The opposing roles of the mTOR signaling pathway in different phases of human umbilical cord blood-derived CD34+ cell erythropoiesis

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Abstract
As an indispensable, even lifesaving practice, red blood cell (RBC) transfusion is challenging due to several issues, including supply shortage, immune incompatibility, and blood-borne infections since donated blood is the only source of RBCs. Although large-scale in vitro production of functional RBCs from human stem cells is a promising alternative, so far, no such system has been reported to produce clinically transfusable RBCs due to the poor understanding of mechanisms of human erythropoiesis, which is essential for the optimization of in vitro erythrocyte generation system. We previously reported that inhibition of mammalian target of rapamycin (mTOR) signaling significantly decreased the percentage of erythroid progenitor cells in the bone marrow of wild-type mice. In contrast, rapamycin treatment remarkably improved terminal maturation of erythroblasts and anemia in a mouse model of β-thalassemia. In the present study, we investigated the effect of mTOR inhibition with rapamycin from different time points on human umbilical cord blood-derived CD34+ cell erythropoiesis in vitro and the underlying mechanisms. Our data showed that rapamycin treatment significantly suppressed erythroid colony formation in the commitment/proliferation phase of erythropoiesis through inhibition of cell-cycle progression and proliferation. In contrast, during the maturation phase of erythropoiesis, mTOR inhibition dramatically...

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promoted enucleation and mitochondrial clearance by enhancing autophagy. Collectively, our results suggest contrasting roles for mTOR in regulating different phases of human erythropoiesis.

**KEYWORDS**

autophagy, erythropoiesis, hematopoietic stem cells, mTOR, rapamycin

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1 | INTRODUCTION

Since the first successful human blood transfusion performed in 1818, blood transfusion therapy has become a mainstay of modern clinical medicine. Blood transfusion refers to the administration of whole blood and blood products, including red blood cells (RBCs), platelets, or plasma. Particularly, transfusion of RBCs can restore the oxygen transport capacity to improve the quality of life in patients with chronic anemia such as thalassemia and sickle cell disease, and can be even lifesaving in several circumstances.

However, as an important therapeutic modality, transfusion of RBCs is challenged by several problems, including shortage of RBC units, the risk of blood borne infections and immune incompatibility because RBCs depend on donation from anonymous volunteers. Therefore, there is an urgent need to find consistent and safe alternatives to RBC transfusion. Large-scale in vitro production of transfusable RBCs from human cells, including hematopoietic stem/progenitor cells (HSPCs), human embryonic stem cells, and the induced pluripotent stem cells under controlled culture conditions could be a promising source to replace RBCs from donors to some extent.

Erythropoiesis in mammals is an intricately regulated, complex, and multiphase process, during which hematopoietic stem cells undergo a series of dramatic morphological changes to generate highly specialized and enucleated erythrocytes. Although significant progress has been made in past few decades, in vitro-generated RBCs are still not clinically available because of several major technical hurdles including limited yield, poor enucleation rate, low level of adult globin expression, and cost-effectiveness. In particular, the erythroid terminal maturation efficiency is a critical rate-limiting step of in vitro RBC production. During terminal differentiation, erythroblasts expel their nuclei, lose 20% to 30% of their cell surface, and eliminate all their organelles. So far, the molecular mechanisms involved in this process have not been fully elucidated.

Autophagy is believed to play an important role in both enucleation and clearance of organelles in erythroblasts. In this homeostatic process, autophagosomes sequester cytosolic components fuse with lysosomes to degrade their cargo. Among the autophagy signaling pathways, mammalian target of rapamycin (mTOR) is the key negative regulator. The physiological function of mTOR in erythroid cell maturation is still unclear, and the use of mTOR inhibitors for the treatment of anemia remains controversial. We previously reported that the inhibition of the mTOR signaling pathway with rapamycin dramatically decreased the percentage of erythroid progenitor cell in bone marrow of wild-type mice, which indicated that the mTOR pathway was necessary for mouse erythroid differentiation and proliferation during early stages of erythropoiesis. Interestingly, in vivo inhibition of mTOR partially corrected the erythroid maturation defect and significantly improved erythroid cell maturation and anemia in a β-thalassemia model. However, the roles of the mTOR signaling pathway in the regulation of human erythropoiesis during different phases and underlying mechanisms remain poorly deciphered.

In the present study, we investigated the roles of mTOR inhibition on human erythropoiesis at different time points in vitro and potential underlying mechanisms. Human umbilical cord blood (UCB)-derived CD34+ cells cultured in a serum- and feeder-free differentiation system were treated with rapamycin, the first mTOR inhibitor approved by the US Food and Drug Administration to prevent acute rejection in transplant recipients. Our findings of divergent inputs from mTOR signaling during commitment/proliferation phase compared to maturation phase may provide a foundation for further optimization of human erythroid differentiation systems in vitro.

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2 | MATERIALS AND METHODS

2.1 | CD34+ cell purification

Human UCB samples were collected from the First Affiliated Hospital of Shantou University Medical College. Informed consent was
obtained from donors, and the study was approved by the hospital's Ethics Committee and Research Ethics Advisory Committee. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (Solarbio, Beijing, China). CD34+ cells were purified using an EasySep Human CD34 Positive Selection Kit (18 056, STEMCELL Technologies, Vancouver, Canada) following manufacturer's instruction. The purity of CD34+ cells was determined by flow cytometry (ranged from 90% to 95%) using anti-human CD34 PE-Cy7 antibody (clone: 4A581G034E3, 4A Biotech Co. Ltd, Beijing, China).

2.2 | Cell culture

Purified CD34+ cells were seeded in a six-well plate at 2 × 10^5 cells/mL in Iscove's modified Dulbecco's medium (IMDM, Thermo Fisher Scientific, Waltham, Massachusetts) containing 20% knockout serum replacement and 2 mM GlutaMAX (Thermo Fisher Scientific), 40 μg/mL insulin, 10 μg/mL folic acid, 90 μg/mL ferric nitrate, 900 ng/mL ferrous sulfate, 25 μg/mL insulin, 150 μg/mL transferrin, and 160 μM monothioglycerol (all from Sigma-Aldrich, St. Louis, Missouri) and were cultured at 37°C in the presence of 5% CO2. From induction day 0 to day 8, the cells were cultured in medium supplemented with 1 nM dexamethasone (Sigma-Aldrich), 100 ng/mL stem cell factor, 3 U/mL erythropoietin (Epo, R&D Systems, Minneapolis, Minnesota). From induction day 8 to 14, expanded erythroblasts were cultured in the presence of 100 ng/mL stem cell factor and 3 U/mL Epo. From day 14, the erythroblasts were resuspended at a density of 1 × 10^9 cells/mL and only 3 U/mL Epo was added. Cells were stained with Wright-Giemsa reagents (Baso, Zuhuai, China) for morphological analysis.

K562 cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts) to identify enucleated cells. To stain mitochondria, cells were stained with PE-conjugated anti-human CD235a and MitoTracker Green (Invitrogen, Carlsbad, California). The mean fluorescence intensity (MFI) of MitoTracker (green) in nucleus. Images were acquired using a Zeiss LSM 800 confocal microscope. The mean fluorescence intensity (MFI) of MitoTracker (green) in CD34+ cells was analyzed using Zen software (Jena, Germany).

2.3 | Rapamycin treatment

According to the in vivo dose of rapamycin used in mice in our previous study and the concentration of in vitro K562 and human erythroid progenitors isolated from normal donors and patients with β-thalassaemia erythroid differentiation model of BFU-E and CFU-E as previously described. Cells were treated with rapamycin (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 50 nM. Media only and DMSO were used as controls. For detection of autophagy marker LC3 by immunoblotting, 1 nM of Bafilomycin A1 (Sigma-Aldrich) was also added into the culture media.

2.4 | Flow cytometry

Samples were analyzed using the BD Accuri C6 flow cytometer (Becton Dickinson, San Jose, California). The following antibodies were used for cell-surface marker detection: phycoerythrin (PE)-conjugated anti-human CD235a (clone: HIR2, Thermo Fisher Scientific), FITC-conjugated anti-human CD36 (clone: CA5-271, 4A Biotech Co. Ltd), and allophycocyanin (APC)-conjugated anti-human CD71 (clone: 4ACY1G4, 4A Biotech Co. Ltd). PE-, FITC-, or APC-conjugated isotype-matched antibodies served as controls. Cells were stained with the nucleic acid stain DRAQ5 (Cell Signaling Technology, Danvers, Massachusetts) to identify enucleated cells. To stain mitochondria, cells were labeled with MitoTracker Green (Invitrogen, Carlsbad, California). Mitochondrial membrane potential (MMP) was measured using MitoProbes JC-1 Assay Kit (Invitrogen). Apoptosis was determined using an Annexin V/Alexa Fluor 488/propidium iodide (PI) Apoptosis Detection Kit (FXP021-100, 4A Biotech Co. Ltd). Cell cycle was determined using a Cell Cycle and Apoptosis Analysis Kit (FXP022-100, 4A Biotech Co. Ltd). Cell proliferation was monitored using a CFDA-SE Cell Proliferation Assay Kit and colchicine-treated (0.05 μg/mL) cells were used as a non-proliferating control (all purchased from Beyotime, Shanghai, China). In addition, cell cycle and proliferation were also measured using an BrdU-APC/7-AAD Kit (BD Biosciences, Franklin Lakes, New Jersey). All staining and detection were performed according to the manufacturers' instructions. Flow cytometry data were obtained using the BD Accuri C6 software and analyzed by the FlowJo 8.0 software (Tree Star, Ashland, Oregon). Cell cycle data were analyzed using ModFit LT 3.0 software (Verity Software House, Topsham, Maine).

2.5 | Confocal microscopy

To analyze the mitochondrial content in erythrocytes, cells were stained with PE-conjugated anti-human CD235a and MitoTracker Green (Invitrogen). LysoTracker-Red and Hoechst 33342 (both purchased from Beyotime) were used to identify the localization of lysosomes and nucleus. Images were acquired using a Zeiss LSM 800 confocal microscope. The mean fluorescence intensity (MFI) of MitoTracker (green) in CD235a+ cells was analyzed using Zen software (Jena, Germany).

2.6 | Colony formation assays

The Colony forming assays were performed to determine the numbers of BFU-E and CFU-E as previously described.

2.7 | Quantitative reverse-transcription PCR (RT-qPCR)

For analysis of gene expression, total RNA was extracted from cultured cells using TRIzol (Invitrogen, 15596–026) according to the manufacturer's instructions. Reverse transcription reactions were performed using PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, Dalian, China) following manufacturer's instructions and real time PCR reactions were performed on CFX96 Real-Time PCR Detection System (Bio-Rad) using SYBR Premix Ex Taq II kit (Takara Bio, Dalian,
China) as previously described. The relative mRNA levels were normalized to those of β-actin. The primer sequences for the real-time PCR are listed in Supporting Information Table S1.

2.8 Western blot analysis

Proteins extraction from cells, separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blotting, and detection were performed as previously described. Primary antibodies against p70S6K (#9208), p-p70S6K-Thr389 (#9234), p-S6-Ser235/236 (#4858), S6 (#2217), LC3 (#12741), p62 (#8025), FIP200 (#12436), ULK1 (#8054), ATG7 (#8558), ATG5 (#12994), ATG12 (#4180), BECLIN (#3495), ATG3 (#3415), ATG101 (#13492), NIX (#12396), and β-actin (#8457) were purchased from Cell Signaling Technology. Anti-p27(F-8) antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas), E2F-2 (ab138515) antibody was purchased from Abcam (Cambridge, UK). HRP-linked anti-rabbit and anti-Mouse IgG were used at 1:5000 (#7074, #7076, Cell Signaling Technology). Band signal intensities were quantified as previously described.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad prism 8.0 software (GraphPad Prism, Inc, La Jolla, California). One-way analysis of variance followed by Dunnett’s multiple comparison test was used to compare means among the various treatment groups. P < .05 was considered significant.

3 RESULTS

3.1 Phenotypical and morphological characterization during erythroid differentiation of human umbilical cord blood-derived CD34+ cells in vitro

Based on our culture conditions developed for the in vitro differentiation of erythroblast from mouse HSPCs, we established a serum-based culture system to induce and monitor the erythroid differentiation of UCB-derived CD34+ cells. Forward scatter, correlated to cell diameter, and side scatter to cell inner complexity, decreased, indicating the reduction of cell size and complexity along with erythroid differentiation (Figure S1A). On days 8 to 10 of induction, cells were mostly pro-erythroblasts and basophilic erythroblasts with large nuclei and a deep violet cytoplasm. On day 19, nucleated cells consisted of polychromatophilic or orthochromic erythroblasts (Figure S1B), whereas on day 14, the cell pellets became reddish indicating the hemoglobinization of erythroid cells (Figure S1C). Obvious enucleation was observed on day 28.

The differentiation was also assessed by the expression of specific erythroid surface antigens. As shown in Figure S2A-C, the expression of CD36, a marker for erythroid progenitors increased steadily and peaked on day 16, followed by a gradual decrease. CD71, the transferrin receptor, is highly expressed on early erythroid cells (BFU-E stage) and disappears during the late reticulocyte stage. The percentage of CD71+ cells reached 94.0% ± 1.0% on day 8 and decreased to 32.9% ± 6.0% by day 72. The expression of CD235a, a mature erythrocyte marker, also increased, and the percentage of CD235a+ cells increased to 70% on day 27 and remained high thereafter. As differentiation progressed, CD235a+ cells started losing CD71. In our serum-free and feeder-free culture system, the percentage of enucleated red cells was 7.6% ± 2.6% on day 27 and 16.6 ± 1.1% on day 72 (Figure S2D). Cytosplasts of DRAQ5+ or DRAQ5− cells sorted by flow cytometry after staining with revealed that DRAQ5-negative cells were enucleated, whereas DRAQ5-positive cells were nucleated, confirming that DRAQ5 staining allowed distinguishing enucleated from nucleated cells (Figure S2E).

At different time points during differentiation, the expression of erythroid differentiation-specific genes and globin genes (GATA1, GATA2, SCL, α-, β-, and γ-globin) was measured by RT-qPCR (Figure S3). GATA1 and SCL expression gradually increased, peaked on day 14 and day 18, and then decreased, whereas GATA2 expression gradually decreased following cell maturation, indicating a GATA switch. Furthermore, mRNA level of globin genes (α-, β-, and γ-globin) elevated and peaked on around day 18, then decreased during maturation phase.

3.2 Rapamycin treatment inhibits human erythroid colony formation and erythroid progenitor cell growth

To study the role of mTOR pathway in the regulation of early erythropoiesis, erythroid colony forming assay was performed with or without rapamycin treatment. May-Grunwald-Giemsa staining results of cells from the colonies provided morphological evidence for BFU-E and CFU-E (Figure S4). The results showed that mTOR inhibition by rapamycin significantly decreased BFU-E number and almost completely inhibited BFU-E formation (Figure 1A-D). These findings suggested that mTOR was required for the formation of BFU-Es and CFU-Es. To further explore the effect of mTOR inhibition on erythroid progenitor cell growth, cells were treated with rapamycin from days 8 and 11 of differentiation, and viable cells were counted 24, 48, 72, and 96 hours following treatment (Figure 1E). Our results showed that rapamycin significantly inhibited the increase in erythroid precursor cell number, especially when added from day 8.

3.3 Rapamycin treatment inhibits human erythroid progenitor cell growth by suppressing proliferation and cell cycle progression instead of inducing apoptosis

Since mTOR signaling is a key controller of erythroid progenitor cell growth, we wanted to know how rapamycin inhibited erythroid
colony formation and cell growth. Our BrdU incorporation assay results showed that rapamycin treatment significantly decreased the proportion of cells in S-phase, whereas the fraction of G0/G1 cells increased (Figure 2A). Next, we performed carboxyfluorescein diacetate succinimidyl ester (CFDA SE) staining to detect cell proliferation by flow cytometry, since cell division can result in decrease of CFDA SE fluorescence. In order to compare proliferating cells on a day-to-day basis, colchicine which can block cell division was added. CFDA SE histograms showed that at indicated time points of differentiation following CFDA SE labeling, the MFI in rapamycin-treated cells was lower than in colchicine-treated cells but much higher than in control cells whether they were labelled from day 8 or 11, and the effect was more pronounced when rapamycin was added from day 8 (Figure 2B,C). These findings confirmed that rapamycin treatment significantly inhibited cell proliferation, and the results were in line with the cell count and the BrdU staining results.

We next evaluated whether mTOR inhibition by rapamycin had any impact on the cell cycle of erythroblast precursors. After erythroblast precursor cells were treated with rapamycin for 72 hours as of day 8 or day 11, the fraction of G0/G1 phase cells was increased (Figure 3A), suggesting that the cell cycle was arrested at the G0/G1 phase, which was consistent with the BrdU staining results (Figure 2A). p27 is an important cell-cycle inhibitor.22 p27 mRNA and protein levels were significantly increased after rapamycin treatment (Figure 3B,C). These results suggested that mTOR signal transduction was necessary for cell-cycle progression. Next, we detected erythroid cell apoptosis by Annexin V/PI staining and flow cytometry. Although, the differentiating erythrocytes showed fairly high apoptotic rates, there was no significant difference between cells treated with rapamycin for 72 hours from differentiation day 8 or day 11 and control groups (Figure S5).

3.4 | Rapamycin treatment does not affect human erythroid differentiation process

Addition of rapamycin at different time points during differentiation (day 8, 11, 18, 22, or 44) did not induce significant changes in CD71+ and CD235a+ cell fractions, indicating that rapamycin had no effect on differentiation antigens (Figure S6A-E). We determined the transcription of erythroid-specific genes and globin genes (Figure S6F,G). Rapamycin did not significantly affect GATA1, GATA2, and SCL mRNA

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**FIGURE 1** Rapamycin treatment inhibits erythroid colony formation and erythroid progenitor cell growth. A-D, Images of BFU-E (A) and CFU-E (C) derived from erythroid progenitor cells in a colony formation assay. Numbers of BFU-E (B) and CFU-E (D) derived from progenitor cells treated with or without rapamycin from day 1. E, Numbers of erythroid progenitor cells on the indicated days after treatment with or without rapamycin from day 8 or day 11. Data are the mean ± SD of technical triplicates from one of several independent experiments. *P < .05, **P < .01. RAPA, rapamycin
expression at most time points. Interestingly, α-, β-, and γ-globin mRNA levels were increased in rapamycin-treated cells, which was consistent with results obtained in other cell lines used as models for studying erythroid differentiation and in erythroid precursors from healthy animals and human subjects, and patients with β-thalassemia.11-13,15,18,23,24 These results suggested that inhibition of mTOR signaling did not affect the process of erythropoiesis. Intriguingly, inhibition of mTOR by rapamycin significantly upregulated the expression of all globin genes including α-, β-, and γ-globin, but not only γ-globin as previously reported.18

### 3.5 | Rapamycin treatment increases the fraction of enucleated cells

To investigate the roles of mTOR in the regulation of terminal maturation of erythrocytes, cells were treated with rapamycin at different time points and stained with DRAQ5 to detect the percentage of enucleated cells. We found that rapamycin treatment from day 11 or day 18 increased enucleation rate dramatically, the enucleation-promotive effect was weakened when rapamycin was added from day 22, and no significant enucleation was induced when rapamycin treatment was added from day 44 (Figure 4A,B; Figure S7A,B). Morphological analyses corroborated that rapamycin treatment from day 18 promoted enucleated cell production (Figure 4C,D). This was confirmed by laser-scanning confocal microscopy analysis of the enucleation rate in CD235a+ cells (Figure S7C). E2F-2 promotes nuclear condensation and enucleation of terminally differentiated erythroblasts.25 We found that rapamycin treatment from day 11 remarkably upregulated the expression of E2F-2 at both mRNA and protein levels (Figure 4E,F).

### 3.6 | Rapamycin treatment promotes mitochondria clearance

The mitochondria are eliminated following nucleus removal during late stage of erythrocyte maturation, so we next sought to determine whether mitochondrial removal was affected in rapamycin-treated erythrocytes at the late stage. First, mitochondria clearance was monitored through MitoTracker Green/CD235a-PE co-staining. Flowcytometry showed that the MFI of mitochondria was gradually decreased during differentiation, particularly in rapamycin-treated group, the MFI of mitochondria was significantly lower than that in control groups (Figure S8). Laser-scanning confocal microscopy also
revealed that rapamycin significantly reduced mitochondrial content (Figure 5A). Mitochondria are engulfed and degraded by lysosomes during mitophagy.26 Lysosome and mitochondria were visualized using LysoTracker-Red and MitoTracker-Green, respectively, under laser confocal microscope. The co-localization of mitochondria and lysosomes in cells is regarded as the formation of autolysosome. Our results showed that lysosome red fluorescence was significantly higher on day 60 in rapamycin treated group than that of control groups and co-localized with mitochondria green fluorescence. Moreover, mitochondria green fluorescence was significantly lower in rapamycin-treated groups than control groups. From these results, we speculated that the decrease of mitochondria content might be the result of mitophagy (Figure 5B). Mitophagy is commonly preceded by the loss of mitochondrial membrane potential (MMP), and thus, MMP is usually used as one of the indicators to evaluate the quality and quantity of mitochondria. As shown in Figure 5C, rapamycin caused a significant decrease in MMP level as seen by using potentiometric probe (JC-1) partly reflecting the quantity and/or function loss of mitochondria. A previous study showed that NIX was required for programmed mitochondrial clearance during reticulocyte maturation.27 Upregulation of NIX leads to the decrease of MMP, which activates autophagy and selective mitochondrial clearance.28 We found that NIX mRNA and protein expression were increased (Figure 5D,E), whereas the MMP was decreased post rapamycin treatment. Mitochondria are the main source of ROS. In mice, rapamycin can scavenge mitochondrial ROS to alleviate hemolytic destruction and anemia.29 Since rapamycin treatment enhanced mitochondrial removal, we next examined whether intracellular ROS level was also decreased in human erythrocytes in response to rapamycin treatment. Indeed, our results showed that intracellular ROS level was significantly decreased after the addition of rapamycin from day 11 (Figure 5F) and day 18, whereas rapamycin did not show a significant effect when added from day 22 or 44. Thus, we speculated that rapamycin suppressed intracellular ROS production by promoting mitochondrial clearance.

3.7 Rapamycin treatment enhances autophagy in human differentiating erythroid cells

We found that mRNA expression of mTOR downstream genes was decreased gradually during the in vitro erythropoiesis (Figure S9A). To investigate the potential mechanism underlying the effect of rapamycin treatment on erythrocyte proliferation and maturation, we

FIGURE 3  Rapamycin induces cell-cycle arrest in erythroid precursor cells. A, Erythroid precursor cells were treated with or without rapamycin from day 8 or day 11 for 72 hours. Then, the cells were stained with propidium iodide and subjected to flow cytometry to analyze DNA content. The right panel shows the fractions (%) of cells in G0/G1, S, and G2/M phases. B, p27 mRNA expression in cells on day 11 and 14 treated with or without rapamycin from day 8 as analyzed by RT-qPCR. Gene expression was normalized to β-actin. C, Representative images of p27 protein expression in cells on days 11 and 14 treated with or without rapamycin from day 8 as determined by western blot analysis. Data are the mean ± SD of technical triplicates from one of several independent experiments. *P < .05, **P < .01. RAPA, rapamycin
FIGURE 4  Rapamycin promotes enucleation. A-B, Cells treated with rapamycin from days 11 (A) and 18 (B) were stained with DRAQ5 (percentages of enucleated cells in the bottom panels). C, Cytospins of cells on days 28, 36, and 66 demonstrating the increase in enucleated cells after rapamycin treatment from day 18. D, The percentage of enucleated cells though counting 100 cells in three distinct areas of the slide. E, E2F-2 mRNA expression in cells on days 14 and 18 treated with or without rapamycin from day 11 as analyzed by RT-qPCR. F, Western blot analysis for E2F-2 in cells on day 18 and day 24 treated with or without rapamycin from day 11. Data are the mean ± SD of technical triplicates from one of several independent experiments. *P < .05, **P < .01. RAPA, rapamycin.
FIGURE 5  Rapamycin promotes mitochondrial removal. A, Confocal laser scanning microscopy images showing CD235a (red) staining with PE-conjugated CD235a antibody and mitochondria (green) staining with MitoTracker in cells on day 70 treated with or without rapamycin from day 18. The right figure indicates the mean fluorescence intensity (MFI) of mitochondrial in CD235a+cells (n = 50 cells from representative of three independent experiments). B, Confocal laser scanning microscopy images showing lysosomes (red) staining with LysoTracker and mitochondria (green) staining with MitoTracker in cells on day 60 treated with or without rapamycin from day 18. C, Mitochondrial membrane potential changes in cells on day 54 treated with rapamycin from day 11. The loss of mitochondrial membrane potential was demonstrated by the change in JC-1 fluorescence from red (JC-1 aggregates) to green (JC-1 monomers). D, NIX mRNA expression in cells on days 14 and 18 treated with or without rapamycin from day 11 as analyzed by RT-qPCR. E, Western blot analysis for NIX in cells on day 18 and day 24 treated with or without rapamycin from day 11. F, Intracellular ROS levels as measured by 2',7'-dichlorodihydrofluorescein diacetate staining treated with rapamycin from day 11. The right figure indicates mean fluorescence intensity as analyzed by flow cytometry. Data are the mean ± SD of technical triplicates from one of several independent experiments. *P < .05, **P < .01. RAPA, rapamycin.
first evaluated the effect of rapamycin treatment as of day 8 or 11 on the mTOR pathway. The timing of rapamycin treatment had no effect on the mRNA expression levels of 4E-BP1 and p70S6K (Figure S9B,C). Furthermore, rapamycin treatment had no effect on total p70S6K and S6 protein levels, whereas it significantly suppressed phosphorylation of p70S6K and of S6 (Figure S10A; Figure 6A).

**FIGURE 6** Rapamycin regulates autophagy by inhibiting the mTOR/p70S6K/S6 signaling pathway and activating autophagy genes. Cells were treated with rapamycin from day 11. Blank and DMSO were used as controls. A, Western blot analysis for mTOR signaling proteins (p-p70S6K [T389], p70S6K p-S6 [S235/236], and S6) in cells on days 14, 18, and 21. B, Western blot analysis for autophagy markers (LC3 and p62) in cells on days 14, 18, and 21. C, Expression of LC3 in erythroid progenitor cells and K562 cells as determined by western blotting. K562 cells were treated with different concentrations of rapamycin and harvested after 48 hours. D, Western blot analysis for autophagy-related proteins (FIP200, ULK1, ATG7, ATG12, ATG5, BECLIN, ATG3, and ATG101) in cells on days 14, 18, and 21. E, Protein expression was quantified by densitometry and normalized to β-actin expression. Data are the mean ± SD of technical triplicates from one of several independent experiments. *P < .05, **P < .01. RAPA, rapamycin.
Although rapamycin is commonly used as an autophagy inducer, the effect of rapamycin on autophagy in erythrocytes has not been studied. We first measured the expression of the autophagy markers LC3 and p62 (Figure S10B; Figure 6B). LC3-II/LC3-I ratio was significantly increased, whereas expression of p62 was significantly decreased in rapamycin-treated compared with control cells, indicating activation of autophagy and lysosomal clearance. These results suggested that rapamycin enhanced autophagy. Next, we compared LC3 expression between erythrocytes and K562 cells (Figure 6C). The LC3-II/LC3-I ratio was significantly higher in differentiating erythroid cells than that in proerythoblastic leukemic K562 cells. Moreover, LC3-II level in K562 cells was positively correlated with the concentration of rapamycin. These data suggested that autophagy was activated during erythroid differentiation.

Next, we measured the mRNA and protein expression of several key autophagy-related genes. Transcript levels of ULK1, BECLIN, and ATG12 were significantly upregulated after rapamycin treatment as of day 11 (Figure S9E). The transcription levels of ULK1, ATG12, and ATG16 were significantly upregulated following rapamycin treatment from day 8 (Figure S9D). Protein levels of FIP200, ULK1, BECLIN, ATG12, ATG5, ATG3, and ATG101 were significantly higher in rapamycin-treated than in control cells after rapamycin treatment as of day 11 (Figure 6D,E). Protein levels of ULK1, BECLIN, and ATG12 were significantly upregulated with rapamycin treatment from day 8 (Figure S10C,D). Collectively, these findings provided the evidence that in human erythropoiesis, autophagy markers were upregulated after rapamycin treatment as of day 11 or 18. Considering that rapamycin treatment as of day 11 had a negative effect on cell proliferation, addition of rapamycin from day 18 is more reasonable. We found that rapamycin promoted enucleation through E2F-2. Upon addition of the glucocorticoid antagonist mifepristone or high doses of iron-saturated transferrin enhanced enucleation has been observed; however, the mechanism has not been studied. We reasoned that rapamycin increased E2F-2 expression to promote enucleation. E2F-2 controls erythroblast enucleation by inducing expression of citron Rho-interacting kinase, a mitotic kinase that regulates astral microtubule length and spindle orientation in dividing cells, participates in nuclear condensation, and is involved in contractile actin ring formation. Before enucleation, erythroblasts exit the mitotic cell cycle which requires sequential control of genes which both accentuate S phase progression (eg, E2F-2) and enable exit from the cell cycle (eg, p27). Our results suggested that rapamycin treatment might increase E2F-2 and p27 production and promote cells exit from the cell cycle, thereby accelerating enucleation. However, further experiments are needed to demonstrate the

4 | DISCUSSION

In the past decades, numerous studies have successfully produced reticulocytes from adult hematopoietic stem and progenitor cells in vitro and demonstrated the feasibility of using in vitro cultured erythrocytes for blood transfusion. However, their clinical application has been hampered by the use of animal serum and/or feeder cells of murine or human origin in the culture medium. Therefore, here we established a serum-free and feeder-free culture system using a chemically defined medium. Erythroid differentiation was evaluated by microscopic observation, Giemsa staining, erythrocyte specific surface marker expression, enucleation rate, and erythroid-specific gene expression, consistent with previous studies on erythroid induction mechanisms. To avoid potential heterogenous and xenogeneic contamination of animal serum, our feeder-free and serum-free system is technically simple, safe, more convenient, and less expensive than conventional methods. It could be an efficient and reliable system for the study of erythropoiesis from human CD34+ HSPCs and even for future in vitro production of transfusable erythrocytes after optimization to achieve complete enucleation.

mTOR is a key regulator of protein synthesis, cell proliferation, and survival. During the proliferative phase of erythropoiesis, mTOR activity is relatively high and appears to be essential for erythroid precursor expansion. We found that BFU-E and CFU-E numbers were significantly decreased after the addition of rapamycin, suggesting that mTOR signal was a regulator of erythroblast proliferation in the early stage of human erythroid differentiation. This is consistent with findings in our previous study on mouse bone marrow erythroid colony formation capacity and a study by Diekmann et al, who observed that rapamycin led to fewer colonies in healthy controls and kidney transplant patients. We demonstrated that rapamycin had an antigrowth effect in erythroid progenitor cells by arresting the cell cycle rather than by inducing apoptosis, thus blocking cell proliferation, ultimately leading to poor colony formation. In our previous study in mice, rapamycin treatment strongly blocked erythroblast cell-cycle progression at the G1/S transition in immature erythroblasts. We found that the mRNA and protein expression of p27, a key cell cycle inhibitor, was upregulated by rapamycin, thereby inhibiting cell-cycle progression and proliferation, which was consistent with findings in other cell types.

Moreover, we noticed the appearance of sub-G1/apoptotic peak during erythroid differentiation process at certain time points (Figure 3A). Interestingly, there was a discrepancy between Annexin V/PI staining results (Figure S5). During mammalian erythropoiesis, the chromatin and nuclear gradually undergo condensation which are thought to be critical for enucleation. Enucleation of erythrocytes was initially thought to be a specific form of apoptosis. However, accumulating evidence supports a model of asymmetric division creating the short-lived nucleated pyrenocyte and the enucleate reticulocyte that matures into the RBC. Together, we believed that the sub-G1 peak found in our result was not a real apoptosis peak, but a natural phenomenon during erythroid enucleation.

Given that mTOR is also the main modulator of mitochondrial and ribosomal biogenesis, although we did not observe a significant effect of rapamycin on the progression of differentiation, early addition of rapamycin may negatively affect not only the quantity, but also the quality of differentiated erythrocytes.
mechanisms and the direct role of E2F-2 or p27 in promoting cell cycle exit or enucleation during erythropoiesis.

Accumulating evidence suggests that autophagy, affecting the proliferation and differentiation of erythroid progenitor cells and promoting the production and release of mature functional erythrocytes into the peripheral blood, is important in the regulation of erythroid maturation and homeostasis both in vitro and in vivo.15 During Atg5/Atg7-dependent canonical autophagy, the conversion of free form LC3-I to phosphatidylethanolamine-conjugated LC3-II is a key regulatory step in autophagosome formation. Thus, elevated LC3-II expression is considered a marker for autophagy activation.10 In addition, a receptor protein, p62, directly interacts with LC3 for degradation. p62 expression is inversely proportional to autophagy activity.46 mTOR is a critical negative regulator of general autophagy. In mammalian cells, rapamycin stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR. Inhibition of mTOR by rapamycin is associated with reduced phosphorylation of two of its downstream effectors, p70S6K and translation initiation factor 4E-BP1.47 Our results showed that as erythropoiesis progressed, the expression of mTOR downstream genes was gradually decreased. Furthermore, rapamycin treatment suppressed mTOR downstream genes p70S6K and S6 phosphorylation. Autophagy-associated genes expression and LC3-II/LC3-I were increased after treatment with rapamycin, whereas p62 expression was decreased. Together, our results suggest that rapamycin inhibits the phosphorylation of p70S6K and S6 downstream of mTOR, enhances the expression of various autophagy genes, enhances LC3-I conversion to LC3-II, reduces p62, and thus promotes autophagy. We observed a dramatic increase in autophagy in differentiating erythrocytes. This finding is consistent with findings by Betin et al, who reported that autophagy was induced at the polychromatic erythroid phase and that autophagosomes remained abundant until enucleation.48 RAB9 is involved in ATG5/ATG7-independent alternative autophagy, which is induced by starvation and treatment with drugs, except rapamycin.49,50 Consistent with these findings, we did not find any significant change in RAB9 mRNA expression upon rapamycin treatment.

The exact role of autophagy in enucleation has not been determined. The nucleus vanishes in the context of autophagy.51 Liu and Yao found that the ATG8 gene was enriched and autophagosomes, which are involved in nuclear degradation, were formed around the nucleus during autophagy, providing clear evidence that autophagy may be the main mechanism of nuclear degradation.52 Our findings corroborate that rapamycin may promote enucleation through active autophagy.

Another interesting finding was that rapamycin treatment promoted mitochondrial clearance. Mitophagy significantly influences mitochondria degradation during hematopoiesis.26 The analysis of mitochondria and lysosome colocalization indicated that the decrease in mitochondria following rapamycin treatment might be the result of mitophagy. The induction of mitophagy is associated with increased NIX expression.48 NIX, which is involved in MMP dispersion, autophagosome formation, and LC3-I-to-LC3-II conversion, is upregulated during reticulocyte maturation.27,28 We found that NIX expression was increased, and the MMP was decreased following treatment with rapamycin. Mitochondria produces the majority of cellular ROS. If mitochondrial clearance is delayed during erythrocyte terminal differentiation, oxidative stress is induced and hemolytic destruction of erythrocytes is enhanced. In agreement with findings in an animal study,29 we found that rapamycin treatment reduced intracellular ROS levels. Thus, rapamycin might promote mitochondrial clearance and reduce intracellular ROS production through upregulating NIX expression. Since the dramatic increase of enucleation rate was between day 30 and day 40 in rapamycin treated groups and mitochondria are supposed to be eliminated after nucleus expelling, it will be more convincing to compare NIX expression levels in late stage of erythrocyte maturation. However, we could not successfully obtain the data because protein and RNA extraction became very challenging after or even around day 30, especially from rapamycin treated cells. It might be due to the chromatin condensation or enucleation which results in the decrease of whole genome transcription level. Furthermore, microanalytical techniques are needed to determine the expression of NIX and other related genes.

Besides its well-known function in organelle clearance, recent data suggest that autophagy contributes to cell-cycle progression.53 p27, which regulates autophagy, cell-cycle arrest, and cell survival, may be an important molecule in the crosstalk between autophagy and the cell cycle.54 In our study, autophagy genes and p27 protein levels were enhanced and G0/G1 arrest was induced in erythroid precursors after rapamycin treatment, corroborating the correlation between autophagy and the cell cycle.

## 5 Conclusion

Our study demonstrated that mTOR played distinct roles at different phases of human erythropoiesis. Rapamycin treatment significantly suppressed the erythroid colony (BFU-E/CFU-E) formation through the inhibition of cell-cycle progression and cell proliferation in the commitment/proliferation phase of erythropoiesis. More importantly, in the maturation phase, rapamycin treatment significantly promoted the enucleation and mitochondria clearance through the inhibition of mTOR signaling and subsequent enhancement of autophagy. This finding provides us an opportunity to optimize the erythroid differentiation protocol for higher percentage of enucleated RBC, which is a significant challenge of transfusible RBC production in vitro. Furthermore, our finding also indicates the potential clinical application to decrease ineffective erythropoiesis in patients with erythroid disorders such as β-thalassemia, sickle cell anemia, and anemia of chronic disease.

**Conflict of Interest**

The authors declared no potential conflicts of interest.

**Author Contributions**

Q.L.: study design, research conduct, data analysis, and manuscript writing; X.Z.: study design, technical assistance, data analysis, supervised the project paper writing, and final approval of manuscript;
L.L.: provision of study material and technical assistance; C.R., M.Z., L.W., B.C., Y.W., S.F., X.H., N.T., S.H., X.H., F.C.: technical assistance and data analysis; S.Y.: research conduct and technical assistance; W.X.: technical assistance and provision of reagents and tools.

ETHICS STATEMENT
The Ethical Committee of First Hospital of Medical College of Shan-tou University approved this study.

DATA AVAILABILITY STATEMENT
The data that supports the findings of this study are available within the main text and supplementary material of this article.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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