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Autonomous control of terminal erythropoiesis *via* physical interactions among erythroid cells

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Abstract *In vitro* erythropoiesis has been studied extensively for its application in the manufacture of transfusable erythrocytes. Unfortunately, culture conditions have not been as effective as *in vivo* growth conditions, where bone marrow macrophages are known to be a key regulator of erythropoiesis. This study focused on the fact that some erythroblasts are detached from macrophages and only contact other erythroblasts. We hypothesized that additional factors regulate erythroblasts, likely through either physical contact or secreted factors. To further elucidate these critical factors, human erythroblasts derived from cord blood were cultured at high density to mimic marrow conditions. This growth condition resulted in a significantly increased erythroid enucleation rate and viability. We found several novel contact-related signals in erythroblasts: intercellular adhesion molecule-4 (ICAM-4) and deleted in liver cancer-1 (DLC-1). DLC-1, a Rho-GTPase-activating protein, has not previously been reported in erythroid cells, but its interaction with ICAM-4 was demonstrated here. We further confirmed the presence of a secreted form of human ICAM-4 for the first time. When soluble ICAM-4 was added to media, cell viability and enucleation increased with decreased nuclear dysplasia, suggesting that ICAM-4 is a key factor in contact between cells. These results highlight potential new mechanisms for autonomous control of erythropoiesis. The application of these procedures to erythrocyte manufacturing could enhance *in vitro* erythrocyte production for clinical use.
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Introduction

Definitive erythropoiesis occurs in the bone marrow, where a set of complex interactions encourages red blood cell (RBC) production. The central macrophage in the erythropoietic niches called erythroblastic islands plays a critical role in controlling the maturation, differentiation, and enucleation of erythroid cells (Bessis, 1958; Chasis and Mohandas, 2008). It is known that the main route of signal transport between macrophages and erythroid cells is physical contact *via* several ligands and receptors (Rhodes et al., 2008; Soni et al., 2006; Spring and Parsons, 2000; Telen, 2000). Various adhesion molecules responsible for maintaining this cell-to-cell contact within the erythroid islands have been identified, mainly through the relationship between macrophages and erythroid cells. However, not every erythroid cell is attached to a macrophage (Rhodes et al., 2008), and some erythroid cells

Abbreviations: Hb, hemoglobin; ICAM-4, intercellular adhesion molecule-4; DLC-1, deleted in liver cancer-1.

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contact only other erythroid cells. Erythroid cells seem to be able to autonomously regulate erythropoiesis, albeit with reduced efficiency (Chasis and Mohandas, 2008). Based on these observations, we speculated that there must be factors regulating erythroid cells that are not based on direct contact with macrophages.

One potential explanation is autonomous regulation among erythroid cells through direct contact, as they reside in clusters within the bone marrow (Neu et al., 2003). However, no secreted factors regulating erythropoiesis or signal transduction between mature erythroid cells have been identified. Fas-FasL is known to be involved in the apoptotic control of pro-erythroblasts *via* mature polychromatic erythroblasts (Allen and Dexter, 1982; Manwani and Bieker, 2008). However, the role of this ligand and receptor has not been studied with regard to the final maturation or enucleation of mature erythroid cells. Erythroblast macrophage protein (Emp) is known to be involved in macrophage–erythroblast attachment and possibly in homotypic erythroblast attachment, but there have been no data establishing this link (Soni et al., 2006). Additionally, there have been no reports that erythropoiesis is controlled *via* an auto-paracrine system (Manwani and Bieker, 2008).

Elucidation of the diverse systems regulating erythroid cells is important not only for understanding basic and pathologic erythropoiesis, but also for developing strategies for generating human RBCs *in vitro* for transfusion. The severe blood supply shortage and the ongoing need for safe blood have underscored efforts to develop RBCs *in vitro* (Chasis, 2006; Sato et al., 1979). However, the process of obtaining final RBC products is still inefficient, mainly due to a low enucleation rate and low viability in the final maturation step, particularly in stromal-cell-free conditions. The enucleation process, where immature erythroid cells extrude their nuclei, is a critical step towards those cells becoming reticulocytes and finally mature RBCs (Sato et al., 1979). Our knowledge regarding which mechanisms regulate these final processes and why enucleation and viability *in vitro* are so markedly low is lacking.

In order to elucidate the missing mechanisms of the regulatory system of erythroid cells and to identify methods to increase RBC production, we focused on the bone marrow microenvironment, in which mature erythroid cells reside in compact clusters. In conventional culture densities (less than 50% confluency) (Miharada et al., 2006), the erythroid cells repel each other in suspension due to strong negative charges on their surface membranes, and do not easily attach as other adhesive cells do. To mimic bone marrow conditions, homogenous erythroid cells were cultured at a high density to increase the chance of contact; the results revealed a significantly increased enucleation rate and viability.

This prompted us to hypothesize that there are adhesion factors that control terminal maturation and enucleation among erythroid cells in the absence of macrophages. To identify candidate genes affecting erythroid cell attachment and enucleation, we searched for genes showing marked changes before and after enucleation *via* microarray. By comparing the candidate gene profiles of erythroid cells cultured at different densities, several density-evoked signals were demonstrated for the first time. Two of these genes are deleted in liver cancer-1 (DLC-1), which has never been studied in primary cells or erythroid cells, and intercellular adhesion molecule-4 (ICAM-4), which is an erythroid cell adhesion protein. Furthermore, we found that ICAM-4 and the Rho-GTPase-activating protein DLC-1 are binding counterparts in erythroid cells. Addition of recombinant ICAM-4 protein to culture media of low-density cells reproduced the results observed at higher densities, such as enhanced viability and enucleation.

In this study, we demonstrate that ICAM-4 is related to the erythropoietic niche between erythroid cells *via* both direct contact and through its secreted form. This study provides new mechanisms by which autonomous control of erythropoiesis *in vivo* occurs and could improve *in vitro* RBC production for clinical use.

Materials and methods

Isolation of CD34$^+$ cells and culture for erythroid differentiation

Cord blood (CB) was collected from healthy pregnant women after obtaining their written informed consent. CD34$^+$ cells were isolated from the CB using the EasySep CD34 isolation kit (StemCell Technologies, Vancouver, Canada). The culture methods are described in a previous report (Baek et al., 2009, 2010). Several cytokines were added to stroma- and serum-free media in a stage-specific manner to induce CD34$^+$ cells to differentiate to a purely erythroid lineage in the absence of feeder cells and serum/plasma. Live cells were counted by trypan blue staining.

Cell lines and culture conditions

The human erythroleukemic cell line K562 was purchased from a Korean cell line bank. This line was grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS; Gibco) in a 5% CO_2 incubator at 37 °C.

Cell-density-dependent growth analysis

The cells were found to expand too rapidly to maintain a constant density. Therefore, cells were maintained at a low cell density from the beginning of culture until day 17. On day 17 of culture, polychromatic and orthochromatic erythroblasts were seeded to reach 50% and 100% confluences in order to mimic the packed cellular environment of bone marrow (Fig. 1A). Fifty percent and 100% confluences were designated as optimal (2.5×10^6 cells/1 ml/2 cm^2) or supraoptimal (5.0×10^6 cells/2 ml/2 cm^2), respectively (Figs. 1B–D). The cells were kept at the same cell density in the media to maintain the same level of nutrients and waste. Cultures were observed under phase contrast microscopy to confirm their confluence, which was determined by cells flocking to the center of the plate. Unless otherwise stated, media were replenished every other day and cell morphology was verified using Wright–Giemsa staining (Sigma Aldrich, St Louis, MO).

Live cell imaging for cell interactions

Cultured primary erythroid cells were stained with a DiO cell-labeling solution and observed using live-cell imaging

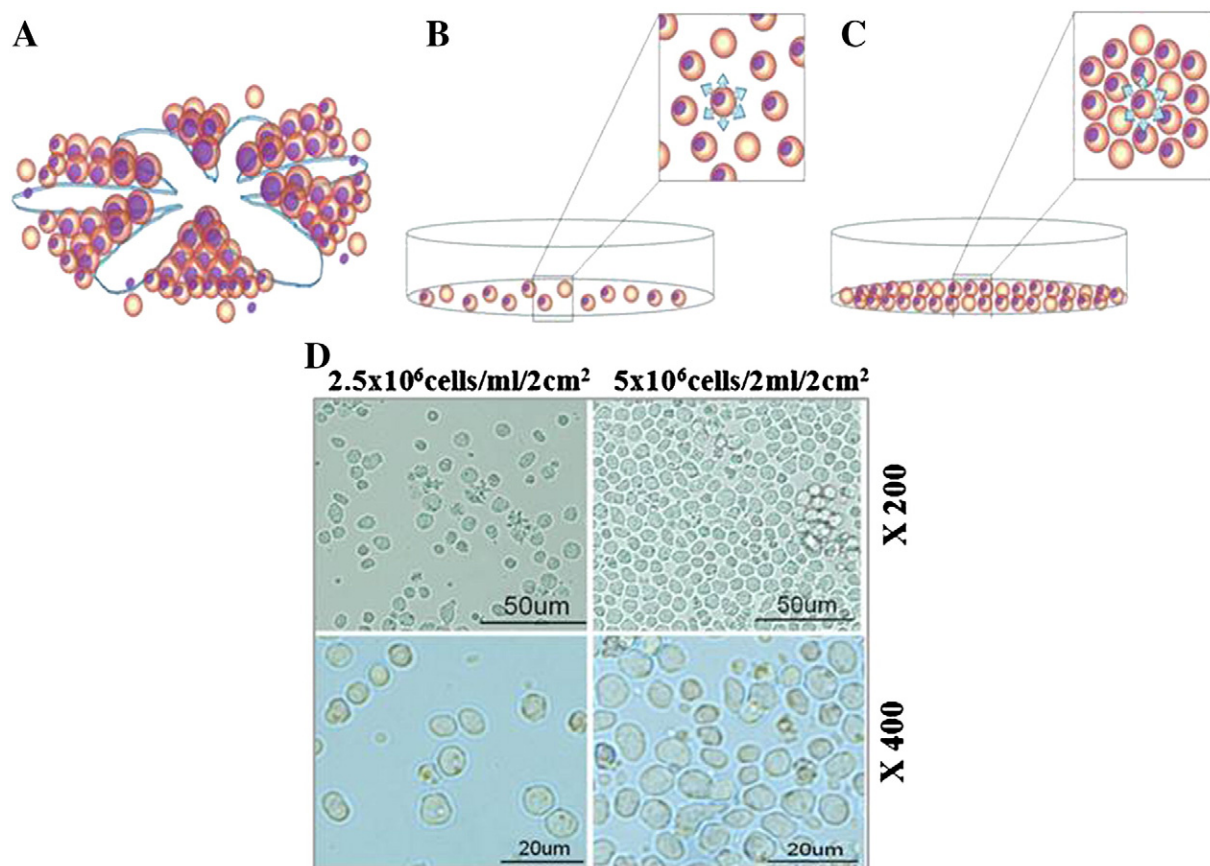

Figure 1 Erythroid cell cultures at different cell densities. Schematic representation of erythroid cell culture (A–C). Human erythroid cells derived from cord blood hematopoietic stem cells were cultured. To mimic erythroblastic niches in the bone marrow, in which erythroid cells are packed without direct contact with macrophages (A), late mature erythroblasts composed of polychromatic and orthochromatic erythroblasts were seeded at (B) conventional optimal confluence (50%; 2.5×10^6 cells/1 ml/2 cm^2) and (C) compact supraoptimal confluences (100%; 5.0×10^6 cells/2 ml/2 cm^2) on day 17 of culture. (D) The cells were observed by phase-contrast microscopy.

microscopy (Deltavision, Applied Precision, Issaquah, WA) according to the manufacture's protocol.

Cell enucleation profiling and viability assay

Cell morphology and enucleation rate experiments were described in a previous study (Baek et al., 2008, 2009, 2010). Cell viability was determined using trypan blue staining. For flow cytometry analysis, cells were stained with AnnexinV-PE and propidium iodide (BD PharMingen, Franklin Lakes, NJ) for 15 min at room temperature.

Analysis of cell surface marker expression by flow cytometry

Cells were labeled with anti-human antibodies glycophorin A (GPA)-FITC (BD PharMingen) and CD71-FITC (eBioscience, San Diego, CA) on days 13 and 17. Immunoglobulin G1 (IgG1)-FITC and IgG1-PE (Beckman Coulter, Miami, FL) were used as isotype controls. FITC-conjugated anti-Hbf (fetal Hb) and PerCP-conjugated anti-Hbβ (adult Hb) (BD PharMingen) were used to measure the intracellular expression of hemoglobin (Hb). Stained cells were analyzed using flow cytometry.

mRNA expression microarray

To select adhesion-related candidate genes expressed just before the enucleation phase, we compared the mRNA expression profiles of proerythroblasts and orthochromatic erythroblasts at several stages of maturation from three individual cord blood samples. RNA expression at each maturation stage was analyzed in triplicate using a NimbleGen human whole genome mRNA expression microarray (Roche, Indianapolis, IN). Adhesion-related signals were sought from among those genes that were upregulated during erythropoiesis, especially at the orthochromatic erythroblasts phase.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen). mRNA levels were then measured in triplicate by qRT-PCR using SYBR

Premix ExTaq (Takara-Bio, Shiga, Japan). The primer sequences were as follows (forward/reverse):

VLA-4, 5′-AGGATGGTGTAAGCGATGGC-3′/5′-TGCTGAAGAATTGGCTGAAGTGGTGG-3′;
DLC-1, 5′-AGTGCGTGCAACAAGCGGGT-3′/5′-TCCGGGTAGCTCTCGCGGTT-3′;
ICAM-4, 5′-CCGGACTAAGCGGGCGCAAA-3′/5′-AGCCACGAACTCCGGGCTCA-3′.

Other primer sequences were as described previously (Baek et al., 2010).

Detection of a secreted form of ICAM-4

Cells were cultured at a density of 1×10^5 cells/well in 24-well plates for 48 h. Supernatants were then collected for measurement of ICAM-4 by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated at 4 °C with the indicated amounts of anti-ICAM-4 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and 10% FBS in PBS. After incubation, the plates were washed three times with 0.05% Tween-20 in PBS. The plates were blocked with 1% BSA in PBS for 2 h at room temperature. The supernatants were then added to the plates and incubated for 2 h. The plates were washed prior to the addition of an HRP-conjugated anti-goat secondary antibody (1:100; Jackson Immunoresearch Laboratories, Avondale, PA). After 1 h of incubation at 37 °C, sICAM-4 was detected with TMB (tetramethyl benzidine), and substrate reagent (Biolegend, San Diego, CA) was added for 30 min. Absorbance was measured at 490 nm using a microplate reader. The absorbance values of the isotype-matched control antibodies were measured as a blank and subtracted from the experimental values.

Immunoblotting and immunoprecipitation

To assess the binding of ICAM-4 and DLC-1, cells were harvested in PBS (phosphate buffered saline) and then lysed as described previously (Baek et al., 2010). For immunoprecipitation, equal amounts of protein were incubated with anti-ICAM-4 antibody for 2 h at 4 °C. Protein A/G agarose beads (Santa Cruz) were added to each tube and incubated overnight at 4 °C. The protein and protein A/G bead complexes were collected and washed three times with lysis buffer. Immunoblotting was performed by modification of the technique described previously (Baek et al., 2010). Immunoblotting analyses were performed with anti-DLC-1 (Santa Cruz), anti-ICAM-4 (Abcam, Cambridge, UK), anti-β-actin (Cell Signaling, Beverly, MA), and anti-GAPDH (Abfrontier, Seoul, Korea) antibodies.

Analysis of soluble ICAM-4 functions in terminal erythropoiesis

To show that secreted ICAM-4 is a factor in modulating the maturation, enucleation, and cell survival regulation of erythroid cells by adjacent erythroid cells, purified recombinant ICAM-4 protein (OriGene Technologies, Baltimore, MD) was added to the culture media at concentrations of 0, 2, 5, and 10 μg/ml. The purified protein was added once the cells had matured to the orthochromatic erythroblast stage at day 17 of culture. After further culturing for 24 h, cell viability, maturation status, enucleation rate, and the level of dysplasia were evaluated on Wright–Giemsa-stained slides by counting more than 400 cells per condition.

Biochemical analysis

At day 19, culture media were collected, and glucose concentrations were determined using an automatic chemical analyzer (Hitachi, Tokyo, Japan). The pH and potassium ion (K+) concentrations were analyzed using RapidSystems blood gas analyzers (Siemens, Medfield, MA). Lactate levels were measured using an enzymatic method with a Cobas Integra 800 (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

Paired t-tests were used to compare the control and experimental groups. *p*-values of less than 0.05 were considered significant.

Results

Cell-to-cell interactions between erythroid cells allow enhanced viability and enucleation

The purity of erythroid cells derived from CD34+ cells was determined using erythroid cell-specific markers. CD71, GPA, HbF, and Hbβ appeared by day 13, and the cultured cells consisted almost solely of poly- and orthochromatic cells with a purity of up to 99% at day 17 (Fig. 2A). Contaminating macrophages were not visible in pictures taken by phase contrast microscopy (Fig. 2B) and Wright–Giemsa-stained slides (Fig. 2C). At higher density, erythroid cell interactions were observed in 17% of the total cells, as measured by recorded movie images and shown in Fig. 1D (Fig. 2B). Enucleation rate and cell integrity, which was defined as whether a cell showed any vacuoles or a destroyed membrane contour, were observed by cell staining (Fig. 2C). When erythroid cells exited the polychromatic stage, cell proliferation declined and the rate of cell death markedly increased. In contrast, cells cultured at 100% (supraoptimal density) and 150% confluences showed better morphology, as indicated by intact cytoplasmic membranes and fewer vacuoles, and showed substantially increased viability and cell number. However, cells at 150% confluence did not show enhanced contact over that seen at 100% confluence, likely because the excess cells could not adhere to the bottom monolayer and were instead floating in the media. Therefore, we compared conventional optimal density (50% confluence) and supraoptimal density (100% confluence). Compared to the optimal condition, the supraoptimal condition demonstrated significantly increased viability (42.9%; Fig. 2D) and enucleation (38.6%; Fig. 2E). As a result, the supraoptimal 100% confluent cultures showed significantly increased RBC production, as calculated by the fold-expansion multiplied by the enucleation rate at the corresponding cell densities (Fig. 2F).

A growing body of evidence indicates that immature and terminal-phase erythroid precursors are sensitive to apoptotic triggering, which is frequently exacerbated in culture (Tsiftsoglou et al., 2009). Therefore, in order to elucidate the effects of supraoptimal culture conditions on apoptotic cell death, we subjected terminal-maturation-stage erythroid cells to flow cytometry analysis using anti-annexin V and anti-caspase-3 antibodies, which are markers of apoptosis. Caspase 3 and annexin V were significantly reduced under the supraoptimal condition (Fig. 2G) compared to the optimal condition. This concomitant increase in cell viability, enucleation, and RBC production and decline in apoptotic cell death suggest that our supraoptimal culture conditions result in efficient erythroid maturation and, in turn, produce more RBCs.

To confirm that the connections shown in Fig. 2B were not due to intercellular bridges at the telophase stage of cytokinesis, we continuously observed two daughter cells that had just completed cell division with real-time fluorescence imaging instead of confocal microscopy. We found that completely separated cells restored contact *via* pseudopod-like connections (Fig. 2H). The connections were maintained for up to 30 min. This is the first report in the literature to show autonomous binding of erythroid cells (Figs. 2B and H).

The culture media were also analyzed for soluble contents by biochemical analysis, and it was found that the culture media did not differ significantly between the low- and high-density cultures over the entire culture period (Fig. 2I).

Physical interaction affects terminal erythroid maturation at the molecular level

Based on the positive effects of supraoptimal density on RBC yield *in vitro*, we explored the possible roles of supraoptimal cultural conditions on erythroid maturation surface markers and signals. The media and culture plates were changed every two days and just before the density experiments to eliminate adherent macrophages. When poly- or orthochromatic erythroid cells at different densities were exposed to erythroid cell markers, the expression levels of membrane-bound CD71 and GPA did not differ between conditions (mean expression value: CD71 > 53.7%, GPA > 87.3%). This was likely because CD71 is expressed until the reticulocytes stage and maturation to mature red blood cells could not be completed within the short culture duration (Fig. 3A). However, mRNA levels of GATA-1 and Hbβ, the expression of which increased along with erythropoiesis, were significantly higher at higher densities (Fig. 3B). Altogether, these results indicate that culture density affects maturation signaling.

Expression of ICAM-4 and VLA-4 induced by homogenous erythroid cell contacts

Of the total 45,034 genes measured by the microarray, 2133 genes were upregulated during erythroid cell maturation. Among the cell adhesion genes, 96 genes were expressed in the orthochromatic erythroblasts at levels more than 1.5-fold those found in proerythroblasts. In particular, DLC-1 gene expression was highest, with a 4.0-fold relative change in expression. We selected the erythroid-specific adhesion marker ICAM-4 and VLA-4 for further study. We first determined the mRNA expression level of ICAM-4 and VLA-4 by RT-PCR (Fig. 4A). Matured erythroid cells grown at different densities were compared using qRT-PCR, and the mRNA levels of ICAM-4 and VLA-4 were found to be markedly increased at higher densities (Fig. 4B). The induction of ICAM-4 mRNA expression was paralleled by an increase in ICAM-4 protein in the erythroid cells that maturated at a higher density (Fig. 4C).

DLC-1 is expressed in human erythroid cells and is associated with physical interactions among erythroid cells

From the mRNA microarray data, it was determined that the DLC-1 mRNA expression levels of basophilic erythroblasts, orthochromatic erythroblasts, and reticulocytes increased 1.60-, 4.0-, and 3.64-fold compared to proerythroblasts. On the basis of the analysis of microarray data, we confirmed the expression of DLC-1 in erythroid cells at culture days 8 and 17 by qRT-PCR (data not shown). In addition, the dose-dependent expression of DLC-1 in human erythroid cells was verified in the human erythroleukemic cell line K562 (Fig. 5A).

To confirm the change in DLC-1 at different cell densities, we performed immunoblotting and qRT-PCR in mature erythroid cells. Compared to the optimal group, the supraoptimal group showed increased levels of DLC-1 mRNA and protein (Figs. 5B and C). These results suggest that DLC-1 is associated with physical interactions among erythroid cells.

DLC-1 is a novel binding counterpart of ICAM-4

Since both DLC-1 and ICAM-4 were expressed at higher levels with increasing physical contact between the human erythroid cells, we investigated whether DLC-1 might interact with ICAM-4 in the K562 cell line. As shown Fig. 6A, the immunoprecipitation of endogenous ICAM-4 with a specific antibody precipitated endogenous DLC-1 as revealed by Western blotting. This result convincingly demonstrates that the association between DLC-1 and ICAM-4 is not only specific, but also physiologically relevant. We also determined the localization of DLC-1 and ICAM-4 by fluorescence (Fig. 6B).

These results suggest that DLC-1 and ICAM-4, the functions of which in erythroblast–erythroblast adhesion were not evaluated, affected terminal erythropoiesis in terms of enhancing viability and enucleation.

ICAM-4 enhances terminal erythropoiesis by decreasing abnormal cytokinesis *in vitro*

In order to evaluate whether ICAM-4 has a soluble form (sICAM-4) in humans, the cell supernatants were collected and analyzed for the presence of sICAM-4 by ELISA. Approximately 40% and 10% greater signals were detected in K562 and mature erythroid cells, respectively, compared to the media alone (Fig. 7A).

To confirm that ICAM-4 could reproduce the results of increased viability and enucleation with decreased dysplasia seen under the supraoptimal density conditions, soluble ICAM-4 protein was added to the terminally matured cells at

various concentrations on day 17 (Fig. 7B). To exclude cell-to-cell contact, the erythroid cells were maintained at a low-density optimal condition. More than 400 cells for each condition were counted after cell staining (Fig. 7B). The results indicate that soluble ICAM-4 increases the number of viable cells in a dose-dependent manner (Fig. 7C). On the other hand, nuclear dysplasia, such as bi-/multi-nucleation and nuclear fragmentation, was markedly diminished in a dose-dependent manner with increased concentrations of ICAM-4 (Fig. 7D). To compare maturation enhancement, the number of cells with different maturation statuses were counted. In addition, the enucleation rate increased more in ICAM-4-treated cells than in untreated cells (11% and 22%, respectively). More mature cells were counted at the higher concentration of ICAM-4. This, taken together with the high enucleation rate, suggests that ICAM-4 has an important role in influencing enucleation and RBC production in terminal erythropoiesis (Figs. 7E and F).

Discussion

Cell-to-cell communication through the attachment of erythroid cells to macrophages is critical for erythroid cell differentiation and maturation (Chasis and Mohandas, 2008). However, during the maturation period, erythroid cells at the outer edge of an erythroblastic island progressively lose their adhesion ligands to the adjacent macrophage (Mohandas and Chasis, 2010). Moreover, some erythroid cells exist outside of these islands and form clusters by themselves (Chasis, 2006). There have been some studies on the interactions between immature erythroid cells (*via* Emp) (Soni et al., 2006) and

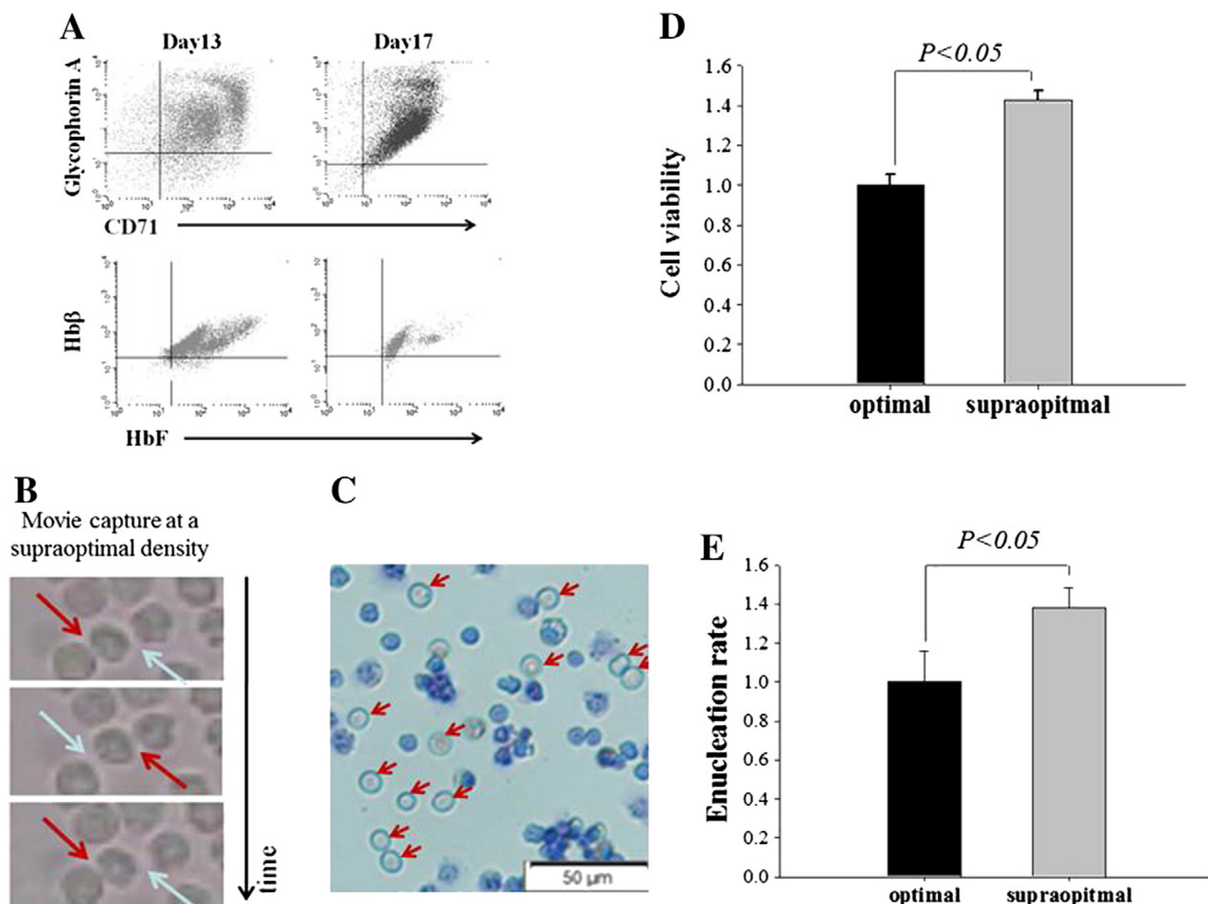

Figure 2 Effects of cell culture density on the terminal maturation of erythroid cells. (A) At day 17 of culture, erythroid cell-specific markers were measured by flow cytometry. (B) The erythroid cells were then distributed to new culture plates at different densities. Erythroid cells could contact each other at a supraoptimal density despite repulsion induced by their negative charges. (C) Erythroid cells stained with Wright–Giemsa. Arrows indicate reticulocytes. Magnification: 200×. (D) The viability of erythroid cells stained with trypan blue was significantly increased at the supraoptimal density. (E) The enucleation rate of maturated erythroid cells was measured in comparison to the optimal condition (n = 6) along with (F) the number of erythrocytes (n = 8). (G) Apoptotic cells at each culture density were quantified using flow cytometry analysis with annexin V staining. The ratio of cells undergoing apoptosis is presented (n = 3). (H) Erythroid cells were cultured for 13 days and were stained with fluorescent DiO for 5 min at room temperature. The upper arrow indicates cell division and the lower arrows indicate cells interacting with each other. (I) Culture media were collected at day 19, and lactate, glucose, the potassium ion (K^+) concentrations, and pH were determined. Results are expressed as mean ± standard error of the mean (SEM). $p < 0.05$, significantly different from the optimal density group.

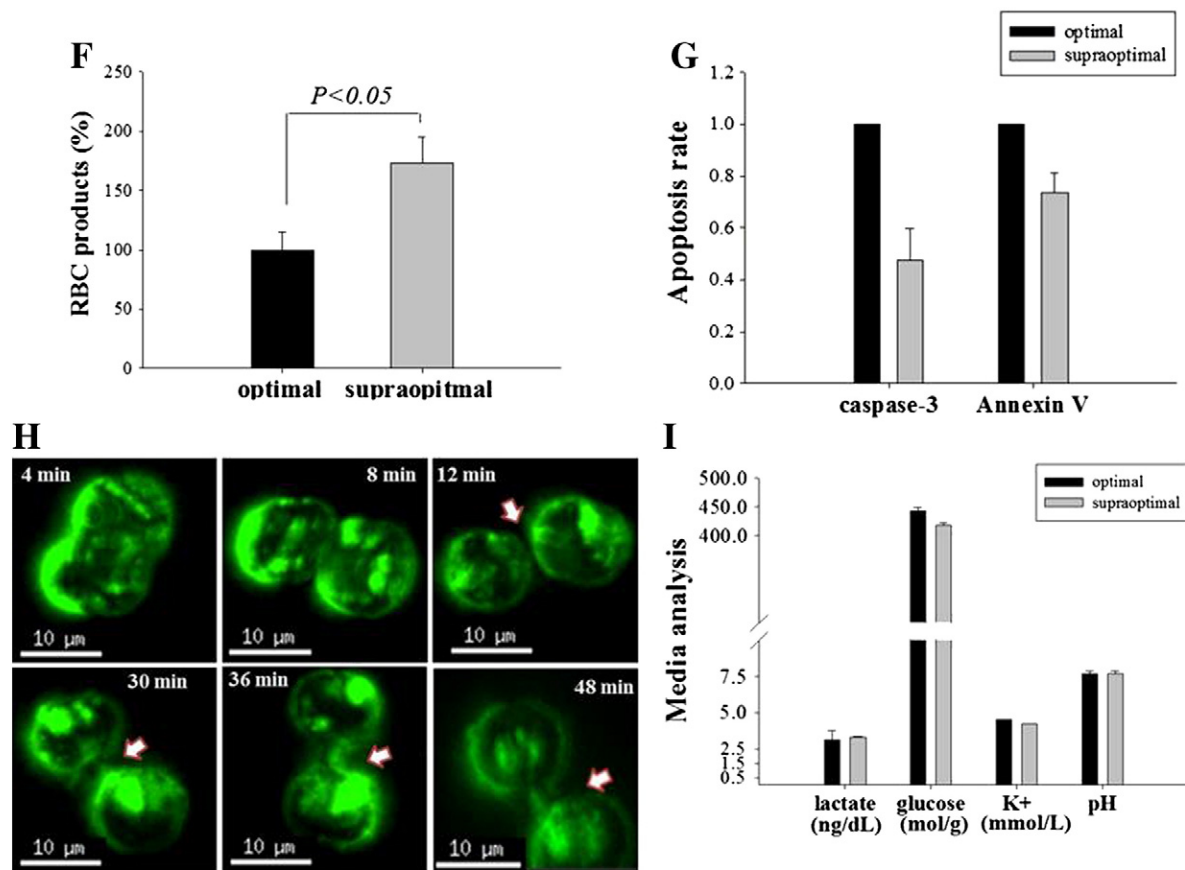

Figure 2 (*continued*).

between immature and mature erythroid cells (*via* ICAM-4-VLA-4 and Fas-FasL proteins) (Chasis, 2006; Manwani and Bieker, 2008; Soni et al., 2006; Hermand et al., 2000; Spring et al., 2001). However, the ligands related to communication within erythroid cells in the late stages of maturation (polychromatic and orthochromatic erythroblasts) have not been studied, nor has any mechanism for autonomous regulation of enucleation and viability been shown. Published reports on this topic are rare, probably due to difficulties in culturing mature human erythroblasts without feeder stromal cells.

Beyond a desire to understand the physiologic and pathologic mechanisms of erythropoiesis regulation, there is a need for methods for producing artificial RBCs from stem

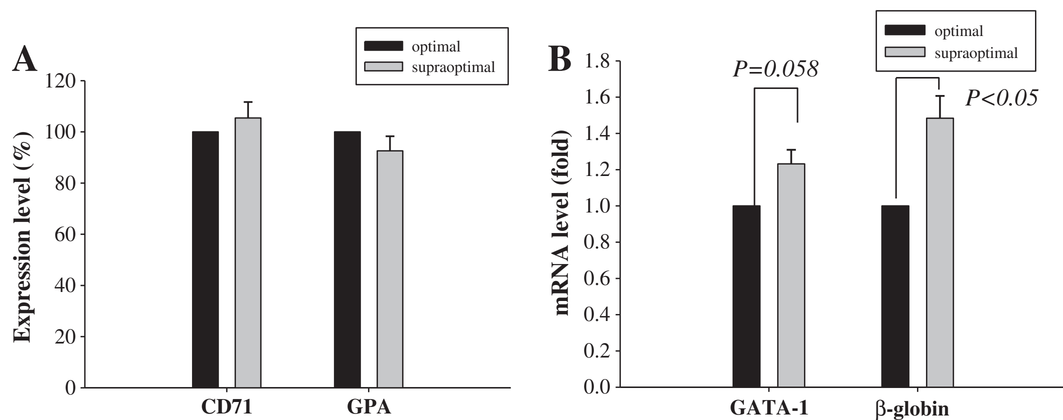

Figure 3 Erythroid cell differentiation at different culture densities. On day 19, erythroid cells were collected from cultures at two densities. (A) Erythroid cell-specific cell surface markers were analyzed for CD71 and Glycophorin A (GPA) expression by flow cytometry, and were found to not differ at the two culture densities. However, GATA-1 and β-globin mRNA levels were increased at the terminal maturation of erythroblasts. The relative levels of GATA-1 and β-globin mRNA were measured by qRT-PCR and normalized by the levels of β-actin transcripts. Results are expressed as the mean ± SEM of the three independent experiments.

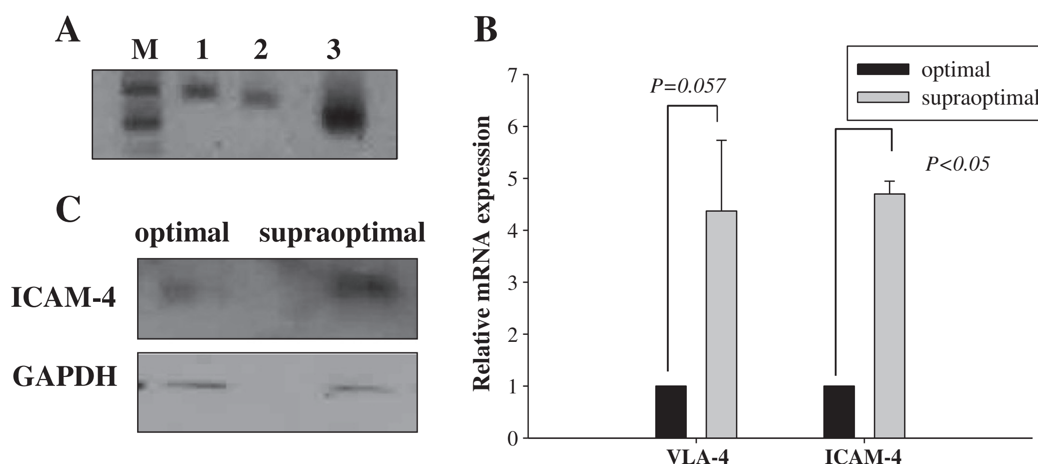

Figure 4 Expression of ICAM-4 in human erythroid cells. Total RNA was isolated from cultured mature erythroid cells over 17 days. (A) ICAM-4 and VLA-4 mRNA levels were measured by RT-PCR (M: marker, 1: VLA-4, 2: ICAM-4, 3: β-actin). (B) RNA was prepared from mature erythroid cells grown at different cell densities. ICAM-4 and VLA-4 mRNA levels were quantified by qRT-PCR. The relative levels of ICAM-4 and VLA-4 mRNA were normalized by the levels of β-actin transcripts. (n = 3, each experiment was done in triplicate) (C) The mature erythroid cell lysates were prepared for immunoblotting using the antibody as indicated. 50 μg of total lysate was loaded on a 12% gel. The same membrane was stripped and re-probed with anti-GAPDH antibody. Expression of GAPDH was used to normalize the intensity of the ICAM-4 band on the immunoblot. Data represent the four independent experiments.

cells. The removal of feeder stromal cells and serum/plasma is critical for any method to be feasible for clinical applications and mass production. However, RBC productivity under these conditions is very low due to the low viability and inefficient enucleation, which is a special form of asymmetric cell division during the cell cycle (Ji et al., 2010). It appears that critical stimuli, such as cell-to-cell contact, are missing in these conventional cultures.

Therefore, we hypothesized that attachment-related signals would be important for these erythroid cells. We previously demonstrated that even electric charges can adversely affect final erythropoiesis maturation steps (Baek et al., 2010). In this report, we reveal that a high-density culture significantly enhances enucleation and cell survival. Even though the erythroid cells were floating in media, we showed that physical interactions occurred *via* thread-like

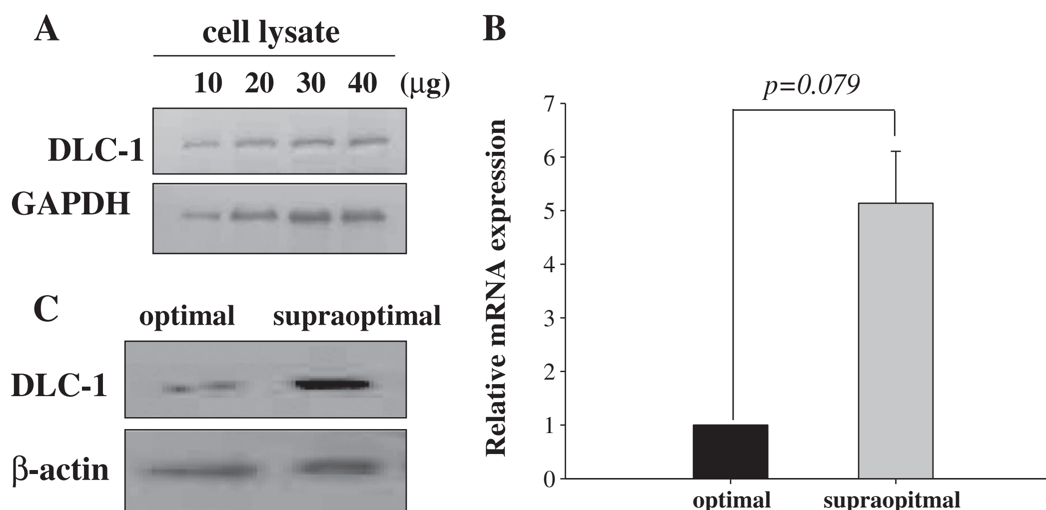

Figure 5 Expression of DLC-1 in human erythroid cells. (A) To confirm the expression of DLC-1 in a human erythroid lineage, K562 cell lysates were prepared for immunoblotting using different amounts of protein. (B) From the erythroid cells cultured at different cell densities, the different expression patterns of DLC-1 were verified using qRT-PCR. Relative expression levels were normalized to the levels of β-actin mRNA (n = 3). (C) Primary erythroid cell lysates from the different culture densities were prepared for Western blotting and probed with anti-DLC-1. The same membrane was stripped and reprobed with anti-β-actin antibody. Expression of β-actin was used to normalize the intensity of the DLC-1 band on the immunoblot. Data represent the three independent experiments.

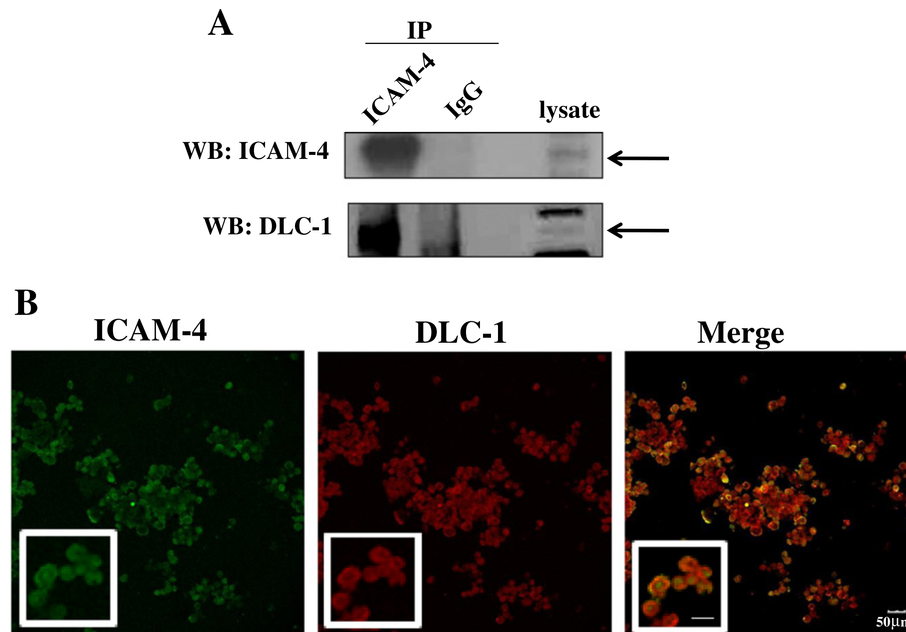

Figure 6 Endogenous interactions between DLC-1 and ICAM-4 in human erythroid cells. (A) Cell lysates were prepared from K562 cells. Immunoprecipitation was performed with anti-ICAM-4 (29 kDa) and anti-IgG (negative control) antibodies and then subjected to immunoblotting to detect interactions between ICAM-4 and anti-DLC-1 (123 kDa). Data represent three independent experiments. (B) Immunofluorescence confocal microscopy was used to explore interactions between DLC-1 (Red) and ICAM-4 (Green) from primary erythroid cells at day 17 of culture. Complexes between endogenous DLC-1 and ICAM-4 were found in the cytosol of cells. Insets show enlarged images (scale bar, 25 μm). The pictures are representative of the three independent experiments.

bridges between erythroid cells, a finding not previously demonstrated in the literature (Figs. 2B and H). We propose that cell-to-cell contact mimics *in vivo* cell maturation conditions, possibly through various stimuli. Therefore, the erythroid cells cultured under high density conditions were found to have diminished numbers of apoptotic cells and higher numbers of viable cells. This indicates that the cells matured under lower stress and with less damage, which can accumulate in normal *in vitro* culture conditions.

In an attempt to identify the responsible key molecules and proteins, we selected several adhesion-related candidate genes from the microarray analysis results from during the enucleation stages. We discovered several enucleation-related signals, such as DLC-1, ICAM-4, and VLA-4. Among these, DLC-1 and ICAM-4 were significantly increased in the high-density cultures, perhaps due to the increased cell-to-cell contact. Even though ICAM-4 is known to be an adhesive ligand in erythroid cells, the expression of DLC-1 in erythroid cells was unexpected. Therefore, the consistent expression of DLC-1 in an erythroid lineage was verified in K562 cells.

DLC-1 was recently studied in various tumor cells, including breast cancer and colon cancer (Healy et al., 2008; Kim et al., 2008a, 2008b; Yuan et al., 2003). This RhoA-specific GTPase-activating protein regulates a variety of cellular processes, including actin cytoskeleton organization, cell adhesion to the extracellular matrix, the cell cycle (Gabet et al., 2011; Kim et al., 2008a, 2008b, 2009), and enhanced protrusion (Kim et al., 2008a, 2008b). Interestingly, all of these processes are required to complete the terminal phase of erythropoiesis and enucleation. Also, the GTPase and GTPase activating protein is known to be very essential for the enucleation process (Ji et al., 2008).

Our finding that DLC-1 expression increased at higher densities concurrent with elevated enucleation rates suggest that DLC-1 is a novel contact-related signal among erythroid cells and might play a role in the enucleation process.

As both DLC-1 and ICAM-4 expression increased at higher densities, we postulate that ICAM-4 might interact with DLC-1 in erythroid cells in a manner similar to the way in which ICAM-1 induces Rho GTPase activation in endothelial cells *via* adhesion-related proteins (Etienne et al., 1998). DLC-1 is also known to co-localize with the adhesion proteins vinculin and tensin in focal adhesions (Kim et al., 2007; Xue et al., 2008; Chan et al., 2009). Focal adhesions form structural links between the actin cytoskeleton and the extracellular matrix and serve as signal transduction centers for cell growth regulation (Burridge and Chrzanowska-Wodnicka, 1996; Xue et al., 2008). This could indicate that cell-to-cell interaction induces DLC-1 activation along with ICAM-4, which also plays an important role during terminal erythropoiesis and enucleation. Further study is needed to identify the roles of DLC-1 in this complicated network.

ICAM-4 is a unique member of the ICAM family expressed on erythroid cells. It binds to VLA-4 during the differentiation of erythroid precursors and the organization of erythroblastic islands (Eshghi et al., 2007; Spring et al., 2001). Even though VLA-4 is present on erythroid cells, the precise relationship and function of VLA-4 on erythroblasts has not been elucidated. Contamination by macrophages, which also express VLA-4, was not observed with phase contrast microscopy or on

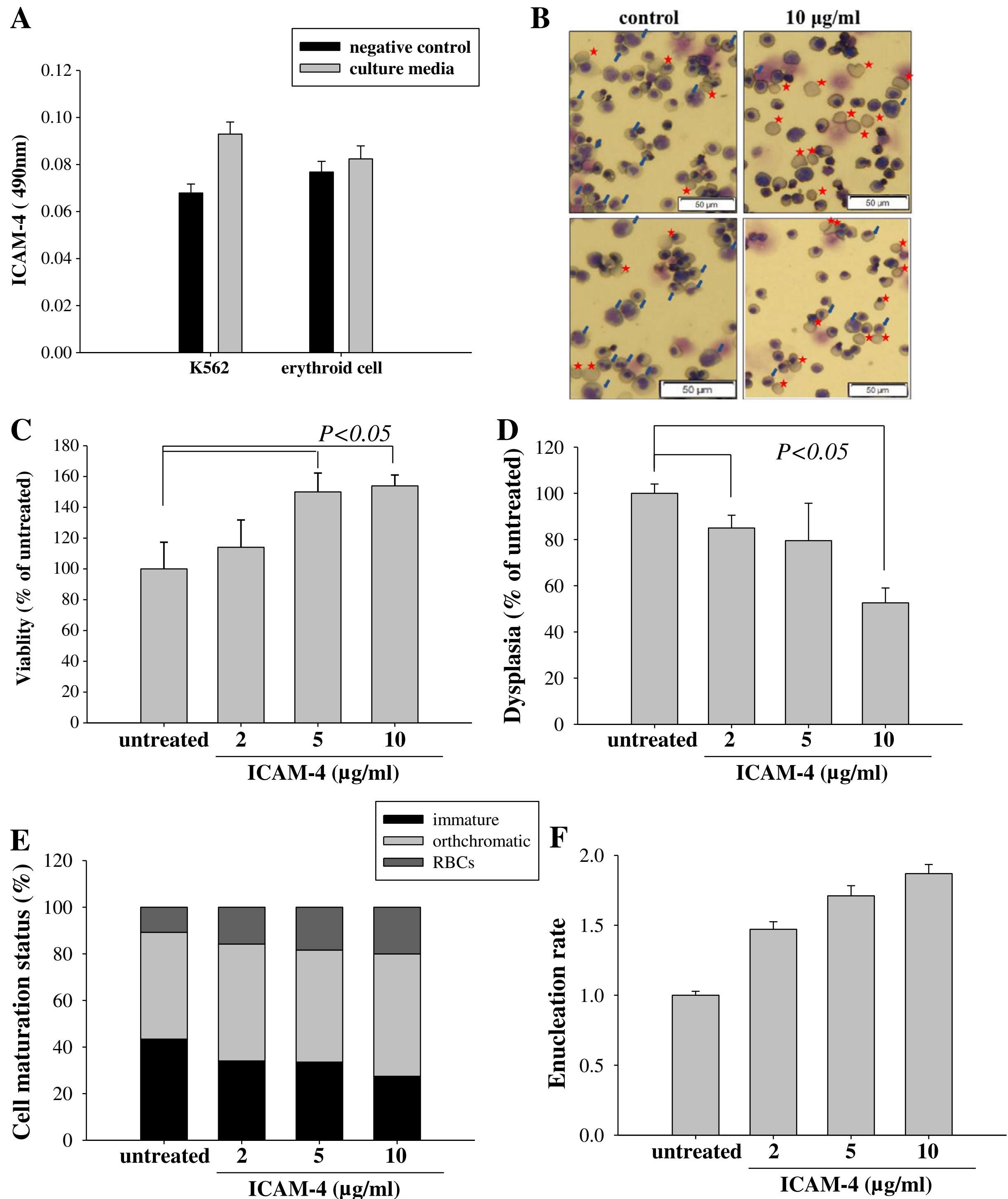

Figure 7 Effects of soluble ICAM-4 on terminal erythropoiesis. (A) The erythroid cells at culture day 17 and K562 cells were prepared to assess the existence of a soluble form of ICAM-4. Culture supernatants of the erythroid cells and K562 cells were collected, and the levels of ICAM-4 were measured by ELISA (n = 3, measured in triplicate). (B) Erythroid cells stained with Wright–Giemsa. Blue arrows indicate dysplastic erythroid cells with multi-nucleation and nuclear protrusions. Red stars indicate enucleated RBCs. Magnification: 200×. With an increase in ICAM-4 concentration, the viability of erythroid cells significantly increased as shown by trypan blue staining (C), nuclear dysplasia decreased (D), maturation status progressed (E), and subsequently, the number of enucleated RBCs increased (F). Data represent three independent experiments, which were performed in duplicate. After pictures were taken, at least 400 cells per condition were counted in a blinded manner.

Wright–Giemsa-stained slides. When the cultured cells were stained with anti-CD11b antibody, which recognizes myeloid cells and monocytes as well as macrophages, and analyzed by flow cytometry, similar percentages of CD11b-positive cells were detected for the optimal and supraoptimal samples (mean 4.6% vs. 4.2%, respectively, n=2; controls were macrophages in ascites from patients with liver cirrhosis). As the purity of erythroid cells was not 100%, there could have been some effects from other cell lineages, including macrophages. However, when erythroid cells were mixed with macrophages, we found that most erythroid cells bound to the macrophages, even at the lower density (data not shown). Therefore, the macrophage–erythroid cell binding effect did not differ significantly between density conditions. More importantly, our results show that erythroid cells have connections, as demonstrated by the presence of bridges between erythroid cells and by the expression of adhesion markers (Figs. 2 and 6). Also, the effects of sICAM-4 could not be devaluated by the presence of a few macrophages and myeloid cells (Fig. 7).

For the first time, we showed that a secreted form of human ICAM-4 (sICAM-4) is made from human erythroblasts. Similarly, erythroblasts derived from murine Friend virus anemia (FVA) cells are known to secrete ICAM-4 (Ihanus et al., 2007; Lee et al., 2003). As the erythroblasts mature, they reside in areas peripheral to macrophages within the erythroblastic island (Bain et al., 1996), and the direct effects of macrophages decrease and the effects of adjacent erythroid cells increase. We therefore speculated that the addition of the sICAM-4 could mimic co-stimulation among erythroblasts.

The addition of recombinant ICAM-4 enhanced cell differentiation to RBCs by decreasing abnormal cytokinesis *in vitro*. Such abnormal cytokinesis is known as nuclear dysplasia, and occurs when the nucleus does not properly divide to form daughter cells. These dysplastic cells show multi-nucleation states, fragmented nuclei, or irregular nuclear contours. Erythroid dysplasia leads to inefficient enucleation and cell death *in vivo* and *in vitro*. The role of DLC-1 in cytokinesis and co-binding with ICAM-4 suggests that the effects induced by ICAM-4 are related to DLC-1. This relationship should be further evaluated in culture systems and in pathologic diseases *in vivo*.

The elucidation of the role of sICAM-4 is also important for practical purposes. The progressive loss of viable cells during successive stages of cell culture is well known, especially in terminally matured erythroblasts, resulting in inefficient generation of RBCs. Rigorous attempts have been made to create a platform for efficient erythrocyte production, but these attempts have met with failure. In our culture system, with its absence of supporting stromal cells and serum/plasma, a supraoptimal density led to significantly enhanced cell survival, enucleation, and finally, to a higher amount of generated RBCs, indicating that erythroblast-erythroblast contact may mimic *in vivo* conditions (Chasis, 2006). Even though these erythroblast contacts do not equal the most powerful effects of macrophages on erythropoiesis, the availability of sICAM-4 for *in vitro* terminal erythropoiesis is valuable. This clearly suggests that our culture system, with its addition of sICAM-4, could be used for mass RBC production using a bioreactor by reducing culture area and enhancing culture efficiency.

Culturing precursor cells of hematopoietic origin at a high density induces a "crowding" effect, which results in a general decline in metabolic activity (Sand et al., 1977). Additional evidence demonstrates that inhibitory cytokines can accumulate in cultures through lactate production (Sand et al., 1977). Therefore, we analyzed conditioned media to investigate the effects of culture density on cell metabolism, and did not observe any variations in metabolic activity or cellular homeostasis.

For the relatively low enucleation rate in this report, the likely reason is that the culture conditions were deprived of serum/plasma and stromal cells. Other reports showing higher enucleation rates of more than 50% were performed in the setting of the addition of serum/plasma or feeder factors.

In summary, our findings indicate the importance of physical contact between erythroid cells during terminal maturation. At a higher density, which increases the chances of cell-to-cell contact, the viability, expansion rate, and enucleation rate of erythroblasts increased significantly. The adhesion-related molecule ICAM-4 and its counterpart VLA-4 were highly expressed under these conditions. DLC-1 was also found to be co-expressed with ICAM-4 in erythroblasts. The presence of the soluble form of ICAM-4 was first confirmed in human erythroblasts. Next, the addition of soluble ICAM-4 during the terminal maturation phase of erythroblasts decreased nuclear dysplasia and elevated cell viability and RBC production. Contact with adjacent erythroblasts might play a crucial role in the formation of terminally matured erythroblasts, which are not in direct contact with macrophages, through ICAM-4- and DLC-1-mediated signaling.

Our findings could be applied to a bioreactor system to effectively and efficiently produce RBCs. Better productivity at high density could enable a reduction in culture area and an increase in cell number at limited culture volumes. The effects of the soluble form of ICAM-4 are readily applicable by simply adding it to any kind of bioreactor, including the agitator type, thus mimicking the highly compacted cellular environment of bone marrow. Since there are currently no known cytokines in terminal erythropoiesis, sICAM-4 would be one of the few reagents available to increase the viability and productivity of RBCs.

Contributors

H.S.C and E.J.B. designed and organized the experiments, analyzed the data, generated the figures, and wrote the manuscript; H.O. K. and E.M.L. performed the culture experiments. M.I.P provided cord blood specimens.

Conflict of interest statements

The authors declare no conflict of interest.

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References

Allen, T.D., Dexter, T.M., 1982. Ultrastructural aspects of erythropoietic differentiation in long-term bone marrow culture. Differentiation 21, 86–94.

Baek, E.J., Kim, H.S., Kim, J.H., Choi, T.Y., Kim, H.O., 2008. *In vitro* clinical-grade generation of red blood cells from human umbilical cord blood CD34+ cells. Transfusion 48, 2235–2245.

Baek, E.J., Kim, H.S., Kim, J.H., Kim, N.J., Kim, H.O., 2009. Stroma-free mass production of clinical grade red blood cells by using poloxamer 188 as a RBC survival enhancer. Transfusion 49, 2285–2295.

Baek, E.J., You, J., Kim, M.S., Lee, S.Y., Cho, S.J., Kim, E., Kim, H.O., 2010. Enhanced production of red blood cells in suspension by electrostatic interactions with culture plates. Tissue Eng. Part C 16, 1325–1334.

Bain, B.J., Clark, D.M., Lampert, I.A., 1996. Bone Marrow Pathology. Blackwell Science, Oxford; Cambridge, Mass., USA.

Bessis, M., 1958. Erythroblastic island, functional unity of bone marrow. Rev. Hematol. 13, 8–11.

Burridge, K., Chrzanowska-Wodnicka, M., 1996. Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol. 12, 463–518.

Chan, L.K., Ko, F.C.F., Ng, I.O.L., Yam, J.W.P., 2009. Deleted in liver cancer 1 (DLC1) utilizes a novel binding site for tensin2 PTB domain interaction and is required for tumor-suppressive function. PLoS One 4, e5572.

Chasis, J.A., 2006. Erythroblastic islands: specialized microenvironmental niches for erythropoiesis. Curr. Opin. Hematol. 13, 137–141.

Chasis, J.A., Mohandas, N., 2008. Erythroblastic islands: niches for erythropoiesis. Blood 112, 470–478.

Eshghi, S., Vogelezang, M.G., Hynes, R.O., Griffith, L.G., Lodish, H.F., 2007. Alpha4beta1 integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development. J. Cell Biol. 177, 871–880.

Etienne, S., Adamson, P., Greenwood, J., Strosberg, A.D., Cazaubon, S., Couraud, P.O., 1998. ICAM-1 signaling pathways associated with rho activation in microvascular brain endothelial cells. J. Immunol. 161, 5755–5761.

Gabet, A.S., Coulon, S., Fricot, A., Vandekerckhove, J., Chang, Y., Ribeil, J.A., Lordier, L., Zermati, Y., Asnafi, V., Belaid, Z., et al., 2011. Caspase-activated ROCK-1 allows erythroblast terminal maturation independently of cytokine-induced Rho signaling. Cell Death Differ. 18, 678–689.

Healy, K.D., Hodgson, L., Kim, T., Shutes, A., Maddileti, S., Juliano, R.L., Hahn, K.M., Harden, T.K., Bang, Y., Der, C.J., 2008. DLC-1 suppresses non-small cell lung cancer growth and invasion by RhoGAP-dependent and independent mechanisms. Mol. Carcinog. 47, 326–337.

Hermand, P., Huet, M., Callebaut, I., Gane, P., Ihanus, E., Gahmberg, C.G., Cartron, J.P., Bailly, P., 2000. Binding sites of leukocyte beta 2 integrins (LFA-1, Mac-1) on the human ICAM-4/LW blood group protein. J. Biol. Chem. 275, 26002–26010.

Ihanus, E., Uotila, L.M., Toivanen, A., Varis, M., Gahmberg, C.G., 2007. Red-cell ICAM-4 is a ligand for the monocyte/macrophage integrin CD11c/CD18: characterization of the binding sites on ICAM-4. Blood 109, 802–810.

Ji, P., Jayapal, S.R., Lodish, H.F., 2008. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. Nat. Cell Biol. 10, 314–321.

Ji, P., Yeh, V., Ramirez, T., Murata Hori, M., Lodish, H.F., 2010. Histone deacetylase 2 is required for chromatin condensation and subsequent enucleation of cultured mouse fetal erythroblasts. Haematology 95, 2013–2021.

Kim, T.Y., Lee, J.W., Kim, H., Jong, H., Jung, M., Bang, Y., 2007. DLC-1, a GTPase-activating protein for Rho, is associated with cell proliferation, morphology, and migration in human hepatocellular carcinoma. Biochem. Biophys. Res. Commun. 355, 72–77.

Kim, T.Y., Healy, K.D., Der, C.J., Sciaky, N., Bang, Y., Juliano, R.L., 2008a. Effects of structure of Rho GTPase-activating protein DLC-1 on cell morphology and migration. J. Cell Biol. 283, 32762–32770.

Kim, T.Y., Kim, I.S., Jong, H.S., Lee, J.W., Jung, M., Bang, Y.J., 2008b. Transcriptional induction of DLC-1 gene through Sp1 sites by histone deacetylase inhibitors in gastric cancer cells. Exp. Mol. Med. 40, 639–646.

Kim, T.Y., Vigil, D., Der, C.J., Juliano, R.L., 2009. Role of DLC-1, a tumor suppressor protein with RhoGAP activity, in regulation of the cytoskeleton and cell motility. Cancer Metastasis Rev. 28, 77–83.

Lee, G., Spring, F.A., Parsons, S.F., Mankelow, T.J., Peters, L., Koury, M.J., Mohandas, N., Anstee, D.J., Chasis, J.A., 2003. Novel secreted isoform of adhesion molecule ICAM-4: potential regulator of membrane-associated ICAM-4 interactions. Blood 101, 1790–1797.

Manwani, D., Bieker, J.J., 2008. The erythroblastic island. Curr. Top. Dev. Biol. 82, 23–53.

Migliaccio, G., Sanchez, M., Masiello, F., Tirelli, V., Varricchio, L., Whitsett, C., Migliaccio, A.R., 2010. Humanized culture medium for clinical expansion of human erythroblasts. Cell Transplant. 19, 453–469.

Miharada, K., Hiroyama, T., Sudo, K., Nagasawa, T., Nakamura, Y., 2006. Efficient enucleation of erythroblasts differentiated *in vitro* from hematopoietic stem and progenitor cells. Nat. Biotechnol. 24, 1255–1256.

Mohandas, N., Chasis, J.A., 2010. The erythroid niche: molecular processes occurring within erythroblastic islands. Transfus. Clin. Biol. 17, 110–111.

Neu, B., Sowemimo-Coker, S.O., Meiselman, H.J., 2003. Cell–cell affinity of senescent human erythrocytes. Biophys. J. 85, 75–84.

Rhodes, M., Kopsombut, P., Bondurant, M.C., Price, J.O., Koury, M.J., 2008. Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. Blood 111, 1700–1708.

Sand, T., Condie, R., Rosenberg, A., 1977. Metabolic crowding effect in suspension of cultured lymphocytes. Blood 50, 337–346.

Sato, C., Kojima, K., Nishizawa, K., Ikawa, Y., 1979. Early decrease in hyaluronidase-sensitive cell surface charge during the differentiation of Friend erythroleukemic cells by dimethyl sulfoxide. Cancer Res. 39, 1113–1117.

Soni, S., Bala, S., Gwynn, B., Sahr, K.E., Peters, L., Hanspal, M., 2006. Absence of erythroblast macrophage protein (Emp) leads to failure of erythroblast nuclear extrusion. J. Biol. Chem. 281, 20181–20189.

Spring, F.A., Parsons, S.F., 2000. Erythroid cell adhesion molecules. Transfus. Med. Rev. 14, 351–363.

Spring, F.A., Parsons, S.F., Ortlepp, S., Olsson, M.L., Sessions, R., Brady, R.L., Anstee, D.J., 2001. Intercellular adhesion molecule-4 binds alpha(4)beta(1) and alpha(V)-family integrins through novel integrin-binding mechanisms. Blood 98, 458–466.

Telen, M.J., 2000. Red blood cell surface adhesion molecules: their possible roles in normal human physiology and disease. Semin. Hematol. 37, 130–142.

Tsiftsoglou, A.S., Vizirianakis, I.S., Strouboulis, J., 2009. Erythropoiesis: model systems, molecular regulators, and developmental programs. IUBMB Life 61, 800–830.

Xue, W., Krasnitz, A., Lucito, R., Sordella, R., Vanaelst, L., Cordon Cardo, C., Singer, S., Kuehnel, F., Wigler, M., Lowe, S.W., et al., 2008. DLC1 is a chromosome 8p tumor suppressor whose loss promotes hepatocellular carcinoma. Genes Dev. 22, 1439–1444.

Yuan, B., Zhou, X., Durkin, M.E., Zimonjic, D.B., Gumundsdottir, K., Eyfjord, J.E., Thorgeirsson, S., Popescu, N.C., 2003. DLC-1 gene inhibits human breast cancer cell growth and *in vivo* tumorigenicity. Oncogene 22, 445–450.