Sirtuin 1 Activator Can Induce Proliferative Erythroblasts from Induced Pluripotent Stem Cells

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This Certifies that the Master's Thesis of Kim, Changyeong is Approved

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Thank my Lord Jesus Christ for His unwavering guidance and grace throughout this journey.

In every challenge, His presence was my strength and peace.

All glory be to Him.

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This achievement is not mine alone, but the result of many hands and hearts

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ABSTRACT

Sirtuin 1 Activator Can Induce Proliferative Erythroblasts from Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) are a promising cell source for regenerative medicine. Clinical applications require a large number of functional red blood cells (RBCs), making it essential to ensure the proliferation of actively dividing, nucleated erythroblasts derived from iPSCs. Small molecules can enhance the efficiency and frequency of iPSC-derived cell differentiation. Sirtuin 1, a key enzyme in multiple biological processes, has been implicated in enhancing iPSC-derived cell differentiation. However, the specific effects of Sirtuin 1 on erythroblast proliferation from iPSCs remain unclear. Here, we developed a protocol to examine the effects of Sirtuin 1 on erythroblasts after endothelial-to-hematopoietic transition (EHT). We found that Sirtuin 1 activation increased the frequency of $CD71^{+}CD235a^{+}$ erythroblasts at the early stage after EHT, suggesting a role for Sirtuin 1 in the proliferation of these specified erythroblasts. These findings reveal that Sirtuin 1 activation benefits erythroblast proliferation and could be considered for translational application in large-scale RBC culture.

Key words : **Induced Pluripotent Stem Cell, Sirtuin 1, $CD71^{+}CD235a^{+}$ erythroblasts, Erythropoiesis**

1. Introduction

Cerdan et al. [1] attempted to differentiate erythropoietic cells from embryonic stem cells (ESCs), and since then, many studies have reported the successful differentiation of functional RBCs from mouse and human induced pluripotent stem cells (iPSCs) [2-6]. Human pluripotent stem cells (hPSCs), including iPSCs, are a promising therapeutic cell source, and the clinical need for their derivatives, red blood cells (RBCs), is rapidly increasing in regenerative medicine and for hematologic malignancies [4, 5, 7]. Recent single-cell transcriptomic profiling of human embryos and yolk sacs (YS) has revealed the ontogeny of erythropoiesis, identifying three developmental waves: primitive erythropoiesis in the YS, hemogenic endothelium (HE)-derived definitive erythropoiesis in the fetal liver, and hematopoietic stem cell-derived erythropoiesis in the bone marrow (BM) [8, 9]. HE, characterized by the $CD34^{dim}CXCR4^{-}CD73^{-}$ phenotype, possesses the bipotent capability to differentiate into endothelial cells and hematopoietic lineage cells, and its specification is crucial for acquiring functional RBCs [10-12]. The large requirement of approximately 2×10^{12} RBCs for clinical applications has driven research to improve ex vivo production methods by achieving a high yield of $CD71^{+}CD235a^{+}$ erythroblasts [13]. Because enucleated RBCs cannot proliferate, obtaining a large number of nucleated erythroblasts is essential for advancing clinical applications [12, 14-16].

Several small molecules, in conjunction with key hematopoietic cytokines, can promote the differentiation of hematopoietic cells, including mature RBCs [17-20]. Sirtuin 1, a histone deacetylase, is involved in cellular activity, aging, and cell differentiation [21-23]. Studies on cell therapeutic approaches have demonstrated biological mechanisms of Sirtuin 1 in aging and its effects on the differentiation of PSC-derived cells, including beta cell-like cells, neuronal progenitors, mesenchymal stem cells, and hematopoietic lineage cells [21, 23-28]. Ou et al. [28] clearly showed defects in hematopoiesis in Sirtuin 1 knockout mice, with delayed development of hematopoiesis in both embryonic and adult stages. Notably, they observed that the low frequency and delayed development of hemangioblasts affected hematopoietic cells but not endothelial cells, suggesting a role for Sirtuin 1 in hematopoiesis. In contrast, Cha et al. [29] demonstrated that high Sirtuin 1 expression in hPSCs helps maintain stem cell pluripotency by silencing differentiation-promoting genes, and Han et al. [30] showed that Sirtuin 1 regulates Nanog expression in ESCs, uncovering a close relationship between Sirtuin 1 and PSC properties.

Therefore, the effects of Sirtuin 1 vary depending on factors such as cell type, developmental stage, and physiological conditions. Sirtuin 1 appears to have functional diversity within cells, promoting both cell differentiation in PSCs and maintenance of stem cell pluripotency. However, there is no clear evidence for the effects of Sirtuin 1 on erythropoiesis using iPSC-derived erythroblasts. To address this gap, we investigated the effects of Sirtuin 1 on the differentiation of

erythroblasts from iPSCs, focusing on the erythroblast stage after EHT. We found that Sirtuin 1 significantly promoted the proliferation of $CD71^{+}CD235a^{+}$ erythroblasts at an early stage. These findings may contribute to establishing a protocol for using Sirtuin 1 to generate clinically applicable RBCs.

2. Materials and methods

2.1. Human PSCs culture into differentiated erythropoietic cells

The human induced pluripotent stem cell (iPSC) line N11 was seeded onto Matrigel-coated plates in mTeSR™1 medium (Stem Cell Technologies, 85850) for differentiation into erythropoietic cells. To induce mesodermal differentiation, cells were cultured in Apel 2 medium (Stem Cell Technologies, 5275) supplemented with 3 μM CHIR-99021 (Selleck Chemicals, S2924), 20 ng/mL vascular endothelial growth factor (VEGF)165 (PeproTech, 100-20), and 25 ng/mL HumanKine® BMP-4 (Proteintech Group Inc., HZ-1045) for 2 days. To induce the formation of HE, cells were treated with Apel 2 medium supplemented with 250 ng/mL stem cell factor (SCF) (PeproTech, 300-07), 20 ng/mL VEGF165 (PeproTech, 100-20) and 25 ng/mL HumanKine® BMP-4 (Proteintech Group Inc., HZ-1045), FMS-like tyrosine kinase 3 (Flt3)-ligand (PeproTech, 300-19), 100 ng/mL Thrombopoietin (TPO) (Peprotech, 300-18), 20 ng/mL erythropoietin (EPO) (PeproTech, 100-64), 50 ng/mL of IGF-1 (Peprotech, AF-100-11), 400 μg/mL of ferric ion (Merck, F3388), 50 ng/mL of transferrin (Roche, 10652202001), and 50 ng/mL of folic acid (Merck, F8758) as described in the previous study. To treat the cells with the Sirtuin 1 activator, SRT2104 (Selleck Chemicals, S7792) was first diluted to a concentration of 3 μM in DMSO and used. (Stem Cell Technologies, 5275) The treatment was applied to the cells from day 8 to day 20, spanning 12 days, as described in a previous study [12, 31-34].

2.2. Human PSCs culture into differentiated erythropoietic cells

To investigate the frequency of hemogenic endothelium and erythroblasts derived from human iPSCs, harvested cells were incubated in MACS buffer (Miltenyi Biotec, 130-092-987) with specific antibodies and analyzed using a BD Verse1 flow cytometer (BD Biosciences). The antibodies used in this study were as follows: FITC-conjugated mouse anti-human CD34 (BD Biosciences, 348053), PE-conjugated mouse anti-human CXCR4 (BD Pharmingen™, 555974), APC-conjugated mouse anti-human CD73 (BioLegend, 344006), APC-conjugated mouse anti-human CD45 (BD Pharmingen™, 555485), FITC-conjugated mouse anti-human CD71 (BD Pharmingen™, 555536), and PE-Cy™7-conjugated mouse anti-human CD235a (BD Pharmingen™, 563666).

2.3. Immunocytochemistry

Human iPSCs and hemogenic endothelium were fixed with 2% PFA and permeabilized with 0.2% Triton X-100 for 30 minutes at room temperature. The cells were then washed with PBS, and non-specific antibody binding was blocked with 10% FBS for 30 minutes at room temperature. Following this, the cells were incubated with primary antibodies, followed by secondary antibodies. The primary antibodies were rat anti- human SRY-Box Transcription Factor 2 (SOX2) (Invitrogen, A24759), mouse anti-human Tumor-related Antigen-1-60 (TRA-1-60) (Invitrogen, A24868), Insulin-like growth factor II (IGF2) (Santa Cruz Biotechnology, sc-515805) and rabbit anti-human Runt-related transcription factor 1 (RUNX1) (Abcam, ab35962). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (VectorLabs, H-1200), and the cells were visualized using a fluorescent microscope (IX73 fluorescent microscope (Olympus) or LSM780 confocal microscope (Carl Zeiss) with LSM780 Image software.

2.4. Blast-forming unit (BFU) and colony-forming unit (CFU) assay

We investigated whether the floating cells collected on days 10 possess the functionality or potential to generate erythropoietic progenitors by incubating 2 x 104 floating cells derived from human iPSCs in 200 μL of MethoCult™ medium (Stem Cell Technologies, H4434) for 7 days. The cells were visualized using an IX73 fluorescent microscope (Olympus), and colony formation was evaluated using a CKX53 microscope (Olympus).

2.5. Quantitative (q) RT-PCR

Total RNA was extracted from human iPSCs using TRIzol™ reagent (Invitrogen™, 15-596-026) following the manufacturer's instructions [35]. cDNA was then synthesized using the SensiFAST™ cDNA Synthesis Kit (Meridian Bioscience, BIO-65054). Fragments were amplified by qRT-PCR using specific primers (Table 1), GoTaq® qPCR Master Mix (Promega, A6001), and the QuantStudio™ 3 System (Thermo Fisher Scientific). The relative mRNA expression of target genes was calculated using the comparative CT method, with all target genes normalized to *GAPDH* in multiplexed triplicate reactions. Differences in CT values were calculated for each target mRNA by subtracting the mean value of *GAPDH*. (relative expression = $2^{-\Delta CT}$)

2.6. Statistical analysis

All results are presented as mean ± s.e.m. Statistical analyses were performed with the Mann-Whitney U tests for comparisons between 2 groups using GraphPad Prism v.9 (GraphPad Software Inc). Values with $p < 0.05$ were considered to denote statistically significant.

3. Results

3.1. Differential Expression of IGF2 and its receptor IGF1R in iPSC lines

To confirm that the generated iPSCs possessed the properties of pluripotent stem cells, we performed immunocytochemistry and qRT-PCR on the iPSC cell lines. As shown in Figure 1, iPSC colonies were detected, and markers for PSCs, including SOX2, TRA-1-60, *NANOG*, and *OCT4*, were strongly expressed in these colonies (Figure 1, 2). These results confirm the successful establishment of iPSCs from mononuclear cells.

A major concern in lineage-specific cell differentiation from iPSCs is the variability in their differentiation capacity into specific lineages. Blood cells are particularly sensitive to this variability. Given a previous study that reported a correlation between high *IGF2* expression and the hematopoietic commitment capacity of human pluripotent stem cells [36], we sought to identify an iPSC line with high *IGF2* expression for efficient differentiation into erythroblasts. To this end, we performed qRT-PCR and immunocytochemistry on the N7, N9, N11, and N12 iPSC lines. The N11 cell line exhibited the highest levels of IGF2 protein (Figure. 3) and transcript levels of both *IGF2* and its receptor, *IGF1R* (Figure 4) and also showed the highest expression of pluripotency markers, despite qualitative expression across most iPSC lines. Therefore, we selected the N11 iPSC line for differentiation into RBCs.

3.2. Hemogenic Endothelium Generation from iPSCs

To investigate the generation of hemogenic endothelium (HE), which serves as a reservoir for blood lineage cells from the mesodermal stage, we examined the frequency of $CD34^{dim}$ HE. The differentiation protocol for HE and erythroblasts with Sirtuin 1 treatment is shown in Figure 5. HE with the $CD34^{dim}CXCR4^{-}CD73^{-}$ phenotype generated after the mesodermal stage, and FACS analysis was performed on cells 1.5 days after mesoderm induction. FACS data showed that $CD34^{+}$ cells were divided into dim and bright populations: $CD34^{dim}$ HE (6.2±0.2%) and $CD34^{bright}$ HE (5.9±0.4%). Both populations contained $CXCR4^{-}CD73^{-}$ undifferentiated cells ($CD34^{dim}$ HE, 30.3±8.7%; $CD34^{bright}$ HE, 20.8±5.5%) (Figure. 6). These results indicate the successful generation of HE with the $CD34^{dim}CXCR4^{-}CD73^{-}$ phenotype under our defined culture conditions. While the role of $CD73^{+}$ mesenchymal stromal cells in generating blood cells remains unclear, our protocol successfully generated an optimized HE population for erythroblast differentiation from N11 iPSCs.

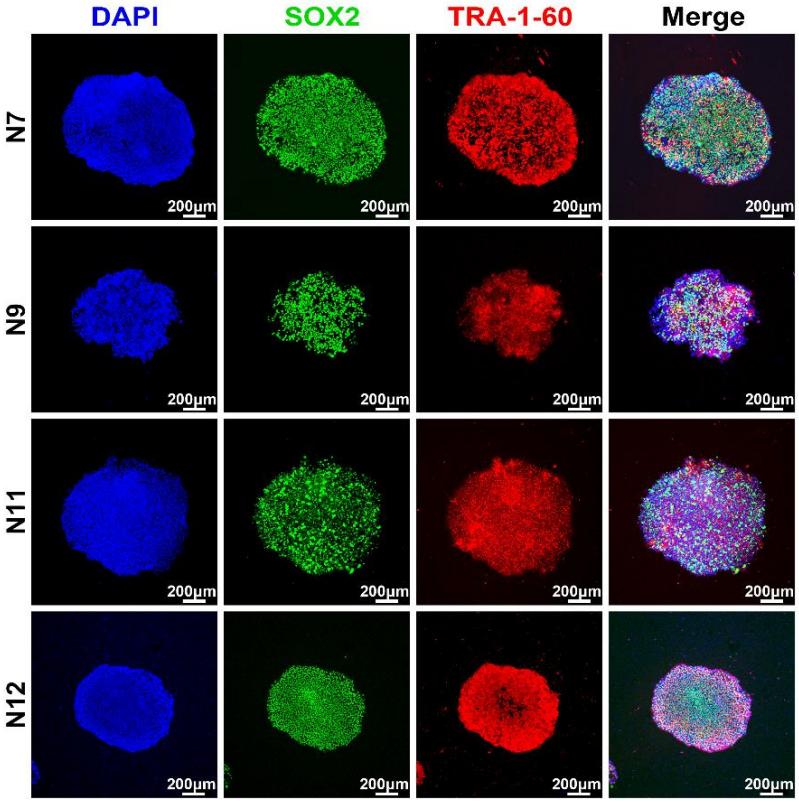

Figure 1. Protein expression of pluripotency markers in hiPSCs. Representative immunofluorescence images showing the expression of pluripotency markers SOX2 (green) and TRA-1-60 (red) in iPSC lines N7, N9, N11, and N12, as determined by immunocytochemistry. Nuclei were counterstained with DAPI (blue). Images were observed under a confocal microscope. Scale bar = 200 μm.

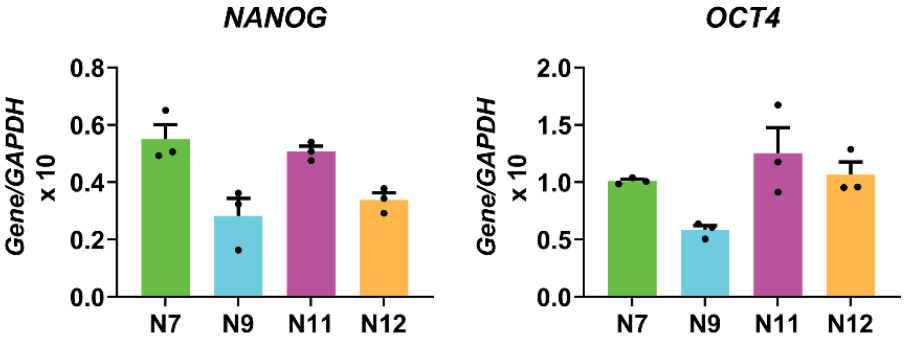

Figure 2. Gene expression of pluripotency markers *NANOG* and *OCT4* in hiPSC. The mRNA expression levels of pluripotency marker, *Nanog and OCT4* in hiPSC lines N7, N9, N11, and N12 were determined by qRT-PCR. *GAPDH* was used as a housekeeping gene. Data are presented as mean ± s.e.m. from 3 independent experiments (n = 3).

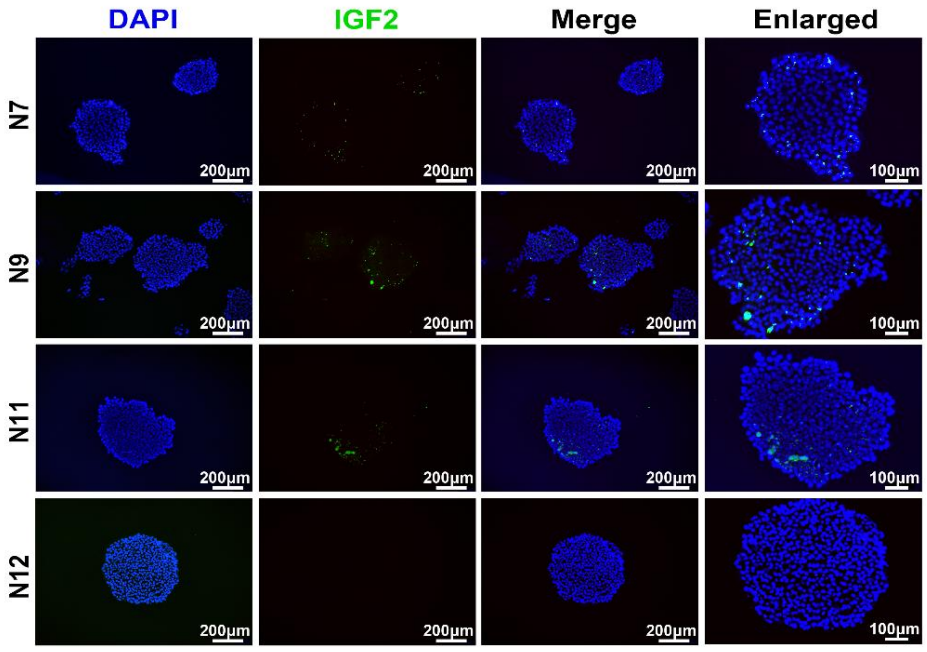

Figure 3. Protein expression of hematopoietic markers in hiPSCs. Representative immunofluorescence images showing the expression of the hematopoietic marker IGF2 (green) in hiPSCs, as determined by immunocytochemistry. Nuclei were counterstained with DAPI (blue). Images were observed under a fluorescence microscope. Scale bar = 200 μm, and 100 μm

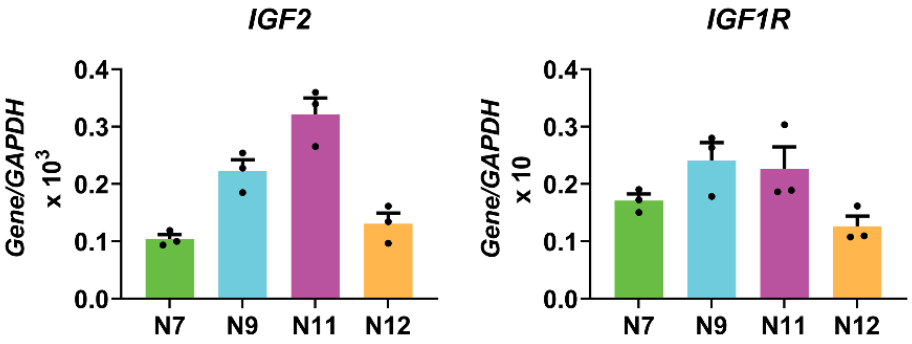

Figure 4. Gene expression of hematopoietic markers *IGF2* and its receptor *IGF1R* in hiPSCs. The mRNA expression levels of *insulin-like growth factor 2 (IGF2)* and its receptor, *insulin-like growth factor 1 receptor (IGF1R)*, in hiPSC lines N7, N9, N11, and N12 were determined by qRT-PCR. *GAPDH* was used as a housekeeping gene. Data are presented as mean ± s.e.m. from 3 independent experiments (n = 3).

Table 1. Primers used in qRT-PCR

Human	Forward primer	Reverse primer
human GAPDH	GGT GGT CTC CTC TGA CTT CAA CA	GTG GTC GTT GAG GGC AAT G
human NANOG	TGA ACC TCA GCT ACA AAC AG	TGG TGG TAG GAA GAG TAA AG
human OCT4	GAA CCG AGT GAG AGG CAA CCT	TCT GCT GCA GTG TGG GTT TC
human IGF2	GAT GCT GGT GCT TCT CAC CT	CAG ACG AAC TGG AGG GTG TC
human IGF1R	ACG TCC TCG ACA ACC AGA AC	CGT CAC TTT CAC AGG AGG CT
human ETV2	CTC AGC TCT CAC CGT TTG CT	ATG GGA CCT CGG TGG TTA GT
human SOX17	ACG CTT TCA TGG TGT GGG CTA AG	GTC AGC GCC TTC CAC GAC TTG
human MEIS1	ATG ACA CGG CAT CTA CTC GTT C	TGT CCA AGC CAT CAC CTT GCT
human CD34	AAA TCC TCT TCC TCT GAG GCT GGA	AAG AGG CAG CTG GTG ATA AGG GTT
human RUNX1	GTA TCC CCG TAG ATG CCA GC	TCG GAA AAG GAC AAG CTC CC
human CD71	ATC GGT TGG TGC CAC TGA ATG G	ACA ACA GTG GGC TGG CAG AAA C
human CD235a	ATA TGC AGC CAC TCC TAG AGC TC	CTG GTT CAG AGA AAT GAT GGG CA
human GATA1	ATC ACA CTG AGC TTG CCA CA	CAG GCC AGG GAA CTC CA

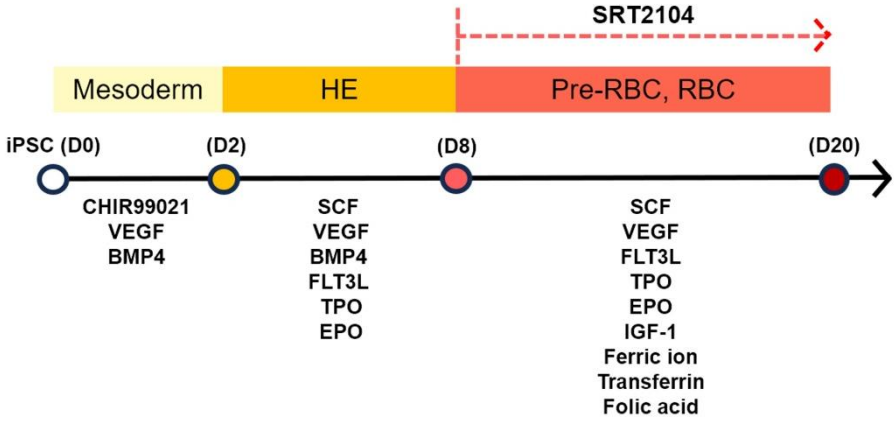

Figure 5. Schematic representation of HE and RBC differentiation. A schematic illustration depicting the differentiation process from iPSCs HE to RBCs. The diagram outlines key stages including mesoderm, HE, Pre-RBC and RBC. The schematic also indicates the stages where hemogenic endothelium and RBCs are generated, with cytokines used at each step specified.

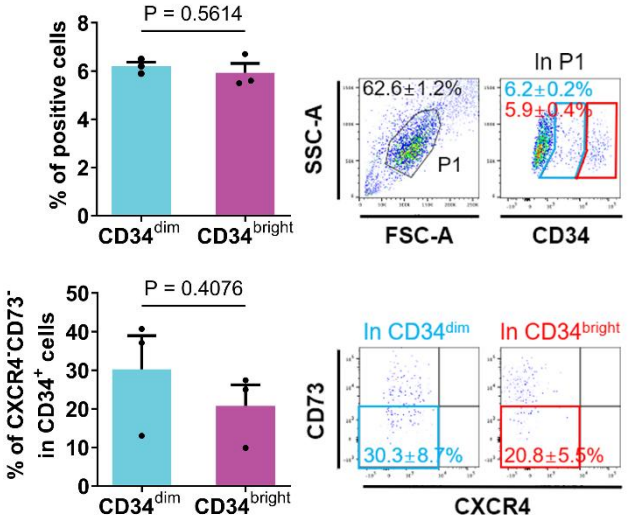

Figure 6. Flow cytometric analysis showing the frequencies of CD34$^{dim/bright}$ and CXCR4^{-}CD73^{-} HE populations. HE were collected at 1.5 days following mesoderm induction. The protein expression of CD34$^{dim/bright}$, CD73, CXCR4 were determined by flow cytometry. Data are presented as mean ± s.e.m. from 3 independent experiments (n = 3).

3.3. Upregulation of Mature Erythroid Cell Markers in Floating Cells by Sirtuin 1 Activator

To examine whether the Sirtuin 1 activator can promote erythroblast differentiation from iPSCs, RBC differentiation via HE was induced using hematopoietic growth factors. For this purpose, SRT2104, a selective activator of Sirtuin 1, was used. Consistent with a previous study (12), no morphological differences were observed in the differentiating cells from the early stage through day 14, regardless of SRT2104 treatment. However, the proliferation of reddish erythroblasts appeared earlier in the Sirtuin 1-treated group on day 18, while few clonal expansions of reddish erythroblasts were observed in the control group until day 20 (Figure 7). To determine if Sirtuin 1 affects HE, qRT-PCR was performed on HE at days 14 and 20. The qRT-PCR data showed that the expression of the mesodermal marker, *ETV2*, was consistently maintained in the presence of Sirtuin 1. The expression of pre-HE and HE markers, such as *SOX17*, *MEIS1*, and *CD34*, did not increase over time, indicating no effect of Sirtuin 1 on HE (Figure 8).

Next, the expression of erythropoiesis-related genes, including *RUNX1*, *GATA1*, *CD71*, and *CD235a*, was examined in HE and floating erythroblasts at day 14. *RUNX1*, a key transcription factor for both hematopoietic cells and HE, was expressed in both cell types. However, *RUNX1* expression was higher in floating cells treated with Sirtuin 1 compared to HE, suggesting that Sirtuin 1 strongly influences commitment to hematopoietic lineages. At the transcript level, the expression of the mature RBC marker, *CD235a*, was significantly increased in floating cells compared to HE under SRT2104 treatment (Figure 9). This result indicates a stronger association of Sirtuin 1 with floating erythroid cells than with HE.

To investigate RUNX1 functionality, immunocytochemistry was performed on HE and floating cells. Although only qualitative images were obtained, strong RUNX1 expression was detected in both cell types, regardless of Sirtuin 1 treatment. Together, our protocol successfully develops RBCs from iPSCs and can provide a platform to study the effects of Sirtuin 1 on erythroblast differentiation (Figure 10).

3.4. Sirtuin 1 Activator Promotes $CD71^{+}CD235a^{+}$ Erythroblasts

To further investigate the functional role of Sirtuin 1 in $RUNX1^{+}$ floating erythroblasts, we performed burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) assays using floating cells after EHT on day 10. BFU-E represents an early stage in RBC commitment with high proliferative capacity, while CFU-E is more specialized for RBC differentiation [37]. Cells differentiated with SRT2104 showed no significant differences in BFU-E and CFU-E colony numbers compared to the control group. Although the number of BFU-E colonies appeared lower with SRT2104 treatment at day 7 (no SRT2104, 30.1±5.5%; SRT2104, 18.4±3.8%),

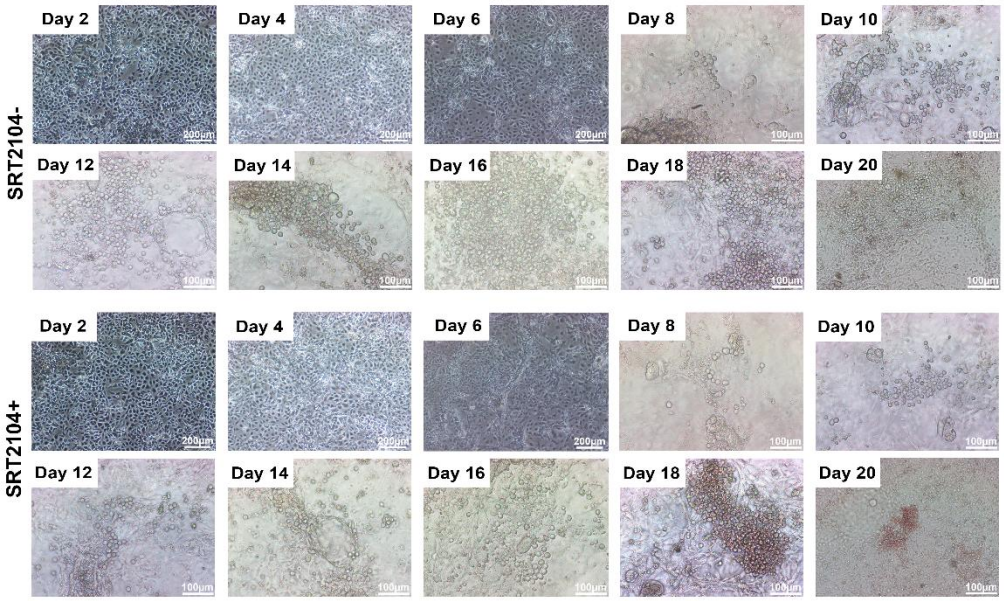

Figure 7. Changes in morphology during differentiation of iPSC-derived HE and erythroblasts with or without SRT2104 treatment. Representative images showing morphological changes during the differentiation of iPSC-derived HE and erythroblasts from day 2 to day 20. Cells were cultured either without ($SRT2104^{-}$, top) or with SRT2104 ($SRT2104^{+}$, bottom), added from day 8. Scale bars: 200 μm, 100 μm.

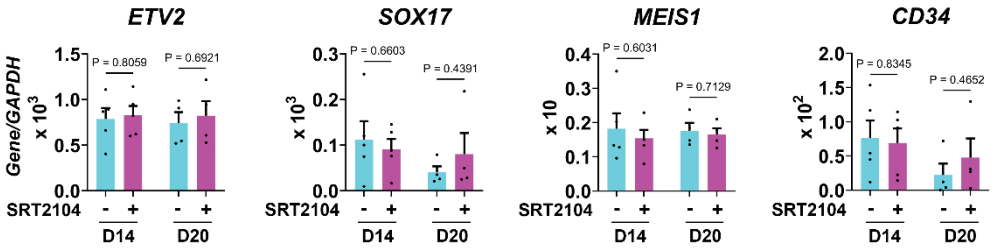

Figure 8. Gene expression of HE markers in HE with or without SRT2104 treatment. The mRNA expression levels of *ETV2, SOX17, MEIS1, CD34* in day14, and day 20 HE were determined by qRT-PCR. *GAPDH* was used as a housekeeping gene. Data are presented as mean ± s.e.m. from 4-5 independent experiments (n = 1-5).

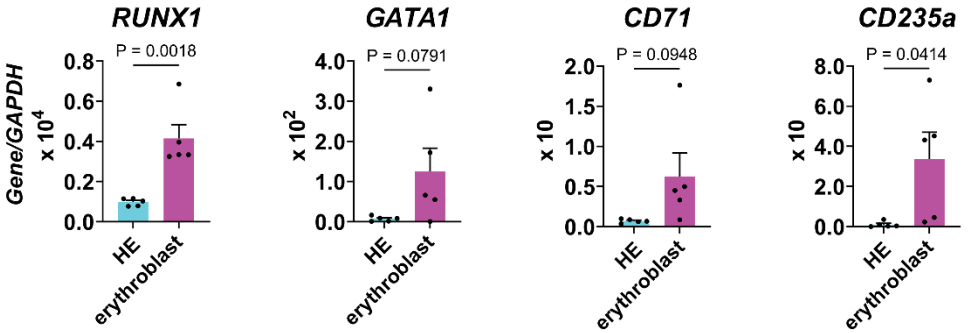

Figure 9. Gene expression of hematopoietic and erythroblast markers in HE and erythroblast with SRT2104 treatment. The mRNA expression levels of *RUNX1 GATA1, CD71, CD235a* in day14 HE and erythroblast were determined by qRT-PCR. *GAPDH* was used as a housekeeping gene. Data are presented as mean ± s.e.m. from 5 independent experiments (n = 2-5).

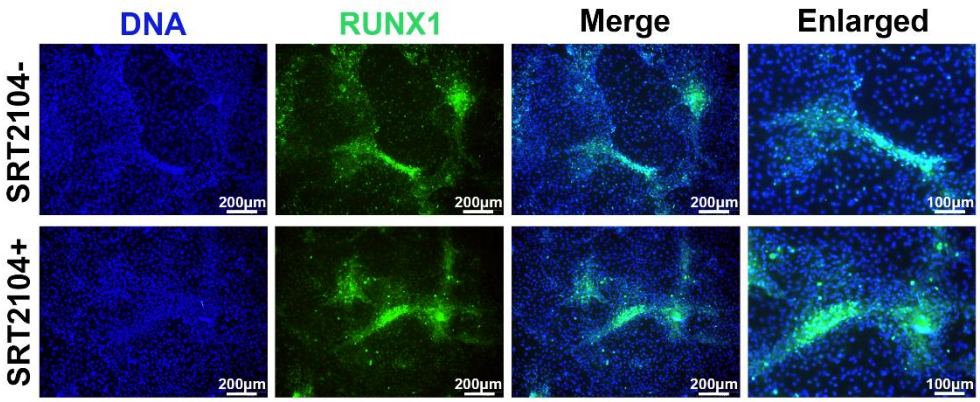

Figure 10. Protein expression of hematopoietic markers in HE and erythroblasts. Representative immunofluorescence images showing the expression of the hematopoietic marker RUNX1 (green) in HE and erythroblast, as determined by immunocytochemistry. Nuclei were counterstained with DAPI (blue). Images were observed under a fluorescence microscope. Scale bar = 200 μm, and 100 μm

this difference was not statistically significant. Similarly, while CFU-E colony numbers showed a trend of increase with SRT2104 treatment (no SRT2104, 27.1±4.7%; SRT2104, 35.7±8.3%), this difference also did not reach statistical significance (Figure 11). These results suggest that Sirtuin 1 does not play a significant role in the early developmental stages of BFU-E and CFU-E formation.

Next, to investigate whether the Sirtuin 1 activator can increase the frequency of erythroblasts, FACS analysis was performed on differentiated cells at day 14. Cells were gated into two populations, P1 (mature erythroblasts) and P2 (immature erythroblasts) (12). No significant differences were observed in the $CD45^-$ mature erythroid cell population between control and SRT2104 treated group (P1: no SRT2104, 93.6±1.4%; SRT2104, 90.5±2.8%; P2: no SRT2104, 78.8±2.4%; SRT2104, 84.9±4.0%) indicating that SRT2104 treatment did not affect the maturation of $CD45^-$ erythroid cells (Figure 12). The frequency of $CD45^+$ progenitor cells also showed no significant differences (data not shown).

To examine the effects of Sirtuin 1 on progenitor stages in detail, FACS analysis was performed using CD71 and CD235a markers to analyze the frequency of RBC progenitors within the P1 and P2 populations. The FACS data displayed all stages of human erythropoiesis based on CD71 and CD235a expression. In the P1 population, the frequencies of $CD71^+CD235a^-$, $CD71^+CD235a^+$, and $CD71^-CD235a^+$ erythroblasts were not significantly increased by SRT2104 at day 14. However, in the immature population P2, the frequency of $CD71^+CD235a^+$ erythroid progenitors was significantly increased by SRT2104 at day 14 ($CD71^+CD235a^+$ cells: no SRT2104, 77.9±6.8%; SRT2104, 88.8±4.3%) (Figure 13). This demonstrates an effective role of Sirtuin 1 in promoting $CD71^+CD235a^+$ erythroblasts at day 14. Based on our data, we conclude that Sirtuin 1 does not significantly affect the early stages of erythropoiesis and HE, but it significantly promotes the expansion of $CD71^+CD235a^+$ erythroblasts following EHT.

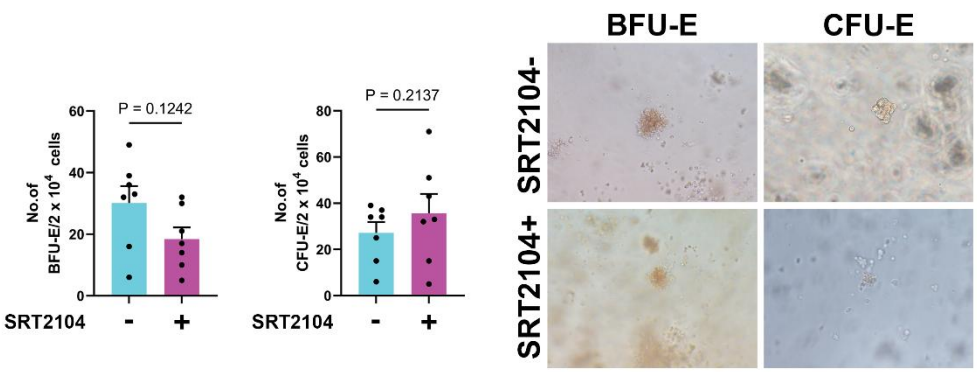

Figure 11. CFU assay of iPSC-derived erythroid cells with or without SRT2104 treatment. Cells were harvested on day 10 and plated in methylcellulose medium. Colonies were assessed after 7 days. Representative images and colony count of CFU-E and BFU-E are shown. Cells were cultured with or without SRT2104 from day 8. Data are presented as mean ± s.e.m. from 2 independent experiments (n = 3-4).

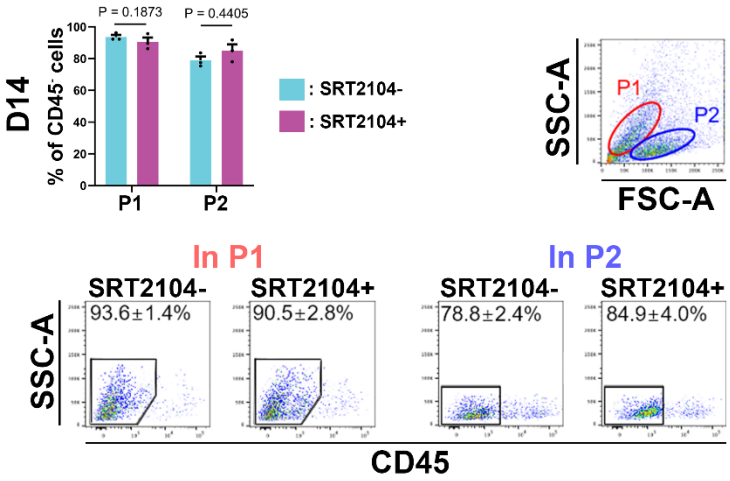

Figure 12. Flow cytometric analysis showing the frequencies of CD45$^-$ cells in mature (P1) and immature (P2) populations with or without SRT2104 treatment. The percentages of CD45$^-$ erythroblasts were analyzed in mature and immature populations at day 14, with or without SRT2104 treatment. Data are presented as mean ± s.e.m. from 3 independent experiments (n = 2-4).

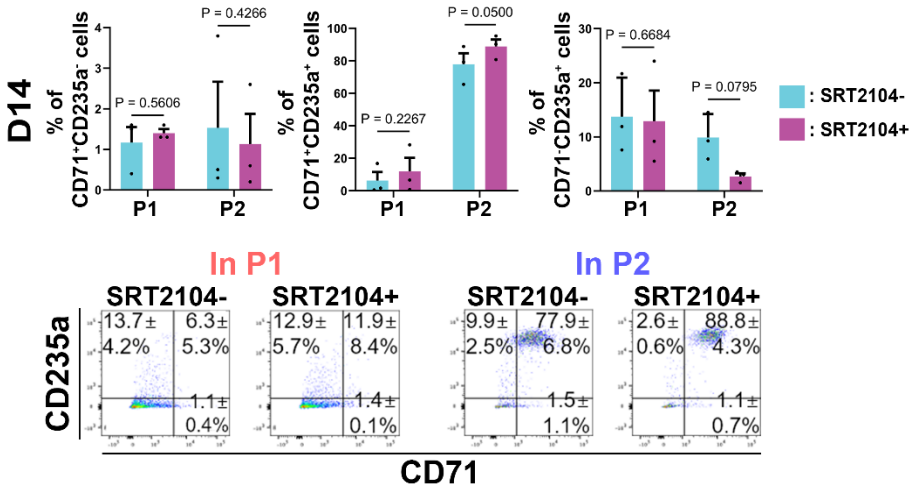

Figure 13. Flow cytometric analysis of CD71$^+$, CD71$^+$CD235a$^+$, and CD235a$^+$ erythroblast populations in CD45$^-$ mature (P1) and immature (P2) cells, with or without SRT2104 treatment. CD71$^+$, CD71$^+$CD235a$^+$, and CD235a$^+$ erythroblast populations were analyzed within the CD45$^-$ cell fraction based on surface marker expression. Data are presented as mean ± s.e.m. from 3 independent experiments (n = 2-4).

4. Disccussion

Sirtuin 1, a NAD^+-dependent histone deacetylase, modulates gene expression and cellular activity, emerging as a promising epigenetic target with activators like resveratrol and SRT2104 in clinical use [38-40]. To investigate the effects of the Sirtuin 1 activator SRT2104 on erythropoiesis, we employed a simplified differentiation protocol encompassing both primitive and definitive erythropoiesis. Primitive erythropoiesis, crucial for early embryonic cardiovascular development [41], and definitive erythropoiesis from $CD34^{dim}CXCR4^-CD73^-$ HE were concurrently induced in our system. While the precise ontogeny of the generated erythroblasts remains to be fully elucidated, we observed a significant increase in erythroblast frequency at differentiation day 14 upon Sirtuin 1 activation. This was further supported by the enhanced proportion of mature erythroblasts ($CD71^+CD235a^+$), which represent polychromatic and orthochromatic stages [42, 43]. This suggests that erythroblasts, which exhibit reduced mitotic capacity, from basophilic- to polychromatic erythroblasts can be stimulated to proliferate by SRT2104, representing a potentially significant finding.

Numerous studies have indicated the role of Sirtuin 1 in suppressing cellular senescence and regulating gene expression through chromatin stabilization [44, 45]. However, the multifaceted functions of the sirtuin family, including Sirtuin 1, have led to ongoing debate [46]. Similarly, conflicting views exist regarding the role of Sirtuin 1 in iPSC differentiation. Some studies suggest it maintains stemness by promoting self-renewal in embryonic stem cells and delaying hematopoietic stem cell aging [23, 29, 30, 44, 47], while a growing body of evidence highlights its critical role in lineage-specific differentiation from PSCs by supporting cell fate determination [48, 49]. Our objective investigation into the effects of a Sirtuin 1 activator on erythroid differentiation from PSCs revealed that erythroblasts were significantly induced after commitment to the RBC lineage, suggesting a promotion of RBC differentiation, consistent with some previous findings [23, 28]. The effects of Sirtuin 1 were prominent at differentiation day 14, a stage characterized by predominantly immature erythroblasts and a limited number of $CD235a^+$ mature RBCs. This suggests that Sirtuin 1 may mitigate cellular senescence and enhance cell proliferation in the early stages following erythroid lineage commitment. In contrast to erythroid cells, Sirtuin 1 minimally or did not affect the frequency of HE, the reservoir for blood cells. This observation supports the hypothesis that the effects of Sirtuin 1 are cell type and developmental stage-dependent.

Clonal variation in PSCs is known to significantly influence iPSC properties, including hematopoietic potential and pluripotency maintenance [36, 50]. These variations can arise from genetic and epigenetic differences linked to donor-specific properties and the origin cells. Nishizawa et al. [36] reported that iPSC differentiation capacity varies between clones, identifying IGF2 as a key factor promoting $CD43^+$ hematopoietic lineage specification via epigenetic modifications. Mills et al. [50] emphasized the importance of selecting iPSC lines with inherent potential for hematopoietic development. Consistent with these findings, we selected the N11 iPSC line, which exhibits high IGF2 and its receptor expression, along with stable stem cell properties, and demonstrated reproducible erythroblast differentiation. However, establishing standardized criteria for lineage

specification selection remains an ongoing challenge. Our observation that Sirtuin 1 treatment from days 3 to 8 had no effect on early-stage cells (mesodermal cells or HE; data not shown) further supports a stage-specific role. Instead, Sirtuin 1 significantly enhanced EHT occurrence and the presence of floating erythroid cells at later stages, suggesting a supportive role in RBC differentiation from HE. However, further research is needed to determine if Sirtuin 1 universally promotes RBC differentiation across different cell lines and clonal variations, highlighting the importance of optimizing the timing and duration of Sirtuin 1 treatment to target the proliferative phase of erythroblasts.

For large-scale GMP-compliant RBC production, obtaining erythroblasts capable of mitotic division is crucial. Since proliferation requires a nucleus, focusing on erythroblasts that can proliferate significantly with iron supplementation is necessary. Additionally, achieving a substantial quantity of $CD71^{+}CD235a^{+}$ mature erythroblasts is critical for overall RBC production, even with relatively reduced proliferation. Our findings indicate that Sirtuin 1 selectively promotes erythroblast proliferation after EHT without affecting HE. Furthermore, we acknowledge the major challenges in large-scale RBC production and suggest that our approach using Sirtuin 1 activation holds promising potential for developing functional RBCs from PSCs for clinical applications and for generating RBC progenitors to support a universal blood bank system.

5 . Conclusion

In this study, we demonstrated that activation of Sirtuin 1 positively influences the proliferation of erythroblasts derived from induced pluripotent stem cells (iPSCs). Specifically, Sirtuin 1 activation led to a significant increase in the frequency of $CD71^{+}CD235a^{+}$ double-positive erythroblasts at the early stage following the endothelial-to-hematopoietic transition (EHT), suggesting a critical role of Sirtuin 1 in promoting erythroblast expansion during early hematopoietic differentiation.

Sirtuin 1 is a well-known enzyme involved in various physiological processes, and our findings extend its functional relevance to iPSC-derived erythroid differentiation. Treatment with SRT2104, a Sirtuin 1 activator, enhanced the generation of proliferative erythroblasts more efficiently than conventional protocols, thereby providing a stronger foundation for subsequent maturation into functional red blood cells.

These results suggest that Sirtuin 1 activation could serve as a useful regulatory strategy for improving the scalability and efficiency of erythroid differentiation from iPSCs. Further investigation into the underlying mechanisms of Sirtuin 1 activation, as well as its long-term safety and reproducibility under extended culture conditions, will be crucial for establishing a robust and clinically applicable platform for large-scale red blood cell production.

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ABSTRACT in Korean

Sirtuin 1 활성화제를 통한 유도만능줄기세포에서 증식성 적혈모세포 유도 연구

유도만능줄기세포(iPSCs)는 재생의학 분야에서 유망한 세포 자원입니다. 임상적 용을 위해서는 기능하는 적혈구(red blood cells. RBCs)를 대량으로 확보하는 것이 필수적이며, 이를 위해서는 iPSC로부터 유래한 활발히 분열하는 핵을 가진 적혈모구(erythroblast)의 증식을 확보하는 것이 중요합니다. Small molecule은 iPSC 유래 세포의 분화 효율성과 빈도를 향상시킬 수 있습니다. 여러 생물학적 과정에서 핵심적인 효소로 알려진 Sirtuin 1은 iPSC 유래 세포의 분화를 촉진하는 것으로 보고된 바 있습니다. 그러나 Sirtuin 1이 iPSC 유래 적혈모구의 증식에 미치는 구체적인 영향은 아직 명확히 밝혀지지 않았습니다. 본 논문은 hemogenic endothelium에서 hematopoietic cell로의 전환 (Endothelial to hematopoietic transition, EHT) 이후 적혈모구에서 Sirtuin 1의 효과를 조사하기 위한 프로토콜을 개발하였습니다. 그 결과, Sirtuin 1 활성화는 EHT 이후 초기 단계에서 $CD71^{+}CD235a^{+}$ 적혈모구의 빈도를 증가시키는 것으로 나타났으며, 이는 Sirtuin 1이 특이적으로 분화된 적혈모구의 증식에 관여함을 시사합니다. 이러한 결과는 Sirtuin 1의 활성화가 적혈모구 증식에 유리하게 작용함을 보여주며, 대량 적혈구 배양을 위한 실용적 적용 가능성을 제시합니다.

핵심 되는 말 : 유도만능줄기세포, Sirtuin 1, $CD71^{+}CD235a^{+}$ 적혈모구세포, 적혈구 생성