facilitate the development of novel therapeutic approaches for treating hematological disorders involving defective erythroid maturation. It may also shed light on the mechanisms underlying β-thalassemia/HbE disease heterogeneity.

Autophagy and mitophagy during erythroblast differentiation.

Erythroid differentiation can be subdivided into three stages: early erythropoiesis, terminal erythroid differentiation, and reticulocyte maturation. Terminal erythroid differentiation includes four stages cells: proerythroblasts, basophilic, polychromatic, and orthochromatic erythroblasts. During terminal erythroid differentiation, increasing in hemoglobinization and dramatic changes in the expression of membrane proteins happened.[53] Glycophorin A (GPA; CD235a) or/and transferrin receptor (CD71) are always used as indicators of erythroid differentiation stages.[54] Basically in normal human erythropoiesis, CD71 is highly express on early erythroid precursors. During maturation to erythrocytes, transferrin receptor will be lost due to down-regulation of hemoglobin synthesis. GPA is an effective marker for normal erythropoiesis because GPA is the major sialoglyeoprotein which highly express on mature red cell membrane. The results from CD34+ progenitor cell cultures in this study showed similarity to *in vivo* erythropoiesis. Erythroid proliferation and maturation were completed by day 14 in vitro erythroblast culture. Flow cytometric results show decrease of CD71 on cultured cell membrane while GPA tends to increase throughout cell differentiation. Conforming to the microscopic results which displayed cultured cell morphology on day 4, 6, 8 11, 14 are prepronormoblasts, basophilic normoblasts, polychromatic normoblast, orthochromatic normoblast and erythroblast, respectively. The flow cytometric results accompany with Giemsa stained cells found that erythroid proliferation and maturation were completed by day 14 of in vitro erythroblast culture.

Apoptosis of erythroid progenitor cells in bone marrow has been shown to be an important mechanism of early erythroid cells destruction in β-thalassemia.[36,38] Apoptosis is

characterized by exposure of phosphatidylserine(s) (PS) at the cell's outer surface and accompanied by characteristically morphologic alteration, biochemical hallmarks and DNA fragmentation. This study shows a significant increase of annexin V positive cells in β-thalassemic erythroid cells when compared to normal control, however, the degree of erythroid apoptosis was relatively low when compared with studies conducted earlier.[55] There are increased evidences indicating the functional relationship between apoptosis and autophagy and also the role of autophagy during normal erythropoiesis.[25,56] In normal erythropoiesis, autophagy is present during the process of erythrocyte maturation which involved in the removal of mitochondria.[25] However, it is still uncertain how it plays a role during the pathogenesis of the diseases, in particular, anemic diseases. The loading and unloading of oxygen by hemoglobin can induce oxidant stress in erythrocytes.[50] The mitochondrion is a major site for the production of reactive oxygen species (ROS) and can function as an apoptotic machinery.[51] In this study, besides apoptosis, the role of mitophagy during stress of erythropoiesis in thalassemic erythroblasts was examined using *ex vivo* CD34+ progenitors culture. Flow cytometry analysis has revealed the lower Mitotracker positive cells in β-thalassemia/HbE than normal erythroblasts the terminal differentiated stage. To confirm the enhanced mitophagy, electron microscopy for detection of the autophagosomes is required. In addition, confocal microscope to investigate the co-localization of LC3 protein, an autophagosome-specific markers; microtubule-associated protein light chain 3, and LAMP-1 protein, a marker of endosomes and lysosomes; a lysosomal-associated membrane proteins, together with the Western blot analysis for amount of LC3-II, a lipid-conjugated autophagosome-associated form of LC3, which is associated with the number of autophagosomes are also in needed. However, because of the time and research budget limitation, these confirmations experiments were not performed yet.

Molecular mechanism underlying mitophagy in thalassemic erythropoiesis.

Autophagy is a process by which cytoplasmic components are sequestered in double membrane vesicles, autophagosomes, and degraded upon autophagosomes fuse with late endosomes or lysosomes that form autolysosomes. These processes are involved with several autophagy-related proteins. This study has focused on the regulation of these genes in the thalassemic erythropoiesis. Expression of eight genes encoding autophagy-related peoteins; ATG5, ATG7, ATG8, BECN1, NIX, PARK2, PINK1, and ULK1, were investigate in β-thalassemia/HbE and normal erythroblasts. NIX and PINK1 transcripts were up-regulated in β-thalassemia/HbE erythroblasts, while the others were not significantly changed. However, this expression level changed still need to be validated by the Western blotting for the protein level.

To examine possible stage-specific effects, an ex vivo expansion system was used to analyze erythroblast development. NIX and PINK1 transcription levels were investigated throughout erythropoiesis. The results revealed that NIX and PINK1 transcripts were up-regulated during normal erythropoiesis, while proteins analysis by Western blot is underway. In addition, NIX and PINK1 transcription levels were increased in late erythroid differentiation in both β-thalassemia/HbE and normal. This finding raises the interesting additional possibility that NIX and PINK1 may exert regulatory effects on erythroblast development.

Role of NIX and PINK1 in thalassemic erythropoiesis.

Several findings indicate that NIX and PINK1 may play a role in autophagy, mitophagy and apoptosis of thalassemic erythropoiesis. Nix, a BH3-only member of the Bcl-2 family, is upregulated in erythroid cells undergoing terminal differentiation.[57] Nix play a role in red blood cell formation depending upon the developmental stage. Nix involves in mitochondrial clearance during erythrocyte maturation[26], and functions as a proapoptotic factor which serves as a mechanism to regulate erythrocyte quantity.[25] Nix mediated apoptotic cell death in erythroblasts, in opposition to EPO induced survival signaling. PINK1, a PTEN-induced putative kinase 1, contains a mitochondrial targeting sequence (MTS) and functions in a common pathway controlling mitochondrial morphology and function.[58] Loss of mitochondrial membrane potential (depolarization) leads to accumulation of PINK1 on the mitochondrial outer membrane, which triggers mitophagy.[31] This study has provided evidence that the NIX and PINK1 transcripts increase in β-thalassemia/HbE erythroblasts, suggesting its role in the thalassemic erythropoiesis. To examine the function of NIX and PINK1 during erythropoiesis, loss-of-function approach was performed in this study. In order to study the effects of gene knockdown using lentivirus carrying shRNA in CD34+ progenitors requires two steps. Present, three different sequences of NIX shRNA, three PINK1 shRNA, and one non-targeted shRNA (control) lentiviral expression vectors were constructed and available for further viral production and transduction. The successful transduction of lentivirus carrying non-targeted shRNA into *in vivo* culture of hematopoietic CD34+ progenitor cells has been shown in this study. For further studies, lentivirus carrying NIX shRNA and PINK1 shRNA will be produced and transfected to CD34+ progenitor cells. The effect of targeted gene knockdown in autophagy, mitophagy and apoptosis of β-thalassemia/HbE will be investigated using this lentivirus. However, because of the time and research budget limitation these experiments are still underway.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

หัวข้อ	Output
1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ	- ยังไม่มีผลงานตีพิมพ์ แต่มี manuscript 2 เรื่องที่เป็นส่วนหนึ่งในงานวิจัยนี้ อยู่ในขั้นตอนการส่งตีพิมพ์ในวารสารวิชาการนานาชาติ ดังนี้ 1. Defect of mitochondria clearance in beta-thalassemia/HbE reticulocytes. 2. Fetal hemoglobin expression is associated with concurrent changes in the nuclear expression of globin gene transcription factors.
2. การนำผลงานวิจัยไปใช้ประโยชน์ 2.1. เชิงพาณิชย์ 2.2. เชิงนโยบาย 2.3. เชิงสาธารณะ 2.4. เชิงวิชาการ	 - ไม่มี - ไม่มี - ไม่มี - มีนักศึกษาระดับบัณฑิตศึกษา 2 คน ร่วมในงานวิจัยนี้ได้แก่ 1. นายศักรินทร์ อาจศิริ นักศึกษาระดับปริญญาโท ภาควิชาภูมิคุ้มกันวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล 2. นางสาวธันวารัตน์ สุริยันต์ นักศึกษาระดับปริญญาเอก ภาควิชาชีวเคมี คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
3. อื่นๆ	- มีผลงานตีพิมพ์ในวารสารวิชาการในประเทศ 1 เรื่อง 1. Ardsiri S, Pattanapanyasat K, Noulsri E, Khuhapinant A, Fucharoen S & Sripichai O. Defective terminal erythroid maturation in beta-thalassemia/HbE disease. Proceedings of the MU Research Expo 2013, Mahidol University, 28-29 January 2014; page 28-35. - มีการเสนอผลงานในที่ประชุมวิชาการ 1 ครั้ง เรื่อง Defective terminal erythroid maturation in beta-thalassemia/HbE disease ในการประชุม MU Research Expo 2013 ณ มหาวิทยาลัยมหิดล, กรุงเทพมหานคร ในวันที่ 28-29 มกราคม 2557 - มีการเชื่อมโยงทางวิชาการกับนักวิชาการอื่น ๆ ในประเทศ ได้แก่

	1. มีการร่วมมือทำงานวิจัยร่วมกับทีมวิจัยของ ศ.ดร.โกวิท พัฒนาปัญญาสัตย์, หน่วยเครื่องมือพิเศษเพื่อการวิจัย, สถานส่งเสริมการวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล 2. มีการนำความรู้เรื่องการเพาะเลี้ยงเม็ดเลือดแดงต้นกำเนิดและตัวอย่างสกัด RNA ไปใช้ในการวิจัยเรื่อง fetal hemoglobin in beta-thalassemia ร่วมกับทีมวิจัยของ ศ.นพ.สุรเดช หงส์อิง, คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล

P-MS004

DEFECTIVE TERMINAL ERYTHROID MATURATION IN BETA-THALASSEMIA/HBE DISEASE

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Abstract

β-Thalassemia/HbE patients display a remarkable variability phenotypic diversity. Anemia in patients is due to an overall reduction in hemoglobin production, ineffective erythropoiesis and hemolysis of mature erythrocytes. Immature reticulocyte is an indicator of stress erythropoiesis and is markedly increased in β-thalassemia/HbE. Red blood cell and reticulocyte indices were determined in 311 non-splenectomized patients including 189 mildly, 86 moderately, and 36 severely affected. In all cases, mature red cells were smaller than reticulocytes (MCV; 59.3 ±5.4 versus 76.6±6.2 fL) with a lower cellular hemoglobin concentration (MCH; 18.2±2.0 versus 20.3±1.6 pg). Significant increases ($P<0.01$) in reticulocyte percentage and immature reticulocyte fraction were observed in severe and moderate cases compared to mild cases, suggesting a higher degree of bone marrow stimulation in the former groups. Among the mild group, lower absolute reticulocyte count, reticulocyte percentage and immature reticulocyte fraction were found in β^+-thalassemia/HbE and β^0-thalassemia/HbE coinheritance with α-thalassemia ($P<0.01$), suggesting that type of β-thalassemia mutation and α-thalassemia interaction are strongly contributes to the lesser degree of stress erythropoiesis. Flow cytometry analysis using anti-CD71 (transferrin receptor) antibody, MitoTracker (cell-permeant mitochondrion-selective dye), and thiazole-orange (RNA-staining dye) demonstrated a more immature phenotype of reticulocyte in regard to CD71-positive, high mitochondria and RNA content level in β-thalassemia/HbE than those from normal or α-thalassemia (HbH disease). In addition, an *ex vivo* reticulocyte culture exhibited delay mitochondrial clearance in β-thalassemia/HbE reticulocytes compared to normal reticulocytes enriched from iron deficiency anemia. These results display the reticulocyte heterogeneity and defect of terminal erythroid maturation in β-thalassemia/HbE that may contribute to the phenotype of patients.

Keywords: thalassemia, HbE, reticulocyte, stress erythropoiesis

Introduction

β-Thalassemia is common in Southeast Asia and it has become a worldwide health problem due to increased population migration (1). HbE acts as a β^+-thalassemia because the synthesis of the β^E-globin chain is reduced due to the mutation, GAG to AAG at codon 26 of the β-globin gene, which activates a cryptic splice site (2). The interaction between HbE and β-thalassemia causes a surprisingly variable degree of anemia and β-Thalassemia/HbE patients have a broad spectrum of clinical severity, including age of presentation, the requirement for transfusion, growth development and other complications (3-5). The mechanism underlying the patient pathophysiology is related to the imbalanced α- to non-α-globin chains synthesis which is an initial cause of red blood cell membrane rigidity, ineffective erythropoiesis, and premature red blood cell destruction results in anemia (6).

During erythropoiesis mature erythroblasts enucleate, becoming reticulocytes which release into peripheral blood from bone marrow and other hematopoietic organs. The maturation of reticulocytes into mature red blood cells (RBCs) is the final stage of erythropoiesis. This maturation

process involves the extensive remodeling of the plasma membrane with progressive loss of various membrane proteins, including transferrin receptor (CD71; mediates the uptake of iron into cells) (7). Equally important is the selectively removal of organelles that are not necessary in RBCs, including mitochondria, Golgi apparatus, endoplasmic reticulum, and endocytic vesicles (8). In basal erythropoiesis conditions, this terminal erythroid maturation occurs over a period of 48 hours in the bone marrow and 24 hours in the circulation (9). The RNAs of reticulocytes is lost upon cell maturation (10). Immature reticulocyte fraction (IRF), contain a higher RNA content than mature reticulocytes reflects the erythropoietic activity of the bone marrow (11, 12). During stress erythropoiesis the reticulocyte count in circulation increases because of an increased release of immature reticulocyte from the bone marrow as responding to increased stimulation of erythroid precursors.

Based on the clinical information provided, the β-thalassemia/HbE patients were classified according to disease severity; mildly, moderately, and severely affected groups. The cohort display markedly increased in IRF and association of the reticulocyte subpopulation with the clinical severity. Moreover, the presence of stress reticulocytes, the most immature fraction, in severely affected patients reflects the greater degree of chronic state of erythroid expansion. Importantly, mitochondria clearance is defect in β-thalassemia/HbE reticulocytes, suggesting that the terminal erythroid maturation is physiologically relevant.

Methodology

Patients

The study cohort included non-spleenectomized β-thalassemia/HbE patients with different degrees of anemia, but excluded patients who recently (within 2 months) have received blood transfusion. Three hundred and eleven patients were recruited in this study underwritten informed consent in accordance with the Declaration of Helsinki, and blood samples were obtained using protocols approved by the Institutional Review Board of Mahidol University. Clinical data was obtained from the patients or their parents medical records and physical examination. Patients were classified into three groups: mild, moderate, and severe, using a β-thalassemia disease severity scoring system (13).

Hematologic Studies

Venous blood was collected into EDTA tubes. Complete blood counts including reticulocyte parameters were determined using an automated blood cell analyzer (ADVIA 120, Bayer, NY, USA). Automated reticulocyte counting was based on the staining of RNA content using oxazine 750. Reticulocytes were further classified into three categories by maturity according to fluorescence intensity: low fluorescence ratio (L-Retic); medium fluorescence ratio (M-Retic); and high fluorescence ratio (H-Retic). Immature reticulocytes fraction (IRF = H-Retic + M-Retic) used for evaluating erythropoietic activity (14).

DNA Analysis

DNA was extracted from peripheral blood leukocytes using the Gentra Puregene blood kit (Qiagen). β-Thalassemia mutations were identified by reverse dot blot hybridization (15). Genotyping of the deletion types of α-thalassemia 1 and α-thalassemia 2 was performed by gapped PCR, while Hb Constant Spring and Hb Pakse mutations were identified by allele-specific PCR (16).

Flow cytometric assay

A total of 2×10^6 red blood cells, approximately 2 μL of blood was diluted with 500 uL of PBS buffer. To stain mitochondria, 500 uL of the dilution were incubated for 30 minutes at 37^{o}C with 200nM MitoTracker deep red (Molecular Probes). The cells were subsequently washed with cold PBS and incubated with thiazole-orange (Sigma) for 40 minutes at room temperature, followed by washed twice with cold PBS. Subsequently MitoTracker and thiazole-orange cells were co-stained with appropriate concentrations of FITC-anti-CD71 (BD Biosciences). Samples were analyzed on a BD FACSCanto using FACSDiva Software v 6.1.3. RBC-gate was set from forward scatter (FSC)/side scatter (SSC). Unstained cells were used as negative controls.

Reticulocytes enrichment and cell culture

Reticulocytes and erythrocytes were separated by centrifugation on a isotonic 70% Percoll, density 0.767 mg/mL (17). Approximately 2 mL of whole blood was layered on top of these gradients and centrifuged at 1200g for 30 minutes without breaking at room temperature. Upon centrifugation, reticulocytes were enriched at the interface, whereas erythrocytes accumulated at the bottom of the tube. The isolated reticulocytes were cultured for three days in Iscove modified Dulbecco medium, 30% fetal bovine serum, 1% bovine albumin, 100 units/mL penicillin, 100 µg/mL streptomycin (18). To monitor the *ex vivo* mitochondrial clearance stressed-erythroid terminal differentiation, the reticulocyte-enriched blood were collected from β-thalassemia, α-thalassemia and healthy normal control.

Statistical analysis

Hematologic data from different severity groups of patients were compared using the chi-squared test for categorical variables and *t*-test for continuous variables. Differences among groups were considered statistically different at P-value less than 0.05.

Results

Heterogeneity of hematologic parameters in β-thalassemia/HbE.

Non-splenectomized β-thalassemia/HbE patients were divided into 5 groups according to disease severity and genetic backgrounds (Table 1). Fifteen patients with β^{+}-thalassemia/HbE and 46 patients who have α-thalassemia interactions with β^{0}-thalassemia/β^{E} genotypes had very mild symptoms with an average hemoglobin (Hb) of 9.2 ± 0.8 and 8.1 ± 1.0 g/dL, respectively. Among the β^{0}-thalassemia/HbE patients who did not inherit α-thalassemia, 128 patients were classified as having mild symptoms: they were transfusion-independent with an average Hb of 7.9 ± 1.2 g/dL. While 86 patients had moderate symptoms and severe symptoms were observed in 36 patients (Hb = 6.3 ± 0.9 and 5.5 ± 0.9 g/dL, respectively). There were no significant differences between the mild, moderate and severe groups for RBC indices, but the RBC number in mild group was significantly higher than moderate and severe groups ($p < 0.001$). In all cases, mature red cells were smaller than reticulocytes (MCV; 59.3 ±5.4 versus 76.6±6.2 fL) with a lower cellular hemoglobin concentration (MCH; 18.2±2.0 versus 20.3±1.6 pg), but the degree of cell size nor Hb content reduction was not significant difference between each groups. Ineffective erythropoiesis were presented in all patients as stated by extremely low reticulocyte hemoglobin content (CHr mean = 20.3 ± 1.6; range 16.7 – 27.9 pg). However the mean in the mild group showed no statistical difference compared to other groups. Significant increases ($P<0.001$) in reticulocyte percentage and immature reticulocyte fraction were observed in severe and moderate cases compared to mild cases.

The reticulocyte counts were increased (5.9 ± 1.8%) with a relative increase in the most immature fractions (H-Retic: 17.1 ± 7.4%) in severe group. Among the mild group, lower absolute reticulocyte counts, reticulocyte percentage and immature reticulocyte fraction were found in β^{+}-thalassemia/HbE and β^{0}-thalassemia/HbE coinheritance with α-thalassemia ($P<0.01$).

Table 1. Distribution of hematologic parameter in different β-thalassemia/HbE disease severity groups.

Characteristics	Mild β^+/β^E	Mild β^0/β^E α-thalassemia	Mild β^0/β^E αα/αα	Moderate β^0/β^E αα/αα	Severe β^0/β^E αα/αα	Total
No. (%)	15 (3.5)	46 (10.7)	128 (29.8)	86 (20.0)	36 (8.4)	311
Sex, female (%)	11 (73.3)	19 (41.3)	76 (59.4)	46 (53.5)	13 (36.1)	165 (53.1)
Age (year)	23.9 ± 23.4 (4 - 87)	16.8 ± 13.3 (3 - 57)	18.6 ± 13.9 (2 - 76)	18.2 ± 13.3 (2 - 49)	11.3 ± 7.1 (3 - 35)	17.6 ± 13.9 (2 - 87)
Red blood cell						
Hb (g/dL)	9.2 ± 0.8 (7.6 - 10.6)	8.1 ± 1.1 (5.6 - 10.0)	7.9 ± 1.2 (4.9 - 12.2)	6.3 ± 0.9 (3.4 - 8.4)	5.5 ± 0.9 (3.8 - 7.4)	7.3 ± 1.5 (3.4 - 12.2)
RBC (10e6/dL)	5.06 ± 0.64 (3.74 - 6.16)	4.85 ± 0.62 (3.27 - 6.21)	4.19 ± 0.55 (2.94 - 6.03)	3.57 ± 0.56 (1.95 - 4.78)	3.19 ± 0.56 (2.21 - 4.87)	4.04 ± 0.79 (1.95 - 6.21)
MCV (fL)	57.2 ± 4.5 (50.8 - 63.1)	54.2 ± 4.0 (45.4 - 65.2)	60.6 ± 5.4 (49.2 - 80.6)	59.9 ± 5.0 (49.9 - 71.2)	60.6 ± 4.6 (51.2 - 72.5)	59.3 ± 5.4 (45.4 - 72.5)
MCH (pg)	18.6 ± 1.6 (16.1 - 21.9)	16.8 ± 1.5 (14.5 - 21.6)	18.9 ± 2.1 (14.8 - 27.9)	18.0 ± 1.7 (14.7 - 22.3)	17.5 ± 1.8 (11.9 - 22.2)	18.2 ± 2.0 (11.9 - 27.9)
MCHC (g/dL)	32.4 ± 1.4 (30.4 - 34.7)	31.1 ± 1.3 (28.4 - 33.9)	31.1 ± 1.7 (27.1 - 35.2)	30.1 ± 1.6 (26.3 - 34.3)	28.9 ± 2.0 (18.9 - 31.3)	30.6 ± 1.8 (18.9 - 35.2)
Reticulocyte						
Retic (10e9/L)	132 ± 42 (64 - 208)	160 ± 42 (72 - 250)	183 ± 49 (80 - 350)	198 ± 117 (51 - 863)	188 ± 45 (101 - 306)	182 ± 75 (51 - 863)
Retic (%)	2.7 ± 1.0 (1.1 - 4.9)	3.4 ± 1.0 (1.6 - 5.1)	4.4 ± 1.2 (1.7 - 7.8)	5.7 ± 4.3 (1.1 - 37.2)	5.9 ± 1.8 (3.5 - 12.8)	4.7 ± 2.6 (1.1 - 37.2)
MCVr (fL)	72.9 ± 6.8 (63.2 - 82.7)	70.9 ± 4.7 (59.8 - 79.3)	77.9 ± 6.0 (65.5 - 96.6)	77.4 ± 6.2 (65.1 - 89.8)	78.9 ± 5.4 (70.4 - 92.6)	76.6 ± 6.4 (59.8 - 96.6)
CHr (pg)	20.5 ± 1.5 (18.2 - 23.3)	19.2 ± 1.4 (16.7 - 23.6)	20.8 ± 1.7 (16.8 - 27.9)	20.2 ± 1.6 (17.3 - 24.6)	20.0 ± 1.5 (17.7 - 24.4)	20.3 ± 1.6 (16.7 - 27.9)
CHCMr (g/dL)	28.5 ± 1.3 (26.4 - 30.5)	27.4 ± 1.3 (24.8 - 30.8)	27.0 ± 1.3 (23.8 - 30.6)	26.6 ± 1.1 (24.1 - 29.2)	25.8 ± 0.8 (24.2 - 27.3)	26.9 ± 1.3 (23.8 - 30.8)
L-Retic (%)	84.6 ± 9.1	77.4 ± 7.7	74.9 ± 7.4	70.0 ± 9.9	65.2 ± 10.6	73.2 ± 9.7
M-Retic (%)	11.1 ± 5.1	15.6 ± 3.7	16.1 ± 3.4	17.3 ± 3.7	17.7 ± 4.0	16.3 ± 3.9
H-Retic (%)	4.4 ± 4.5	7.1 ± 4.6	9.1 ± 4.8	12.7 ± 6.9	17.1 ± 7.4	10.5 ± 6.6

Hematological analyses of β-thalassemia/HbE patients was performed using a Bayer Advia 120 hematology analyzer. Hb, hemoglobin; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Retic, reticulocyte; MCVr, reticulocyte mean corpuscular volume; CHr, reticulocyte hemoglobin content; CHCMr, reticulocyte mean corpuscular hemoglobin concentration; L-Retic, low fluorescence ratio reticulocyte; M-retic, medium fluorescence ratio reticulocyte; H-Retic, high fluorescence reticulocyte. Data are presented as mean±s.d. and range.

Immature reticulocyte fraction in β-thalassemia/HbE.

Analysis of β-thalassemia/HbE erythrocytes by thiazol orange staining and flow cytometry confirmed the presence of marked reticulocytosis with elevated numbers of IRF. As representatively shown in Figure 1, second panel, RNA content levels in β-thalassemia/HbE erythrocytes were higher than those of α-thalassemia (HbH disease) and normal control. In addition to RNA content levels, the intensity of MitoTracker was also shown to be enhanced in thalassemia patients (Figure 1, third panel). Transferrin receptor positive reticulocytes are the youngest red blood cell cohort in blood (19). Immature and transferrin receptor positive reticulocytes were observed in all cases (Figure 1, right panel). In these analyses, transferrin receptor positive cells were always thiazol-orange and MitoTracker positive.

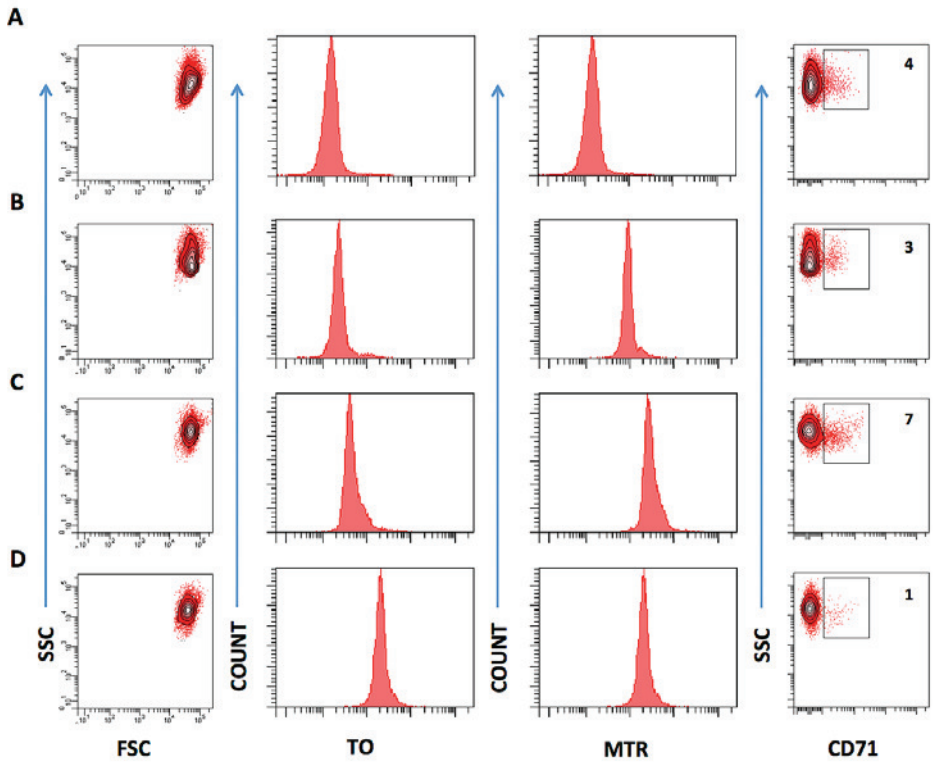

Figure 1. Flow cytometry analysis of erythroid cells from the peripheral blood. (A) Normal control, (B) α-thalassemia (HbH disease), (C) β-thalassemia/HbE with high reticulocyte count, and (D) β-thalassemia/HbE with low reticulocyte count. Left panel: contour plots of FSC versus SSC dot plot showing the erythrocyte population. Second panel: histogram plot showing thiazol-orange (TO) stained erythrocytes. Third panel: histogram plots showing MitoTracker (MTR) stained erythrocytes. Right panel: contour plots of CD71 versus SSC showing the percentages of CD71 erythrocytes positive population.

Delay mitochondrial clearance in β-thalassemia/HbE reticulocytes.

In general, the amount of RNA in reticulocytes decreases as cells mature, and in older reticulocytes the various organelles decrease in number, usually mitochondria will be disappeared first and ribosomal last. The higher MitoTracker intensity in thalassemia erythrocytes (Figure 1, third panel) leading to further examination whether β-thalassemia/HbE reticulocytes might be defective in eliminating the mitochondria. Notably, less RNA and mitochondria contents were observed in freshly isolated β-thalassemia/HbE reticulocytes (Figure 2, left panel). In contrast, fewer normal reticulocytes were negative for mitochondrial staining (Figure 2A, left panel). After culture for *in vitro* maturation for three days, RNA and mitochondria in normal reticulocytes were completely removed (Figure 2A, right panel). While a slightly decline of MitoTracker and thiazol-orange double positive reticulocytes was observed in β-thalassemia/HbE reticulocytes (Figure 2B and C).

Discussion and Conclusion

With the life span of reticulocytes in the circulation being only 24-48 hours, reticulocyte cellular characteristics provide useful information of marrow erythropoietic activity. In the present study, the amount of reticulocytes was measured as a function of effective erythropoiesis in β-thalassemia/HbE. Patients clearly reveal the significant heterogeneity in the reticulocyte population. An increase in the percentage of reticulocyte was related to degree of anemia in the patients, as significantly higher in the severe compared to mild cases ($P<0.001$, Table 1). The absolute reticulocyte count, percentage of reticulocyte, and percentage of IMF were lower in β^{+}-thalassemia/HbE and β^{0}-thalassemia/HbE coinheritance with α-thalassemia ($P<0.001$) than those of β^{0}-thalassemia/HbE without α-thalassemia interaction, suggesting that type of β-thalassemia mutation and coinheritance of

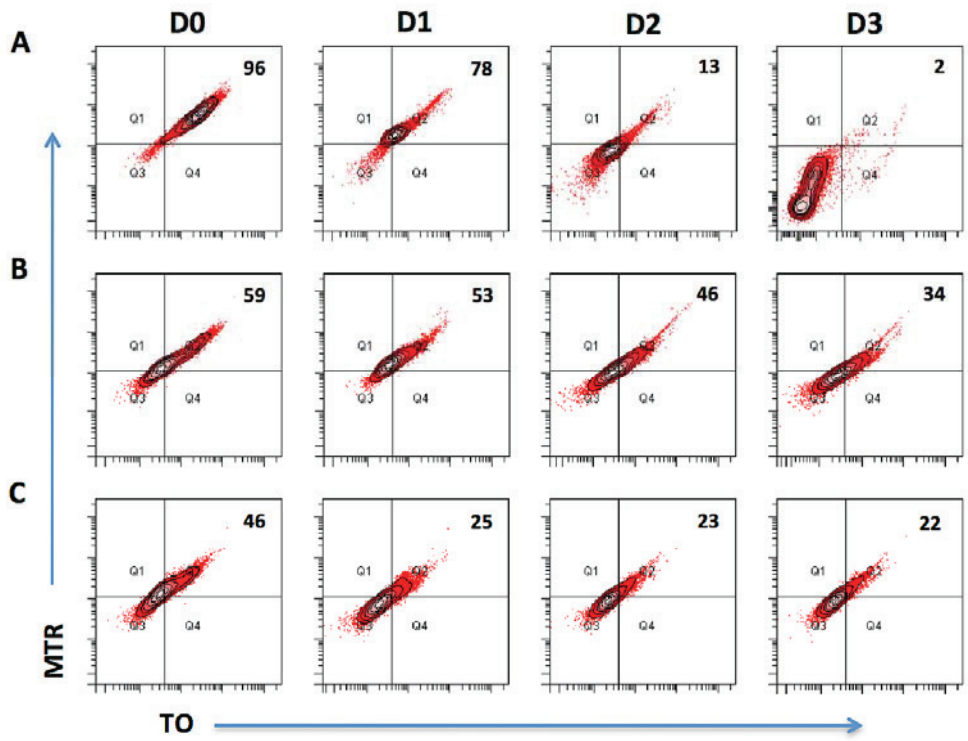

Figure 2. Flow cytometry analysis of the RNA and mitochondria contents in reticulocytes. Thiazol-orange (TO) and MitoTracker (MTR) contour plots of (A) non-thalassemic control, (B) β-thalassemia/HbE with high reticulocyte count, and (C) β-thalassemia/HbE with low reticulocyte count at *in vitro* cultured days 0, 1, 2, and 3.

α-thalassemia are strongly contributes to a lesser degree of stress erythropoiesis. Accelerated erythropoiesis may result in arrival of immature stress reticulocytes in peripheral blood (20). In severely affected patients, absolute reticulocyte counts showed no increase despite the rise in immature reticulocytes. This pattern of reticulocyte counts and shift in reticulocyte fractions may reflect ineffective erythropoiesis in this group of patients.

The apparent discrepancy between reticulocyte counts and IMF can likewisely be explained by neocytolysis and apoptosis of immature reticulocytes (21, 22). In order to circulate through capillaries and respond to a range of shear stresses, mature RBCs must have the capacity to undergo marked membrane deformation and shape changes. During terminal erythroid maturation, changes in both protein content and membrane organization occur. Specifically, it is known that membrane vesiculation leads to approximately 20% loss of surface area (23, 24) and that membranes of young reticulocytes are mechanically much less stable than those of mature cells (25, 26). As globular shapes confer a lesser deformability than biconcave shapes, the present of the biconcave-like geometries first detected in the low expression of CD71 reticulocytes population contribute to its increased flexibility (27). In addition to shape effect, later stages of reticulocyte development also shows significant increases to deformability and mechanical stability (28). Under basal erythropoiesis, reticulocytes have a lifespan in the systemic circulation of approximately 24 hours before developing into mature RBCs, however, the reticulocyte lifespan in the circulation increases to an estimated 2-3 days during stress erythropoiesis (29). An early release of less flexibility and deformability immature reticulocyte from bone marrow into the blood circulation may lead to hemolysis of these cells.

In mammalian erythroids, the expulsion of the nucleus followed by the removal of other organelles is necessary for maturation. Mitochondria are the most abundant organelles to be cleared for the completion of erythropoiesis. Since approximately 30% of hemoglobin is produced in reticulocytes, and heme is synthesized in the mitochondria these organelles are among the last to be eliminated (30). The loading and unloading of oxygen by hemoglobin can induce oxidant stress in RBCs (31). The mitochondrion is a major site for the production of reactive oxygen species (ROS) and can function as an apoptotic machinery (32). Therefore, timely elimination of mitochondria is

essential for erythropoiesis and survival of RBCs. Within 2 to 3 days of enucleation, the mitochondria are eliminated through an autophagy-related process. After culture for *in vitro* maturation, β-thalassemia/HbE reticulocytes showed significant delays in the removal of the mitochondria compared to normal controls, clearly showing that mitochondrial clearance is defective. Recent studies in erythroid cells have shown that autophagy is an important process for the physiological clearance of mitochondria during terminal erythroid differentiation (33, 34). Defective autophagic removal of mitochondria during maturation of erythroid cells have been linked to anemia (35). Therefore, understanding the mechanisms for mitochondrial autophagy in erythroid cells should facilitate the development of novel therapeutic approaches for treating hematological disorders involving defective erythroid maturation. It may also shed light on the mechanisms underlying β-thalassemia/HbE disease heterogeneity.

In summary, the present study demonstrates the reticulocyte heterogeneity and defective in clearing mitochondria during maturation. Reticulocytes undergo extensive membrane remodeling, volume changes, and elimination of all intracellular organelles, to ensure that critical function, such as hemoglobin production, oxygen transportation, and deformability are optimized in mature RBCs. Hence, defect of terminal erythroid maturation in β-thalassemia/HbE may contribute to the phenotype of patients.

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การศึกษาบทบาทของไมโตฟาจีในกระบวนการสร้างเม็ดเลือดแดง

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Thalassemia syndromes represent a major worldwide health problem and are amongst the most common monogenic diseases. Thalassemias cause from defective globin synthesis which result in globin chain imbalanced in erythroid cells. Consequently, the patients have chronic anemia, jaundice, hepatosplenomegaly, iron overload and many complications in various organs. In addition to diminished hemoglobinization of individual erythrocytes, anemia in thalassemia is due to two major factors which are early erythroid precursor cell destruction (ineffective erythropoiesis) and shortened survival of the circulating erythrocytes. An accumulation of excess non-α-globin chains in the α-thalassemia erythrocytes leads to oxidative damage of red cell membrane and hemolysis. While in β-thalassemia, ineffective erythropoiesis with accelerated apoptosis of the erythroid precursors leads to severe anemia and extensive erythroid expansion.

β-Thalassemia/HbE patients display a remarkable variability phenotypic diversity. Anemia in patients is due to an overall reduction in hemoglobin production, ineffective erythropoiesis and hemolysis of mature erythrocytes. Immature reticulocyte is an indicator of stress erythropoiesis and is markedly increased in β-thalassemia/HbE. Red blood cell and reticulocyte indices were determined in the patient cohort including 189 mildly, 86 moderately, and 155 severely affected. Significant increases ($P<0.01$) in reticulocyte percentage and

immature reticulocyte fraction were observed in severe and moderate cases compared to mild cases, suggesting a higher degree of bone marrow stimulation in the former groups.

Autophagy is the cellular homeostatic pathway that delivers large cytosolic components for degradation in the lysosome. In addition to the well-documented role of autophagy in cell survival mechanism during nutrient or growth factor deprivation, a function for autophagy in cell death has been described. The presence of autophagic structures in dying cells suggesting a causative role of autophagy in autophagic cell death. Apoptosis and autophagic cell death are associated either through complementary pathways of activation, or by the presence of both active caspase and autophagy in a common dying cell. β-Thalassemia erythroblasts show features of enhanced autophagy at an earlier stage of erythroid differentiation than in normal control. Whether the enhanced autophagy is a cell protective mechanism response to stress condition or a cause of apoptosis in β-thalassemic erythrobalsts, and whether this phenomenon occurs in α-thalassemia are the questions that merits investigation.

Autophagy also plays an important role in removing damaged and dysfunctional mitochondria. Mitochondrion is the site of aerobic energy production in eukaryotic cells, of which reactive oxygen species (ROS) are an inevitable by-product. ROS can lead to mitochondrial DNA mutations, dysfunction, and apoptosis. Therefore, clearance of damaged mitochondria by autophagy was necessary to protect cells from the potential harm of damaged mitochondria and release of proapoptotic proteins. Hence the autophagic clearance of dysfunctional mitochondria might be altered in erythroblasts from thalassemia patients in response to stress erythropoiesis. Several line of evidence indicated that autophagy participates in the degradation of mitochondria (mitophagy) during terminal erythroid differentiation. In mammalian erythroids, the expulsion of the nucleus followed by the removal of other organelles is necessary for maturation. Mitochondria are the most abundant organelles to be cleared for the completion of erythropoiesis. However, the production of red cell hemoglobin is depended on heme synthesis in mitochondria, therefore, timely elimination of mitochondria is essential for erythropoiesis. Flow cytometry analysis using anti-CD71 (transferrin receptor) antibody, MitoTracker (cell-permeant mitochondrion-selective dye), and thiazole-orange (RNA-staining dye) demonstrated a more immature phenotype of reticulocyte in regard to CD71-positive, high mitochondria and RNA content level in β-thalassemia/HbE than those from normal or α-thalassemia (HbH disease). In addition, an *ex vivo* reticulocyte culture exhibited delay mitochondrial clearance in β-thalassemia/HbE reticulocytes compared to normal reticulocytes enriched from iron deficiency anemia. This study demonstrates the reticulocyte heterogeneity and defective in clearing mitochondria during maturation. Reticulocytes undergo extensive

membrane remodeling, volume changes, and elimination of all intracellular organelles, to ensure that critical function, such as hemoglobin production, oxygen transportation, and deformability are optimized in mature RBCs. Hence, defect of terminal erythroid maturation in β-thalassemia/HbE may contribute to the phenotype of patients.

Due to the association with the thalassemia disease pathophysiology, thus, it is interesting to further explore the autophagy in thalassemia. Although the upregulation of autophagy have been reported in β-thalassemia but the characterization of the autophagy-related genes expression pattern during erythropoiesis of thalassemic erythroblasts which may explain its molecular mechanism has not been study. Mitochondria are important regulators of both survival and cell death of erythroblasts, therefore dysregulation of mitophagy may involve in early death of erythroid precursor cells. Inductions of autophagy in early stage of differentiation were observed in β-thalassemia/HbE erythroblast. The stimulation of autophagy was concomitant with the transcriptional upregulation of several mitophagy-related genes, in which modulation of its levels affects number of mitochondria and the amount of cell death. The mechanisms responsible for the mitophagy in developing erythroid cells are still being elucidated.