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REGULAR SUBMISSION

ATP produced by anaerobic glycolysis is essential for enucleation of human erythroblasts

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More than 2 million human erythroblasts extrude their nuclei every second in bone marrow under hypoxic conditions (<7% O_2). Enucleation requires specific signal transduction pathways and the local assembly of contractile actomyosin rings. However, the energy source driving these events has not yet been identified. We examined whether different O_2 environments (hypoxic [5% O_2] and normoxic [21% O_2] conditions) affected human $CD34^+$ cell erythroblast differentiation. We also investigated the regulatory mechanisms underlying energy production in erythroblasts during terminal differentiation under 5% or 21% O_2 conditions. The results obtained revealed that the enucleation ratio and intracellular levels of adenosine triphosphate (ATP), lactate dehydrogenase (LDH) M_3H, and hypoxia-inducible factor 1α in erythroblasts during terminal differentiation were higher under the 5% O_2 condition than under the 21% O_2 condition. We also found that the enzymatic inhibition of glyceraldehyde 3-phosphate dehydrogenase and LDH, key enzymes in anaerobic glycolysis, blocked the proliferation of colony-forming units−erythroid and enucleation of erythroblasts, and also reduced ATP levels in erythroblasts under both hypoxic and normoxic conditions. Under both conditions, phosphorylation of the Ser232, Ser293, and Ser300 residues in pyruvate dehydrogenase (inactive state of the enzyme) in erythroblasts was involved in regulating the pathway governing energy metabolism during erythroid terminal differentiation. This reaction may be mediated by pyruvate dehydrogenase kinase (PDK) 4, the major PDK isozyme expressed in erythroblasts undergoing enucleation. Collectively, these results suggest that ATP produced by anaerobic glycolysis is the main source of energy for human erythroblast enucleation in the hypoxic bone marrow environment. **© 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

Mammalian erythropoiesis culminates in enucleation, a still partially understood process that entails the expulsion of the nucleus from the cytoplasm of erythroblasts. During erythropoiesis, stem cells undergo lineage-specific commitment and generate erythroid progenitor cells through cellular division events, which include nuclear (mitosis) and cytoplasmic (cytokinesis) components. These progenitor cells consist of burst-forming units −erythroid (BFU-Es) and their progeny, colony-forming

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units−erythroid (CFU-Es) [1,2]. Over the course of an additional 6 to 7 days, human CFU-Es proliferate and differentiate into mature erythroblasts [3−5]. Mammalian erythroblasts then undergo enucleation, become reticulocytes, and, finally, mature erythrocytes. Expelled nuclei are phagocytosed by macrophages [6].

More than 2 million erythroblasts extrude their nuclei every second [7]. The process of enucleation may be similar to cytokinesis, and many of the general principles of cytokinesis apply to enucleation. In cytokinesis and enucleation, the cytoskeleton plays a key role in the positioning of the division site. Once this site is selected, the local assembly of contractile actomyosin rings occurs, during which nonmuscle myosin IIB remodels the plasma membrane [4]. Trafficking of the necessary components to the division site and membrane fusion lead to the physical separation of daughter cells [8,9]. Moreover, several key signal transduction effectors, such as phosphoinositide 3-kinase [10], contribute to the enucleation process mainly through phosphorylation events. These findings strongly support enucleation requiring adenosine triphosphate (ATP) as an energy source. The concentration of oxygen in the bone marrow environment, in which erythroblasts develop, differentiate, and extrude their nucleus, is less than 7% [11,12]. This feature implies that erythroblasts extrude their nuclei under hypoxic conditions and suggests that the ATP necessary for enucleation is produced by anaerobic glycolysis rather than by oxidative phosphorylation in mitochondria. This assumption appears to be supported by the following observations on other processes that occur in the hypoxic bone marrow environment: Simsek et al. [13] and Kocabas et al. [14] reported that mouse and human long-term hematopoietic stem cells (LT-HSCs) use glycolysis instead of mitochondrial oxidative phosphorylation as their main energy source. These unique metabolic properties of LT-HSCs are considered to depend on hypoxia-inducible factor (HIF)-1α−driven metabolic pathways [13,14].

Glycolysis is a series of metabolic processes by which one molecule of glucose is catabolized to two molecules of pyruvate with a net gain of two ATP [15]. Under anaerobic conditions, glycolysis is the main energy source in living cells, and nicotinamide adenine dinucleotide (NAD$^+$ [oxidized form]), which is essential for the catalytic reaction of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is regenerated from NADH (reduced form) by the reduction of pyruvate to lactate catalyzed by lactate dehydrogenase (LDH) [15]. In anaerobic glycolysis, monocarboxylate transporters (MCTs), in which MCT1 is ubiquitously expressed, are essential for the transport of increased lactate across the plasma membranes [16]. To generate ATP through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria, dephosphorylated pyruvate dehydrogenase (PDH) catalyzes the irreversible oxidative decarboxylation of pyruvate into acetyl-coenzyme A (CoA) [15,17]. The phosphorylation of PDH is accomplished by PDH kinases (PDKs), which consequently inactivate the enzymatic activity of PDH [17]. Three phosphorylation sites have been identified on the α subunit of human PDH: Ser232, Ser293, and Ser300 [18].

The superfamily of glucose transporters (GLUTs) comprises 14 isoforms in the human genome. Among them, GLUT1 is the main functional transporter in various hematopoietic cell lineages, including erythroblasts and mature erythrocytes [19,20]. However, limited information is currently available on the regulation and function of GLUT1 during erythropoiesis. According to the findings reported by Montel-Hagen et al. [21], glucose transport actually decreases during human erythropoiesis despite an increase in GLUT1 transcripts, which naturally raises the question of the nature and regulation of energy metabolism in human erythroblasts. Limited information is currently available on ex vivo models of the regulation of erythropoiesis by low O_2 concentrations [22].

In the present study, we examined whether different O_2 environments (hypoxic [5% O_2] and normoxic [21% O_2] conditions) affect human CD34$^+$ cell erythroblast differentiation. We also investigated the regulatory mechanisms underlying energy production in erythroblasts during terminal differentiation under hypoxic or normoxic conditions. The results obtained revealed the acceleration of erythroblast enucleation under hypoxic conditions and led us to propose mechanisms for the production of ATP via anaerobic glycolysis during the terminal differentiation of erythroblasts including enucleation.

Methods

Reagents and inhibitors

Bovine serum albumin (BSA) and Iscove's Modified Dulbecco's Medium (IMDM) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium was from Life Technologies (Carlsbad, CA). Fetal calf serum (FCS) was from Flow Laboratories (McLean, VA) and Hyclone Laboratories (Logan, UT). Penicillin and streptomycin were from Flow Laboratories. Insulin was from Wako Pure Chemical Industries (Osaka, Japan). Interleukin-3 (IL-3) and stem cell factor (SCF) were gifts from Kirin Brewery (Tokyo, Japan). Erythropoietin (EPO) was from Chugai Pharmaceutical (Tokyo, Japan). Vitamin B_{12} was from Eisai (Tokyo, Japan), and folic acid was from Takeda Pharmaceutical (Osaka, Japan).

The GAPDH inhibitor koningic acid (KA) [23] was from Adipogen (San Diego, CA). The LDH inhibitor stiripentol (STP) [24] was from Tokyo Kasei (Tokyo, Japan).

Cell preparations

Granulocyte colony-stimulating factor (G-CSF)−mobilized human peripheral blood CD34$^+$ cells were purified as previously described [25] and stored in liquid nitrogen until further use. Informed consent was obtained from all participants prior to their entry into this study, which was approved by the Akita University Graduate School of Medicine Committee for the Protection of Human Subjects.

To generate erythroid progenitor cells, CD34$^+$ cells prepared from the same individual were thawed and cultured as previously described [3−5]. Briefly, cells were cultured in phase I medium (IMDM erythroid medium containing 20% FCS, 10% heat-inactivated pooled human AB serum, 1% BSA, 10 μg/mL insulin, 0.5 μg/mL vitamin B_{12}, 15 μg/mL folic acid, 50 nmol/L β-mercaptoethanol, 50 U/mL penicillin, 50 μg/mL streptomycin in the presence of 50 ng/mL IL-3, 50 ng/mL SCF, and 2 IU/mL EPO). Cells were maintained in a 5% CO_2 incubator (MCO-170AICUVH, PHC Holdings, Tokyo, Japan) at 37°C under 21% O_2 conditions or in a Multi-Gas Incubator (MCO-5M, PHC Holdings) at 37°C under 5% CO_2 and 5% O_2 conditions. After 7 days in culture, cells were harvested and washed three times with IMDM containing 0.3% BSA. The maturation stage of day 7 cells was similar to that of CFU-Es [3−5]. Hereafter, day 7 cells were referred to as CFU-Es. Aliquots of CFU-Es (1×10^5 cells) were then cultured in phase II medium (erythroid medium with EPO alone, without β-mercaptoethanol, IL-3, or SCF), as previously described [3−5] under 5% or 21% O_2 conditions, with or without the inhibitors KA and STP dissolved in dimethyl sulfoxide (DMSO).

Evaluation of enucleation

Enucleation was evaluated as previously described [4,5]. Briefly, cultured cells were spun onto slides using Cytospin 3 (Shandon Lipshaw, Pittsburgh, PA) and were stained with May−Grünwald−Giemsa reagent. Enucleation was defined as the expulsion of the nucleus to the outside of the reticulocyte. Reticulocytes touching expelled nuclei or with a thin connecting strand between the reticulocyte and nucleus were considered to be the earliest enucleated cells. The enucleation fraction among cytospin cells was similar to that among cells prepared without mechanical force (without centrifugation).

The enucleation fraction was calculated using the formula (erythrocytes/[erythrocytes + erythroblasts]) × 100 (%), with 300 cells, including erythrocytes and erythroblasts, for each slide. Yield and viability were assessed based on dye exclusion using 0.2% trypan blue. Triplicate cultures were used at each time point.

Flow cytometry

Flow cytometry was performed as previously described [5]. Briefly, cells collected from cultures were washed twice with IMDM containing 0.3% BSA and incubated with a phycoerythrin-conjugated mouse monoclonal antibody to human CD71 (transferrin receptor; BD Biosciences, Franklin Lakes, NJ) and fluorescein isothiocyanate-conjugated mouse monoclonal antibody to human glycophorin A (GPA; Dako, Santa Clara, CA). Cells were then washed twice with 10 mmol/L sodium phosphate buffer, pH 7.4, 0.15 mol/L NaCl (phosphate-buffered saline [PBS]) containing 0.5% BSA and analyzed using a FACSCanto II (BD Biosciences).

Measurement of intracellular ATP levels

Intracellular ATP levels in erythroblasts were measured based on the luciferin−luciferase reaction. Briefly, 1×10^4 cultured cells per sample were resuspended in 100 μL of IMDM and placed in each well of a 96-well plate. Light emission was recorded in triplicate using a luminometer (Tecan Group, Männedorf, Switzerland) after the addition of 100 μL of the “Cellno” ATP assay reagent (TOYO B-Net, Tokyo, Japan).

Glucose uptake

During the terminal differentiation of erythroblasts, 1×10^5 cultured cells were incubated in serum- and glucose-free RPMI-1640 medium for 30 min. After serum and glucose starvation, glucose uptake was initiated by the addition of 2-deoxy-D-[1,2-^{3}H]glucose (PerkinElmer, Waltham, MA) to a final concentration of 2.5 μmol/L (74 kBq). Uptake assays were performed for 30 min. Cells were then washed twice with PBS and resuspended in 6 mL of AQUASOL-II (PerkinElmer Japan, Yokohama, Japan). The uptake of 2-deoxy-D-[1,2-^{3}H]glucose was assessed in triplicate using the liquid scintillation counter LSC-8000 (Hitachi Aloka Medical, Tokyo, Japan).

Real-time PCR analysis

Real-time PCR was performed as previously described [5]. Briefly, total RNA was extracted from 2×10^4 cultured cells per sample using TRIzol reagent (Invitrogen, Carlsbad, CA) or an RNeasy Mini kit (QIAGEN, Hilden, Germany). Extracted RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). The resultant cDNA was then subjected to real-time PCR using LightCycler 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany).

PCR primer sequences for the *GLUT1* [26], *HIF1A* [26], *MCT1* [27], *PDK1* [28], *PDK2* [28], *PDK3* [28], and *PDK4* [28] genes are described in Table 1. Relative gene expression levels were normalized with the *28S rRNA* gene as previously described [29]. Each sample was amplified in triplicate.

Electrophoresis

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE for LDH isozyme separation, and the LDH staining of gels were performed as previously described [30,31].

Immunoblot analysis

An immunoblot analysis was performed as previously described [4,5]. In the analysis of HIF-1α expression, cells were treated with 100 μmol/L $CoCl_2$, which is a chemical stability enhancer of HIF-1α [32], at 37°C for 24 hours before harvesting, as described in a previous study [27]. The primary and secondary antibodies used in the present study are listed in Table 2. ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) was used for color development. The intensities of immunoreactive protein bands were quantified using the ChemiDoc XRS imaging system (Bio-Rad, Tokyo,

Table 1. Primers used in real-time polymerase chain reaction analyses

Gene	Orientation	Sequence
GLUT1	Forward	5′-ATACTCATGACCATCGCGCTAG-3′
GLUT1	Reverse	5′-AAAGAAGGCCACAAAGCCAAAG-3′
HIF1A	Forward	5′-GAAAGCGCAAGTCTTCAAAG-3′
HIF1A	Reverse	5′-TGGGTAGGAGATGGAGATGC-3′
MCT1	Forward	5′-CATGCCACCACCAGCGAAG-3′
MCT1	Reverse	5′-TGACAAGCAGCCACCAACAATC-3′
PDK1	Forward	5′-CCGCTCTCCATGAAGCAGTT-3′
PDK1	Reverse	5′-TTGCCGCAGAAACATAAATGAG-3′
PDK2	Forward	5′-CCGCTGTCCATGAAGCAGTT-3′
PDK2	Reverse	5′-TGCCTGAGGAAGGTGAAGGA-3′
PDK3	Forward	5′-CAAGCAGATCGAGCGCTACTC-3′
PDK3	Reverse	5′-CGAAGTCCAGGAATTGTTTGATG-3′
PDK4	Forward	5′-CCCGAGAGGTGGAGCATTT-3′
PDK4	Reverse	5′-GCATTTTCTGAACCAAAGTCCAGTA-3′
28S rRNA	Forward	5′-TGGGTTTTAAGCAGGAGGTG-3′
28S rRNA	Reverse	5′-CCAGCTCACGTTCCCTATTA-3′

GLUT=Glucose transporter; *HIF*=hypoxia-inducible factor; *LDH*=lactate dehydrogenase; *MCT*=monocarboxylate transporter; *PDH*=pyruvate dehydrogenase; *PDK*=pyruvate dehydrogenase kinase.

Japan). The relative expression levels of the analyzed proteins were normalized with α-tubulin expression.

Statistical analysis

Statistical analyses were performed using Student's *t* test for parametric data and the Mann−Whitney *U* test for nonparametric data. *P* values < 0.05 were considered to be significant in all analyses.

Results

Proliferation of erythroid progenitor cells and erythroblast proliferation or enucleation under hypoxic or normoxic conditions

To evaluate the effects of O_2 concentrations on $CD34^+$ cell erythroblast differentiation, we examined the proliferation of erythroid progenitor cells and erythroblast proliferation or enucleation under 5% or 21% O_2 conditions.

The cell number of erythroid progenitor cells during phase I of the culture was significantly higher under the 21% O_2 condition than under the 5% O_2 condition (Fig. 1A). Aliquots of day 7 CFU-Es (1×10^5 cells) were then cultured in phase II medium. During phase II of the culture, no significant differences were observed in the cell number of day 7 CFU-Es cultured for up to 48 hours between the 5% and 21% O_2 conditions (Fig. 1B), whereas the ratio of enucleation of day 10 erythroblasts cultured for up to 48 hours was significantly higher under the 5% O_2 condition than under the 21% O_2 condition (Fig. 1C).

Table 2. Antibodies used in immunoblot analyses

Antigen[a]	Host animal/Polyclonal or monoclonal[b]	Vendor information
α-tubulin	Mouse/mono	Sigma-Aldrich, T9026
GLUT1	Rabbit/poly	Millipore, 07-1401
HIF-1α	Rabbit/poly	Cell Signaling, 3716
LDHA	Rabbit/poly	Cell Signaling, 3582
LDHB	Rabbit/poly	GeneTex, GTX101747
MCT1	Rabbit/poly	Millipore, AB3538P
PDH	Mouse/mono	Abcam, ab110330
PDH phosphorylated at Ser232	Rabbit/poly	Millipore, AP1063
PDH phosphorylated at Ser293	Rabbit/poly	Millipore, ABS204
PDH phosphorylated at Ser300	Rabbit/poly	Millipore, ABS194
PDK1	Rabbit/poly	Sigma-Aldrich, D9320
PDK2	Rabbit/poly	Bioworld, BS3913
PDK3	Mouse/mono	GeneTex, GTXab55579
PDK4	Rabbit/poly	Sigma-Aldrich, K4980
Mouse IgG	Goat/poly	HRP-linked, KPL, 074-1809
Rabbit IgG	Goat/poly	HRP-linked, Cell Signaling, 7074

GLUT=glucose transporter; *HIF*=hypoxia-inducible factor; *HRP*=horseradish peroxidase; *IgG*, immunoglobulin G; *LDH*=lactate dehydrogenase; *MCT*=monocarboxylate transporter; *PDH*=pyruvate dehydrogenase; *PDK*=pyruvate dehydrogenase kinase.

[a] The amino acid sequences of all antigens were derived from human resources.

[b] Poly or mono indicates a polyclonal or monoclonal antibody, respectively.

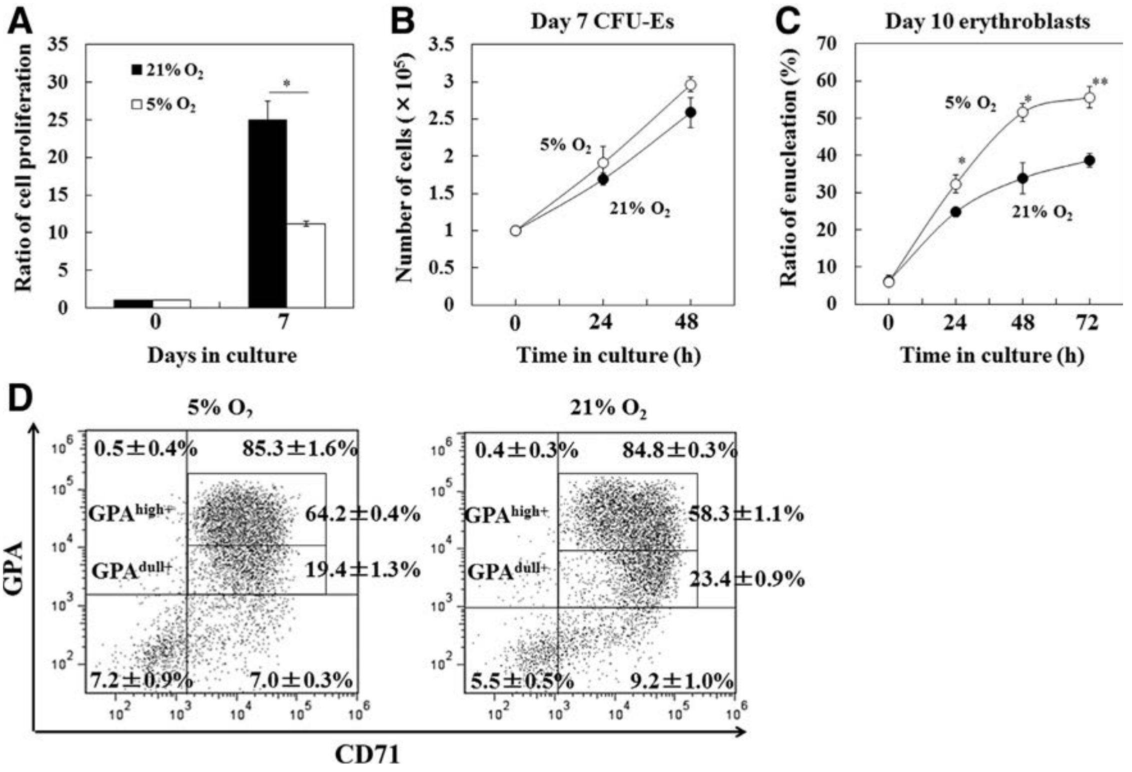

Figure 1. Effects of O_2 concentrations on the proliferation of erythroid progenitor cells and erythroblast proliferation/enucleation during terminal differentiation. (**A**) $CD34^+$ cells were cultured in phase I medium under 5% or 21% O_2 conditions. Cell expansion on day 7 was estimated as a fold increase in cell numbers. Results are expressed as the mean ± SD of three independent experiments. 5% O_2 vs. 21% O_2, $*p < 0.05$. (**B**) Day 7 CFU-Es (1×10^5 cells) were cultured in phase II medium for up to 48 hours under 5% or 21% O_2 conditions. Cell expansion was evaluated. Results are expressed as the means ± SD of three independent experiments. (**C**) Day 10 erythroblasts (1×10^5 cells) were cultured in phase II medium for up to 96 hours under 5% or 21% O_2 conditions. Erythroblast enucleation was evaluated. Results are expressed as the mean ± SD of three independent experiments. 5% O_2 vs. 21% O_2, $*p < 0.05$ and $**p < 0.01$. (**D**) $CD34^+$ cells were cultured in phase I medium under 5% or 21% O_2 conditions. Day 7 CFU-E populations expressing CD71 and GPA were analyzed using flow cytometry. GPA^{high+} ($>10^4$) and GPA^{dull+} (cutoff line − 10^4) subsets are indicated by lines. *CFU-Es*=Colony-forming units−erythroid; *GPA*=glycophorin A.

Ex vivo−generated erythroblasts may be divided using flow cytometry into several maturation classes based on CD71 and GPA expression levels, which are low and high, respectively, in mature erythroblasts [33]. In the flow cytometric analysis, the percentages of $CD71^+$ GPA^+ cells on day 7 were similar (~85%) under the 5% and 21% O_2 conditions, whereas those of $CD71^+$ GPA^{high+} subset cells on day 7 under the 5% and 21% O_2 conditions were 64.2 ± 0.4% and 58.3 ± 1.1%, respectively (Fig. 1D). The $CD71^+$ mean fluorescence intensities of $CD71^+$ GPA^{high+} subset cells and $CD71^+$ GPA^{dull+} subset cells were similar for the two conditions (data not shown).

The acceleration of erythroid differentiation under 5% O_2 conditions during phase II of the culture, as illustrated in Figure 1C, was confirmed by monitoring the evolution of cell morphology in the culture using May−Grünwald−Giemsa staining (Fig. 2). On days 7−9, the majority of cells cultured under both 5% and 21% O_2 conditions were early erythroblasts. Mature erythroblasts and enucleated cells were recognizable from day 10 of the culture, and a larger proportion of enucleated cells were observed in cultures under 5% O_2 conditions than under 21% O_2 conditions.

Changes in intracellular ATP levels in, and glucose uptake by, CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

To evaluate changes in ATP levels in CFU-Es and erythroblasts undergoing terminal differentiation, we measured intracellular ATP levels in these cells under 5% or 21% O_2 conditions.

Intracellular ATP levels in day 7 CFU-Es were ~0.20−0.25 μmol/10^4 cells under 5% and 21% O_2 conditions, but decreased in day 13 cells to ~0.04−0.065 μmol/10^4 cells under 5% and 21% O_2 conditions (Fig. 3A). Intracellular ATP levels were significantly higher in day 7 cells cultured under the 21% O_2 condition than under the 5% O_2 condition and in day 13 cells cultured under the 5% O_2 condition than under the 21% O_2 condition (Fig. 3A).

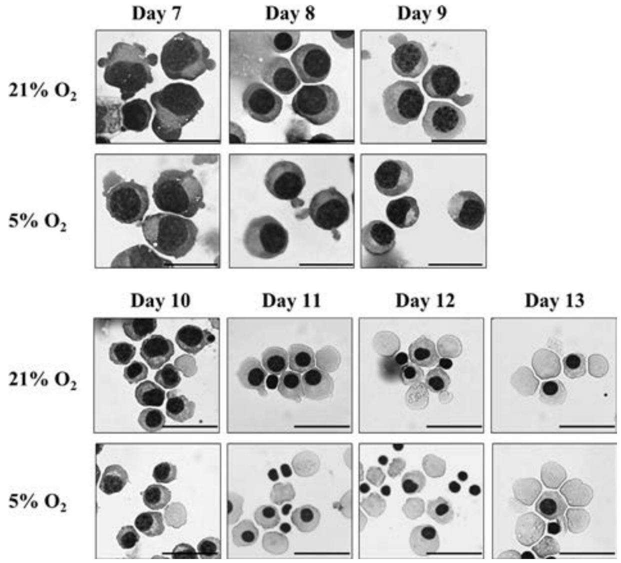

Figure 2. Typical morphologies of CFU-Es and erythroblasts at various stages of terminal differentiation. Cells were cultured up to day 13 under 5% or 21% O_2 conditions (May-Grünwald-Giemsa staining). Bar = 20 μm. *CFU-Es*=Colony-forming units−erythroid.

To evaluate changes in glucose uptake by CFU-Es and erythroblasts undergoing terminal differentiation, we measured the uptake of radiolabeled 2-deoxyglucose by CFU-Es and erythroblasts cultured under 5% or 21% O_2 conditions.

Similar to the results obtained for intracellular ATP levels, glucose uptake also markedly decreased during differentiation, with the most prominent reduction occurring between days 7 and 11 (Fig. 3B). These changes in glucose uptake were independent of O_2 concentrations (Fig. 3B).

Expression of GLUT1 and MCT1 in CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

GLUT1 is a main erythroid glucose transporter [20], and MCT1 is a ubiquitously expressed lactate transporter [16]. To clarify the expression patterns of GLUT1 and MCT1 in CFU-Es and erythroblasts undergoing terminal differentiation, we analyzed the expression of GLUT1 and MCT1 in these cells under 5% or 21% O_2 conditions.

Under 5% O_2 conditions, the real-time PCR analysis revealed that *GLUT1* relative mRNA levels increased in erythroblasts during terminal differentiation (Fig. 3C). In the immunoblot analysis, GLUT1 protein levels increased in erythroblasts during terminal differentiation, with a “smear-like” pattern, as described in a previous study [21] (Fig. 3C). In contrast, *MCT1* relative mRNA levels remained mostly unchanged in erythroblasts during terminal differentiation (Fig. 3D). In the immunoblot analysis, the intensities of MCT1 protein bands remained mostly unchanged, and normalized protein levels of MCT1 increased in erythroblasts during terminal differentiation (Fig. 3D).

Under 21% O_2 conditions, the results obtained were similar to those from experiments performed under 5% O_2 conditions (data not shown).

Effects of GAPDH inhibition on CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

GAPDH is one of the key enzymes in anaerobic glycolysis [15]. To evaluate the importance of GAPDH activity and associated ATP production in the erythroblast enucleation process, we investigated the effects of the inhibition of GAPDH activity by the specific inhibitor KA [23] on CFU-E proliferation, the enucleation ratio, and intracellular ATP levels in erythroblast terminal differentiation under 5% or 21% O_2 conditions.

Under 5% O_2 conditions, the incubation of day 7 CFU-Es with 0.25−4.0 μg/mL KA significantly inhibited their proliferation in a concentration-dependent manner (Fig. 4A). Additionally, intracellular ATP levels were significantly decreased in CFU-Es treated with 4 μg/mL KA (Fig. 4A). Similarly, the incubation of day 10 erythroblasts with 0.25−4.0 μg/mL KA significantly inhibited enucleation (Fig. 4B). Moreover, when day 10 erythroblasts were incubated with 4 μg/mL KA, intracellular ATP levels significantly decreased (Fig. 4B). Figure 4C illustrates the morphology of differentiating cells incubated in the presence or absence of 4.0 μg/mL KA. The morphological changes observed in cells incubated with 4 μg/mL KA were consistent with the results depicted in Figure 4A and B.

Under 21% O_2 conditions, the results obtained were similar to those from experiments performed under 5% O_2 conditions (data not shown).

LDH isozymes in, and effects of LDH inhibition on, CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

LDH, one of the key enzymes in anaerobic glycolysis, is a tetramer of A and B subunits [15]. To clarify the expression patterns of LDH isozymes in CFU-Es and erythroblasts undergoing terminal differentiation, we analyzed the expression of LDH isozymes in these cells under 5% or 21% O_2 conditions.

Under 5% O_2 conditions, native PAGE analysis identified homo- and heterotetramers consisting of LDHA or LDHB monomers encoded by the *LDHA* (skeletal muscle type, M) and *LDHB* (heart type, H) genes. These tetramers included mainly M_3H, M_2H_2, and MH_3 (Fig. 5A). Expression of the LDHA and LDHB isozymes was confirmed by the immunoblot analysis (Fig. 5B).

To evaluate the importance of LDH activity and associated ATP production in the erythroblast

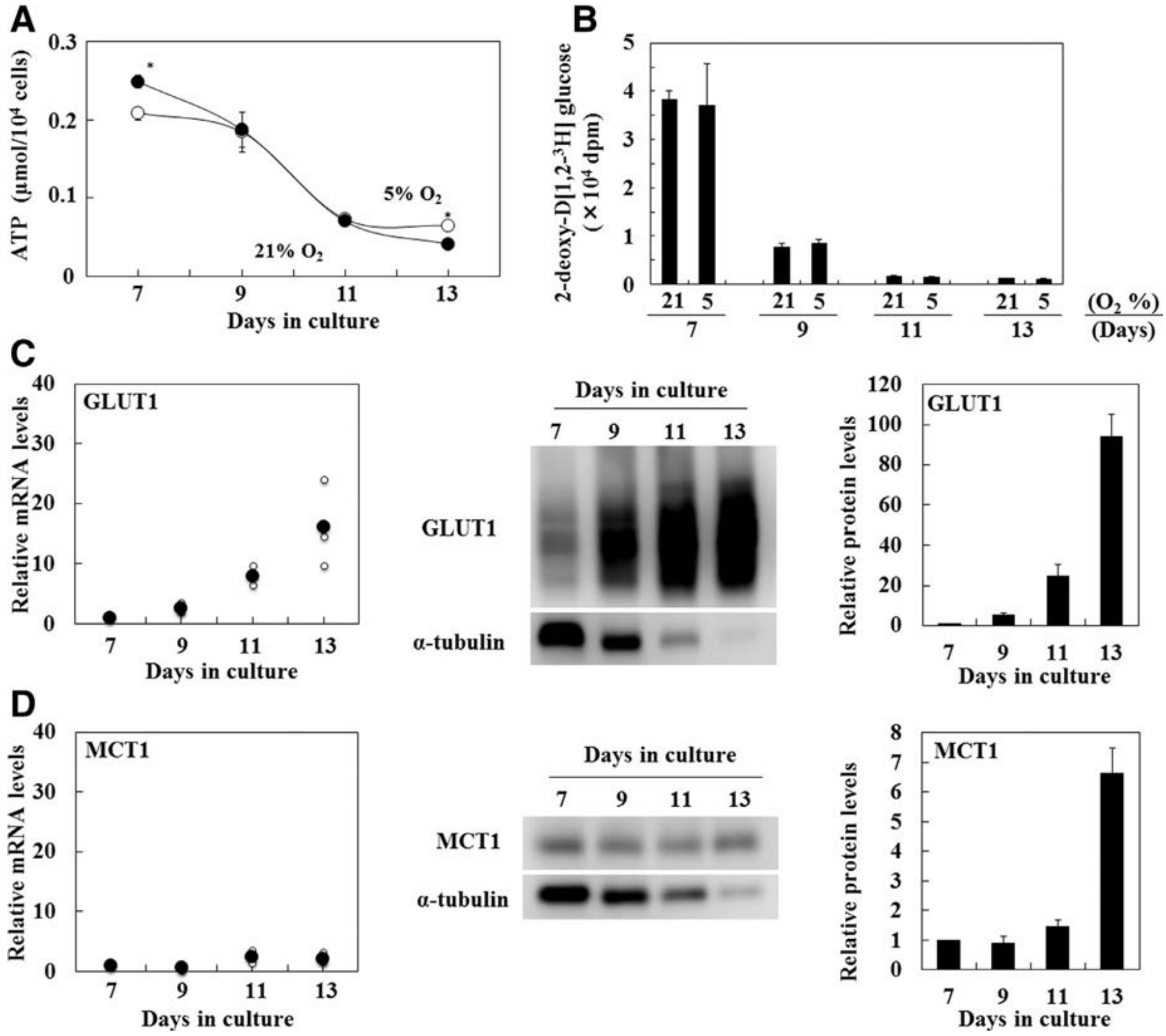

Figure 3. Effects of O_2 concentrations on intracellular ATP levels and 2-deoxy-D-[1,2-^{3}H]glucose uptake in CFU-Es and erythroblasts and changes in GLUT1 and MCT1 expression in CFU-Es and erythroblasts under hypoxic conditions. (**A**) Day 7 CFU-Es (1×10^5 cells) were cultured for up to 48 hours under 5% or 21% O_2 conditions. Intracellular ATP levels were evaluated. Results are expressed as the mean ± SD of three independent experiments. 5% O_2 vs. 21% O_2, $*p < 0.05$. (**B**) Day 7 CFU-Es (1×10^5 cells) were cultured up to day 13 under 5% or 21% O_2 conditions, referred to as "5" and "21," respectively. Cellular glucose uptake was evaluated. Results are expressed as the mean ± SD of three independent experiments. (**C, D**) CFU-Es and erythroblasts were cultured under 5% O_2 conditions. Cultured cells were harvested at the indicated time points and were extracted for real-time PCR analysis of *GLUT1* and *MCT1* (left panels) or immunoblot analysis of GLUT1 and MCT1 (middle panels). In real-time PCR analysis, *28S rRNA* was used as a control. All values were normalized to the value on day 7. Raw data (◯) and mean values (•) are shown ($n = 3$). In the immunoblot analysis, the relative expression levels of GLUT1 and MCT1 were normalized with α-tubulin expression. A representative result of three independent experiments is shown. *CFU-Es*=Colony-forming units−erythroid; *GLUT1*=glucose transporter 1; *MCT1*=monocarboxylate transporter 1; *PCR*=polymerase chain reaction.

enucleation process, we investigated the effects of the inhibition of LDH activity by the specific inhibitor STP [24] on CFU-E proliferation, the enucleation ratio, and intracellular ATP levels in erythroblast terminal differentiation under 5% or 21% O_2 conditions.

Under 5% O_2 conditions, the incubation of CFU-Es with 0.5−1.0 mM STP significantly inhibited their proliferation in a concentration-dependent manner (Fig. 5C). Additionally, intracellular ATP levels were significantly decreased in CFU-Es treated with 1 mM STP (Fig. 5C). Similarly, the incubation of day 10 erythroblasts with 0.5−1.0 mM STP significantly inhibited enucleation, as reflected by the greater decrease in the enucleation ratio with increases in STP concentrations (Fig. 5D). Moreover, when day 10 erythroblasts were incubated with 1 mM STP, intracellular ATP levels significantly decreased (Fig. 5D). Figure 5E illustrates the morphology of differentiating cells incubated in the presence or absence of 1 mM STP. Morphological changes in cells incubated with 1 mM STP were consistent with the results shown in Figure 5C and D.

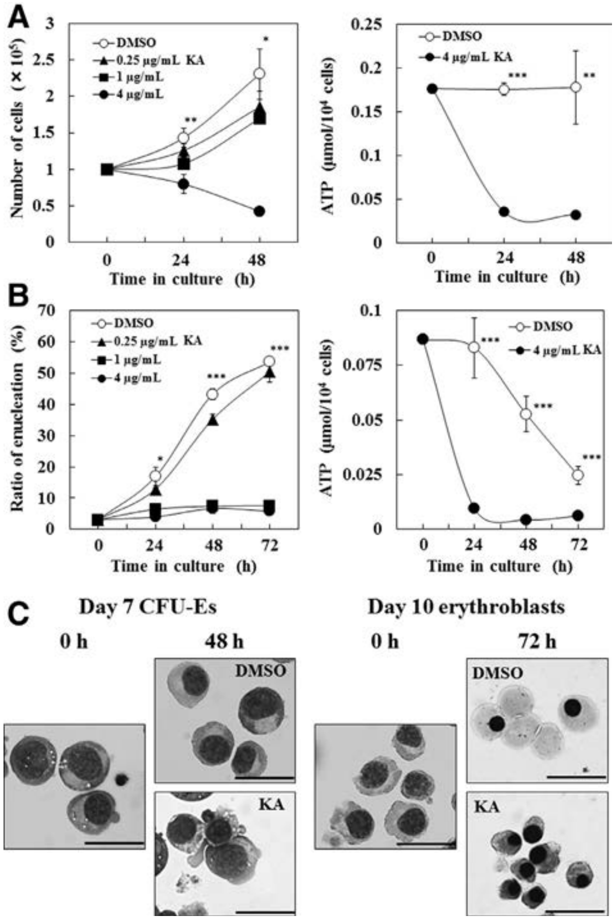

Figure 4. Effects of the inhibition of GAPDH enzymatic activity by KA on CFU-E proliferation and the ratio of enucleation under hypoxic conditions. (**A**) Effects of the inhibition of GAPDH by KA on CFU-E proliferation. Day 7 CFU-Es (1×10^5) were cultured in the presence of different concentrations of KA (0.25, 1, or 4 μg/mL) or in its absence (DMSO) under 5% O_2 conditions, and were harvested 24 and 48 hours after the addition of KA. The numbers of cells are shown in the left panel. Intracellular ATP levels are shown in the right panel. Data are the mean ± SD of three independent experiments. DMSO vs. 4 μg/mL KA, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (**B**) Effects of the inhibition of GAPDH by KA on the ratio of enucleation in erythroblasts. Day 10 erythroblasts (1×10^5) were cultured in the presence of different concentrations of KA (0.25, 1, or 4 μg/mL) or in its absence (DMSO) under 5% O_2 conditions, and were harvested 24, 48, and 72 hours after the addition of KA. Ratios of enucleation are shown in the left panel. Intracellular ATP levels are shown in the right panel. Data are shown as the mean ± SD of three independent experiments. DMSO vs. 4 μg/mL KA, $*p < 0.05$, $***p < 0.001$. (**C**) Day 7 CFU-Es were cultured for up to 48 hours in the absence of KA (DMSO) or the presence of 4 μg/mL KA under 5% O_2 conditions. Day 10 erythroblasts were cultured for up to 72 hours in the absence of KA (DMSO) or the presence of 4 μg/mL KA under 5% O_2 conditions. Cells were stained with May−Grünwald−Giemsa reagent. Bar = 20 µm. *CFU-Es*=Colony-forming units−erythroid; *DMSO*=dimethyl sulfoxide; *KA*=koningic acid;

Under 21% O_2 conditions, the results obtained were similar to those from experiments performed under 5% O_2 conditions, except that LDH tetramers included mainly M_2H_2, MH_3, and H_4 (Supplementary Figure E1, online only, available at www.exphem.org).

Phosphorylated PDH and expression of PDK isozymes in CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

Oxidative phosphorylation is suppressed via the phosphorylation of PDH by PDKs [17]. To clarify PDH phosphorylation accomplished by PDKs in CFU-Es and erythroblasts undergoing terminal differentiation, we analyzed PDH phosphorylation and the expression of PDK1, PDK2, PDK3, and PDK4 in these cells under 5% or 21% O_2 conditions.

Under 5% O_2 conditions, the immunoblot analysis revealed that PDH phosphorylated at residues Ser232, Ser293, and Ser300 was stably expressed in erythroblasts during terminal differentiation (Fig. 6A). The real-time PCR analysis revealed that *PDK1* and *PDK4* relative mRNA levels increased in erythroblasts during terminal differentiation, whereas those of *PDK2* and *PDK3* did not (Fig. 6B). The immunoblot analysis indicated that PDK4 was expressed at high levels in erythroblasts at all stages of differentiation, whereas PDK1 expression levels were lower and decreased over time (Fig. 6C). Consistent with the results of the real-time PCR analysis, the immunoblot analysis revealed that the expression of PDK2 and PDK3 was not detectable in erythroblasts at any stage (Fig. 6C).

Under 21% O_2 conditions, the results obtained were similar to those from experiments performed under 5% O_2 conditions (data not shown).

Expression of HIF-1α in CFU-Es and erythroblasts during terminal differentiation under hypoxic or normoxic conditions

HIF-1α is a key regulator of hypoxic metabolism in mouse and human HSCs [13,14]. The HIF-1α protein is stabilized mainly during hypoxia, whereas it undergoes ubiquitin-dependent degradation under normoxia in most cells [13,14]. To clarify the expression pattern of HIF-1α in CFU-Es and erythroblasts undergoing terminal differentiation, we analyzed the expression of HIF-1α in these cells under 5% or 21% O_2 conditions.

The real-time PCR analysis revealed that *HIF-1α* relative mRNA levels were almost unchanged in erythroblasts during terminal differentiation under both 5% and 21% O_2 conditions (data not shown). The immunoblot analysis revealed that protein expression was stronger in CFU-Es and erythroblasts cultured under the 5% O_2 condition than under the 21% O_2 condition (Fig. 7).

Discussion

In the present study, we found that different O_2 environments affected human $CD34^+$ cell erythroblast

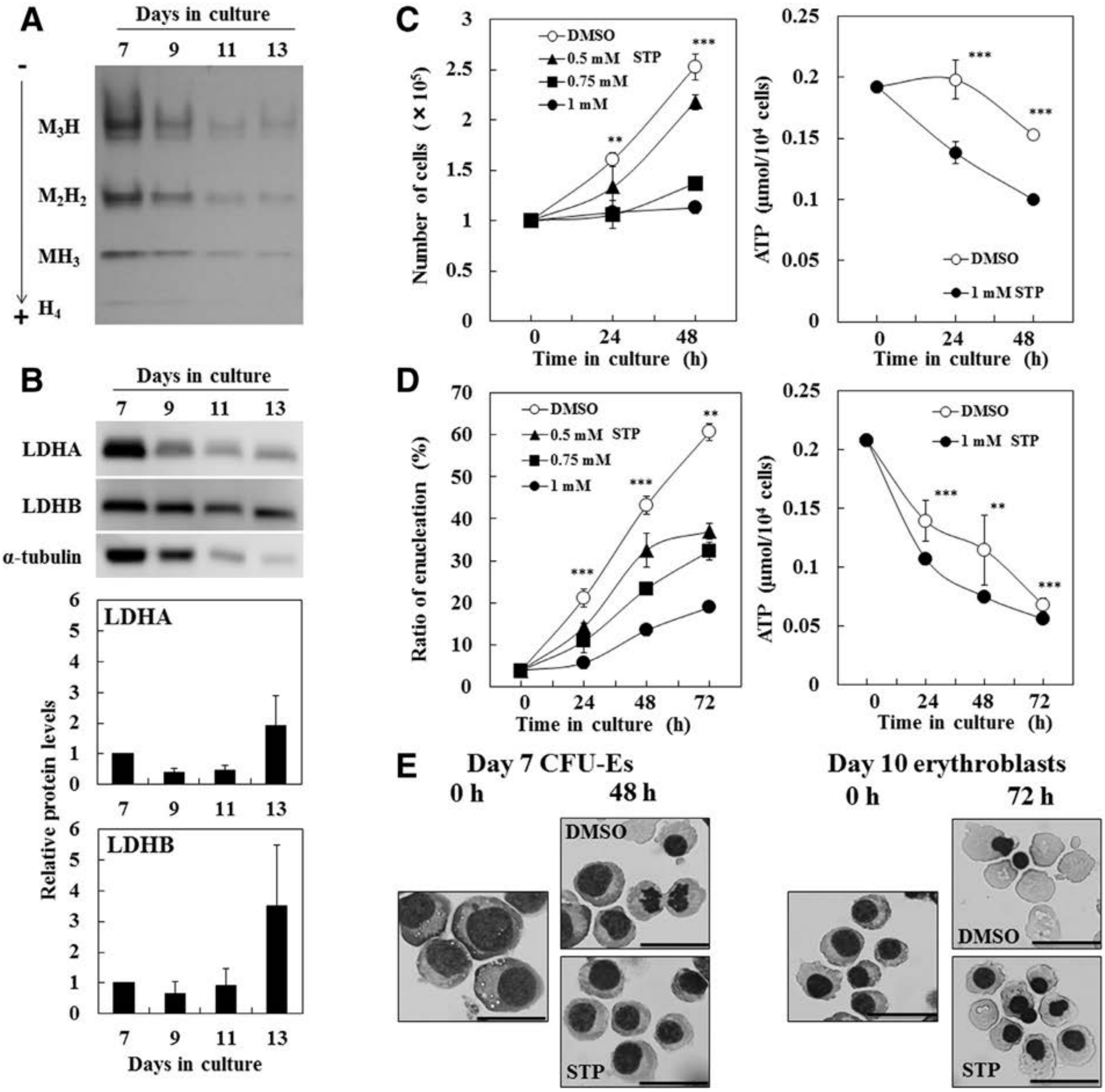

Figure 5. LDH isozymes in CFU-Es and erythroblasts during terminal differentiation and effects of the inhibition of LDH enzymatic activity by STP on CFU-E proliferation and the ratio of enucleation under hypoxic conditions. (**A**) Native PAGE analysis of the expression of LDH isozymes in CFU-Es and erythroblasts during terminal differentiation. Day 7 CFU-Es were cultured up to day 13 under 5% O_2 conditions. A representative result of three independent experiments is shown. (**B**) Immunoblot analysis of the expression of LDHA and LDHB. The relative expression levels of LDHA and LDHB were normalized with α-tubulin expression. A representative result of three independent experiments is shown. (**C**) Effects of the inhibition of LDH by STP on CFU-E proliferation. Day 7 CFU-Es (1×10^5) were cultured in the presence of different concentrations of STP (0.5, 0.75, or 1 mM) or in its absence (DMSO) under 5% O_2 conditions, and were harvested 24 and 48 hours after the addition of STP. The numbers of cells are shown in the left panel. Intracellular ATP levels are shown in the right panel. Data are the mean ± SD of three independent experiments. DMSO vs. 1 mM STP, $^{**}p < 0.01$, $^{***}p < 0.001$. (**D**) Effects of the inhibition of LDH by STP on the ratio of enucleation. Day 10 erythroblasts (1×10^5) were cultured in the presence of different concentrations of STP (0.5, 0.75, or 1 mM) or in its absence (DMSO) under 5% O_2 conditions, and were harvested 24, 48, and 72 hours after the addition of STP. Ratios of enucleation are shown in the left panel. Intracellular ATP levels are shown in the right panel. Data are the mean ± SD of three independent experiments. DMSO vs. 1 mM STP, $^{**}p < 0.01$, $^{***}p < 0.001$. (**E**) Day 7 CFU-Es were cultured for up to 48 hours in the absence of STP (DMSO) or the presence of 1 mM STP under 5% O_2 conditions. Day 10 erythroblasts were cultured for up to 72 hours in the absence of STP (DMSO) or the presence of 1 mM STP under 5% O_2 conditions. Cells were stained with May−Grünwald−Giemsa reagent. Bar = 20 µm. *CFU-Es*=Colony-forming units−erythroid; *DMSO*=dimethyl sulfoxide; *LDH*=lactate dehydrogenase; *PAGE*=polyacrylamide gel electrophoresis; *STP*=stiripentol.

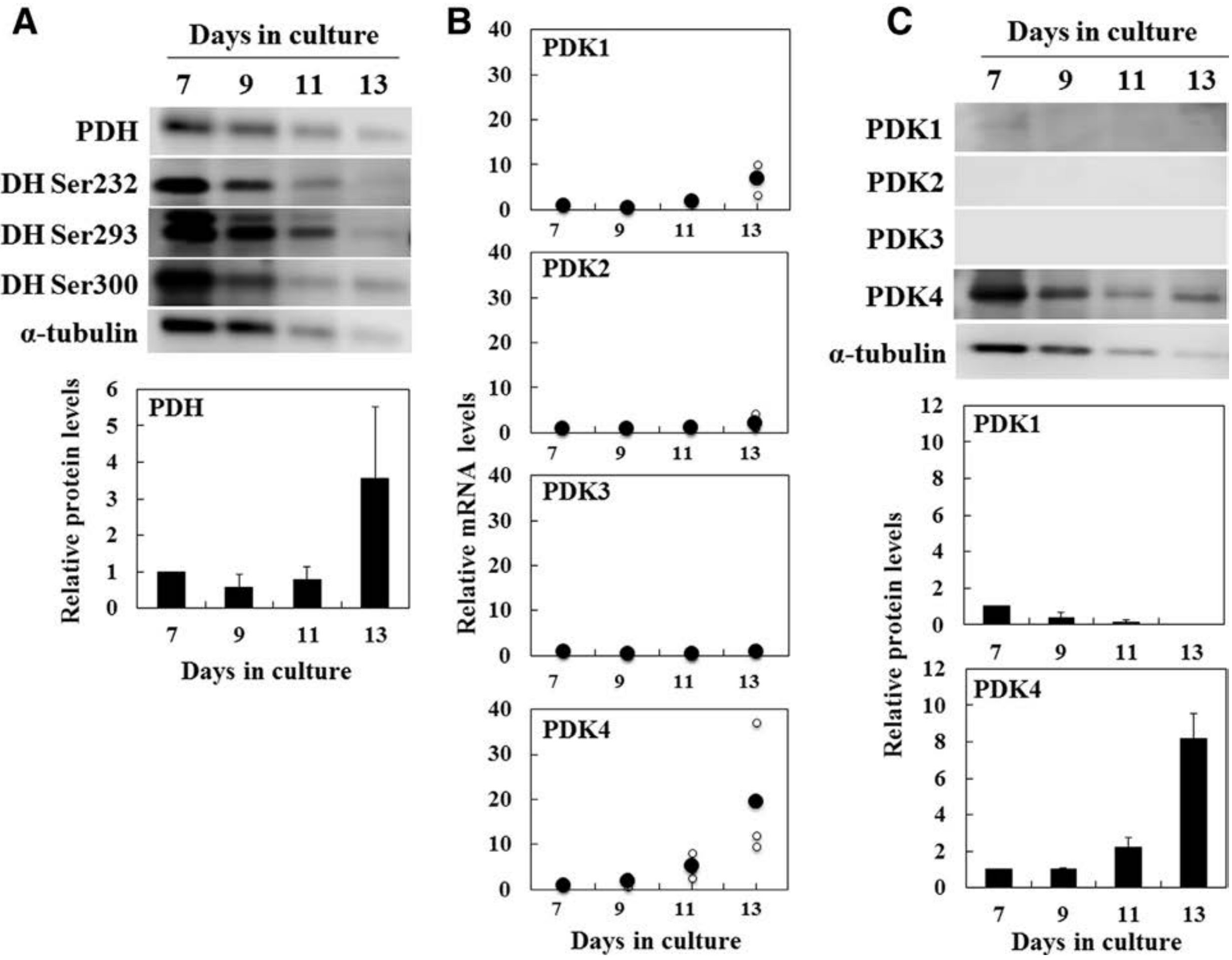

Figure 6. Phosphorylation of Ser232, Ser293, and Ser300 residues in PDH and changes in mRNA and protein expression levels of PDK isozymes in CFU-Es and erythroblasts during terminal differentiation under hypoxic conditions. CFU-Es and erythroblasts cultured under 5% O_2 conditions were harvested at the indicated time points and were extracted for an immunoblot or real-time PCR analysis. (**A**) Immunoblot analysis using an antibody specific for PDH or antibodies specific for PDH phosphorylated at Ser232, Ser293, and Ser300 (PDH Ser232, PDH Ser293, and PDH Ser300). The relative expression levels of PDH were normalized with α-tubulin expression. A representative result of three independent experiments is shown. (**B**) Real-time PCR analysis of *PDK* isozymes; *28S rRNA* was used as a control. All values were normalized to the value on day 7. Raw data (○) and mean values (•) are shown ($n = 3$). (**C**) Immunoblot analysis of PDK isozymes. The relative expression levels of PDK1 and PDK4 were normalized with α-tubulin expression. A representative result of three independent experiments is shown. *CFU-Es*=Colony-forming units−erythroid; *PCR*=polymerase chain reaction; *PDH*=pyruvate dehydrogenase; *PDK*=pyruvate dehydrogenase kinase.

differentiation. The enucleation ratio and intracellular levels of ATP and HIF-1α in erythroblasts during terminal differentiation were higher under hypoxic conditions than under normoxic conditions. In addition, the LDH isozyme pattern differed between the two conditions. We also investigated the potential regulatory mechanisms underlying energy metabolism during the terminal differentiation of human erythroblasts under hypoxic and normoxic conditions. The enzymatic inhibition of GAPDH and LDH, enzymes involved in anaerobic glycolysis, blocked the proliferation of CFU-Es and enucleation of erythroblasts, and reduced ATP levels in erythroblasts, under both hypoxic and normoxic conditions. A key result was that the phosphorylation of PDH at residues Ser232, Ser293, and Ser300, possibly by PDK4, constitutes an important event during the terminal differentiation of human erythroblasts. Two major consequences of PDH phosphorylation are a reduction in its catalytic activity and a decrease in the production of acetyl-CoA [17]. Furthermore, we observed increased *GLUT1* mRNA expression levels and GLUT1 protein levels as well as the sustained expression of the MCT1 protein in erythroblasts during terminal differentiation. Collectively, the present results strongly support human erythroblasts using primarily ATP produced by anaerobic glycolysis during terminal differentiation (Fig. 8).

A previous study by Montel-Hagen et al. [21] indicated that glucose uptake markedly decreased despite the increase in GLUT1 expression in human erythroblasts

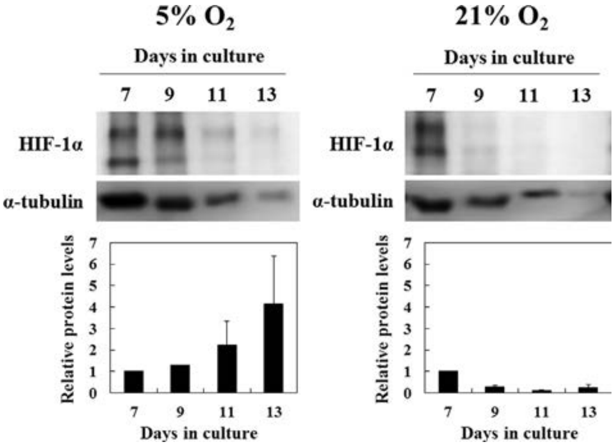

Figure 7. Changes in protein expression levels of HIF-1*α* during the terminal differentiation of human erythroblasts. Day 7 CFU-Es were cultured up to day 13 under 5% or 21% O_2 conditions and were harvested at the indicated time points. In the immunoblot analysis, the lower bands observed in the panels of HIF-1*α* are considered to be degradation products (antibody vendor information). The relative expression levels of HIF-1*α* (upper and lower bands) were normalized with *α*-tubulin expression. A representative result of three independent experiments is shown. *CFU-Es*=colony-forming units −erythroid; *HIF-1α*=hypoxia-inducible factor-1*α*.

during terminal differentiation. They identified stomatin, an integral erythrocyte membrane protein, as a regulator of the switch from glucose to dehydroascorbic acid. After membrane translocation, stomatin binds to GLUT1 and switches GLUT1 glucose transport activity to dehydroascorbic acid transport activity [21,34]. The decrease observed in glucose uptake in the present study and reported previously [21] further supports a decrease in oxidative phosphorylation in erythroblasts during terminal differentiation. The simultaneous increase in dehydroascorbic acid transport suggests that terminally differentiated erythrocytes opt for energy metabolism that reduces intracellular oxidation processes and, thus, increases the lifespan of mature erythrocytes.

In the glycolytic pathway, GAPDH catalyzes an essential redox reaction: The conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate coupled the reduction of NAD^+ to NADH [15]. LDH catalyzes NADH oxidation in parallel with the reduction of pyruvate to lactate, thereby providing a supply of NAD^+ for glycolytic-dependent ATP production [17]. As illustrated in the present study, the enzymatic inhibition of GAPDH and LDH very effectively inhibited CFU-E proliferation and erythroblast enucleation, which further supports the importance of glycolytic-dependent ATP production in differentiating erythroblasts.

We found that PDH phosphorylated at residues Ser232, Ser293, and Ser300 was stably expressed during the terminal differentiation of human erythroblasts. The stable expression of phosphorylated PDH suggests that the inhibition of PDH enzymatic activity constitutes an important event leading to the blockage of oxidative phosphorylation in mitochondria during the terminal differentiation of erythroblasts. Consistent with the stable phosphorylation of PDH, intracellular ATP levels decreased during the terminal differentiation of human erythroblasts.

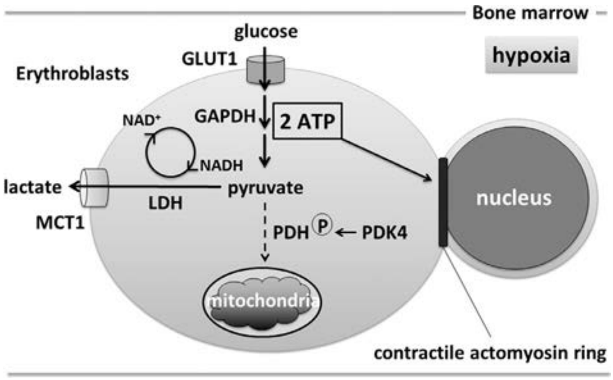

Figure 8. Proposed model for the regulation of energy metabolism in human erythroblasts. In the hypoxic bone marrow environment, erythroblasts take up glucose through GLUT1, and produce ATP by LDH-mediated glycolysis of pyruvate into lactate, which is, in turn, extruded through MCT1. This glycolytic reaction is favored by PDH phosphorylation at Ser232, Ser293, and Ser300 via PDK4. *GLUT1*=Glucose transporter 1; *MCT1*=monocarboxylate transporter 1; *PDH*=pyruvate dehydrogenase; *PDK4*=pyruvate dehydrogenase kinase 4.

We also found that PDK4 was strongly expressed among PDK isozymes during the terminal differentiation of human erythroblasts. The sustained expression of PDK4 in differentiating erythroblasts may result in the stable expression of phosphorylated PDH, which, in turn, programs glucose metabolism from oxidative glycolysis to aerobic glycolysis [35]. Energy metabolism in HSCs has been investigated. In murine HSCs, the regulation of glycolysis by PDK2 and PDK4 functions as a metabolic checkpoint for cell cycle quiescence [36,37]. On the basis of our results showing the strong expression of PDK4 in differentiating human erythroblasts, PDK4 appears to be responsible for the phosphorylation of PDH during hematopoietic differentiation across species. We suggest that oxidative phosphorylation as an energy source is suppressed via PDH phosphorylation by PDK4 during the terminal differentiation of erythroblasts in a hypoxic bone marrow environment. It is also tempting to propose that this adaptation may provide a protective mechanism to minimize oxidative processes after enucleation.

We confirmed that HIF-1*α* expression levels were higher in CFU-Es and erythroblasts cultured under the 5% O_2 condition than under the 21% condition. HIF-1*α* shifts metabolism from oxidative phosphorylation to anaerobic glycolysis. The two major targets of HIF-1*α* that decrease pyruvate conversion to acetyl-CoA are LDHA and PDKs (PDK1 and PDK4) [38]. In the present study, the LDH isozymes in CFU-Es and erythroblasts cultured under 5% O_2 conditions were the heterotetramers M_3H, M_2H_2, and MH_3, whereas the LDH isozymes in these cells cultured under 21% O_2 conditions were the heterotetramers M_2H_2, MH_3, and H_4. This may be explained by differences in the control of the expression of the *LDHA* (M type) and *LDHB* (H type) genes. The *LDHA* gene is controlled by HIF-1α under hypoxic conditions, whereas the *LDHB* gene is not regulated by low O_2 concentrations [15]. The HIF-1α−mediated expression of PDKs is also considered to be a metabolic switch required for cellular adaptation to hypoxia [39]. In the present study, PDK4 was strongly expressed and PDH was phosphorylated at residues Ser232, Ser293, and Ser300 in erythroblasts during terminal differentiation.

Vlaski et al. [22] reported that low O_2 concentrations promoted the proliferation of erythroid progenitors and differentiation of human cord blood or peripheral blood CD34$^+$ cells into erythroid progenitors. They showed that low O_2 concentrations enhanced or accelerated the production of BFU-Es, the production of CFU-Es, and CFU-E proliferation and differentiation. In their study, in which culture conditions differed from those in the present study (e.g., 1.5−5% O_2 during days 0−8 and 13−20% O_2 during days 9−27), a larger proportion of enucleated cells were recognizable from day 20 of the culture at 13% O_2 than at 20% O_2. In our culture system using G-CSF−mobilized human peripheral blood CD34$^+$ cells, the ratio of erythroblast enucleation was higher under 5% O_2 conditions than under 21% conditions from day 11 of the culture. As a relevant result, the flow cytometric analysis revealed an increase in the percentage of CD71$^+$ GPA^{high+} subset cells on day 7 under the 5% O_2 conditions. Furthermore, we characterized the regulatory mechanisms underlying energy production in erythroblasts during terminal differentiation under 5% or 21% O_2 conditions, as previously discussed. Regarding the mechanisms underlying regulation of erythropoiesis by low O_2 concentrations, Zhang et al. [40] reported that HIF-1α−mediated GATA1 upregulation promoted erythropoiesis. Vlaski et al. [22] also found that hypoxia (1.5% O_2) enhanced GATA-1 expression in cord blood cell cultures. Their findings and the present results suggest that HIF-1α is needed to accelerate erythroid differentiation.

To the best of our knowledge, the regulation and function of MCTs during human erythropoiesis have not yet been examined. Aoi et al. [41] investigated the effects of sustained swimming exercise on increases in MCT1 concentrations and its ability to regulate pH homeostasis in rat erythrocytes. Their findings in rats suggested that erythrocyte MCT1 was increased during erythropoiesis in bone marrow, which facilitated lactate transport through the sustained swimming exercise. In the present study, we observed sustained expression of MCT1 in erythroblasts during terminal differentiation. Therefore, MCT1 appears to be a key transporter of lactate in human erythrocytes.

In conclusion, human erythroblasts may produce ATP as the main source of energy for enucleation via LDH-driven glycolysis during terminal differentiation. This may be related to the hypoxic bone marrow environment.

Acknowledgments

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Conflict of interest disclosure

The authors declare no competing financial interests.

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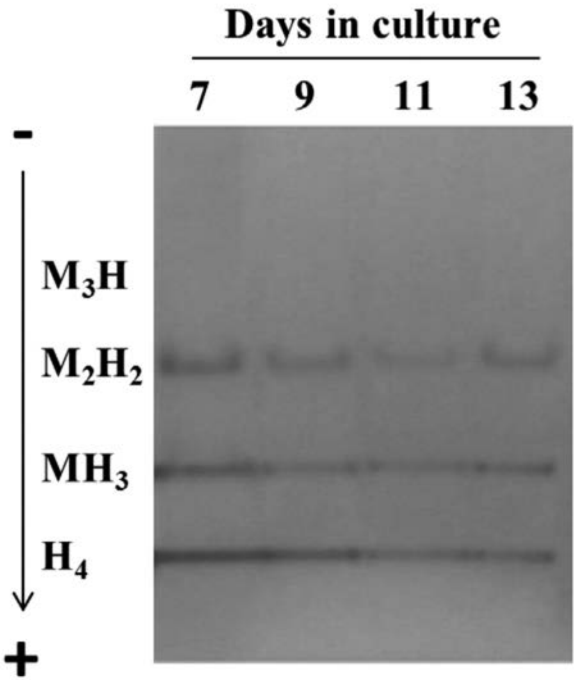
Supplemental Material

Supplementary Figure E1



Supplementary Figure E1. LDH isozymes in CFU-Es and erythroblasts during terminal differentiation under normoxic conditions. Native PAGE analysis of the expression of LDH isozymes in CFU-Es and erythroblasts during terminal differentiation. Day 7 CFU-Es were cultured up to day 13 under 21% O_2 conditions. A representative result of three independent experiments is shown.