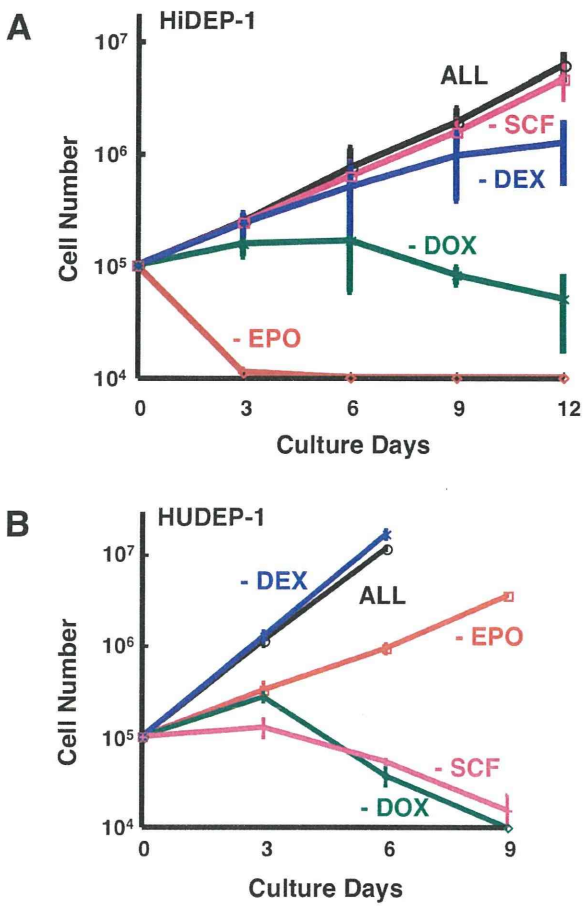

Figure 3. Dependency of the established erythroid progenitor cell lines on externally supplied culture factors. (A) The survival and proliferation of HiDEP-1 cells are dependent on DOX (HPV16-E6/E7) and EPO and partially dependent on DEX. (B) The survival and proliferation of HUDEP-1 cells are dependent on DOX (HPV16-E6/E7) and SCF and partially dependent on EPO. (A, B) DOX, doxycycline; expression of HPV16-E6/E7 is induced by DOX. SCF, stem cell factor. EPO, erythropoietin. DEX, dexamethasone. ALL, cells were cultured in the presence of DOX, SCF, EPO and DEX. –DOX, –SCF, –EPO, –DEX, cells were cultured after deprivation of DOX, SCF, EPO and DEX, respectively. Dependencies of other cell lines on externally supplied culture factors are summarized in Table S1.
doi:10.1371/journal.pone.0059890.g003

expression of TAL1 in HUDEP-1 cells at 7 days after induction of HPV16-E6/E7 expression and established the cell line HUDEP-1-TAL1. However, the phenotype and characteristics of HUDEP-1-TAL1 cells were quite similar to those of HUDEP-1 cells (Figure 4B and Figure S3B).

Induction of Differentiation

Next, we examined whether HiDEP and HUDEP cells could differentiate into more mature stages and produce enucleated RBCs. We found that differentiation could be induced by culturing the HiDEP and HUDEP cells in an erythroid differentiation medium in the presence of EPO alone (see Materials and methods).

Production of Hemoglobin

Upon centrifugation, the HiDEP-1 cells produced a red cell pellet even before the induction of differentiation (Figure 5A) suggesting a continuous and abundant production of hemoglobin. In contrast, upon centrifugation, the HUDEP-1 cells yielded a light orange cell pellet before the induction of differentiation and a red cell pellet after the induction of differentiation (Figure 5B) suggesting that hemoglobin synthesis was upregulated following differentiation.

Functional Analysis of Hemoglobin Produced in HiDEP and HUDEP Cells

We used a Hemox-Analyzer to measure the oxygen binding and dissociation abilities of the hemoglobin produced in mature cells derived from the HiDEP and HUDEP cells [20,21]. Although the oxygen binding and dissociation curves obtained with HiDEP- and HUDEP-derived cells differed between cell lines, they showed similar curves to those obtained with adult peripheral blood or umbilical cord blood (Figure 6). The variation among the curves may be due to the types of hemoglobin expressed in each cell line, i.e., adult type hemoglobin, fetal type hemoglobin or mixture of both types (see below). Apart from this variation, the data indicate that HiDEP and HUDEP cells can produce hemoglobin with oxygen binding and dissociation abilities equivalent to red blood cells produced in vivo.

Analysis of Gene Expression

Gene expression profiles were analyzed by quantitative RT-PCR (qRT-PCR) before and after the induction of differentiation in the HiDEP and HUDEP cells lines. The erythroid-specific markers GATA1, EKLF, GFI1B, TAL1 and EPOR were detected in all cell lines and their expression profiles before and after differentiation were similar to those of cultured erythroid cells derived from umbilical cord blood (Figure 7). This was also the case for c-MYB and SOX6, which are markers of definitive erythroid cells [22,23,24]. In addition, we detected upregulation of the erythroid membrane genes, Band 3, Band 4.1, Ankyrin-1 and α-Spectrin in all cell lines after differentiation (Figure 7).

After differentiation, we found upregulation of α-globin in all cell lines, upregulation of β-globin only in the HUDEP-2 cell line, and upregulation of γ-globin in all cell lines except HUDEP-2. Of note, BCL11A, a repressor of γ-globin expression [25] and critical mediator of globin switching [26], was abundantly detected only in the HUDEP-2 cell line in which β-globin was up-regulated after differentiation (Figure 7).

Cell Viability and Cell Size

After the induction of differentiation in HiDEP-1 cells, the proportion of viable cells decreased but more than half of the cells were still viable 14 days after differentiation (Figure 8A). The average cell size after differentiation decreased compared to undifferentiated cells and the proportion of cells with a diameter less than 10 μm increased from about 20% to approximately 40% (Figure 8A) indicating the increase of very mature erythroid cells.

After the induction of differentiation in HUDEP-1 cells, the frequency of viable cells was considerably reduced compared to HiDEP-1 cells (Figure 8B). However, among viable cells average cell sizes decreased rapidly for two days after differentiation compared to undifferentiated cells, and the proportion of cells with a diameter less than 10 μm increased from about 10% to about 50% (Figure 8B) indicating the increase of very mature erythroid cells. To improve viability, the cells were cultured on OP9 feeder cells and a morphological analysis was performed.

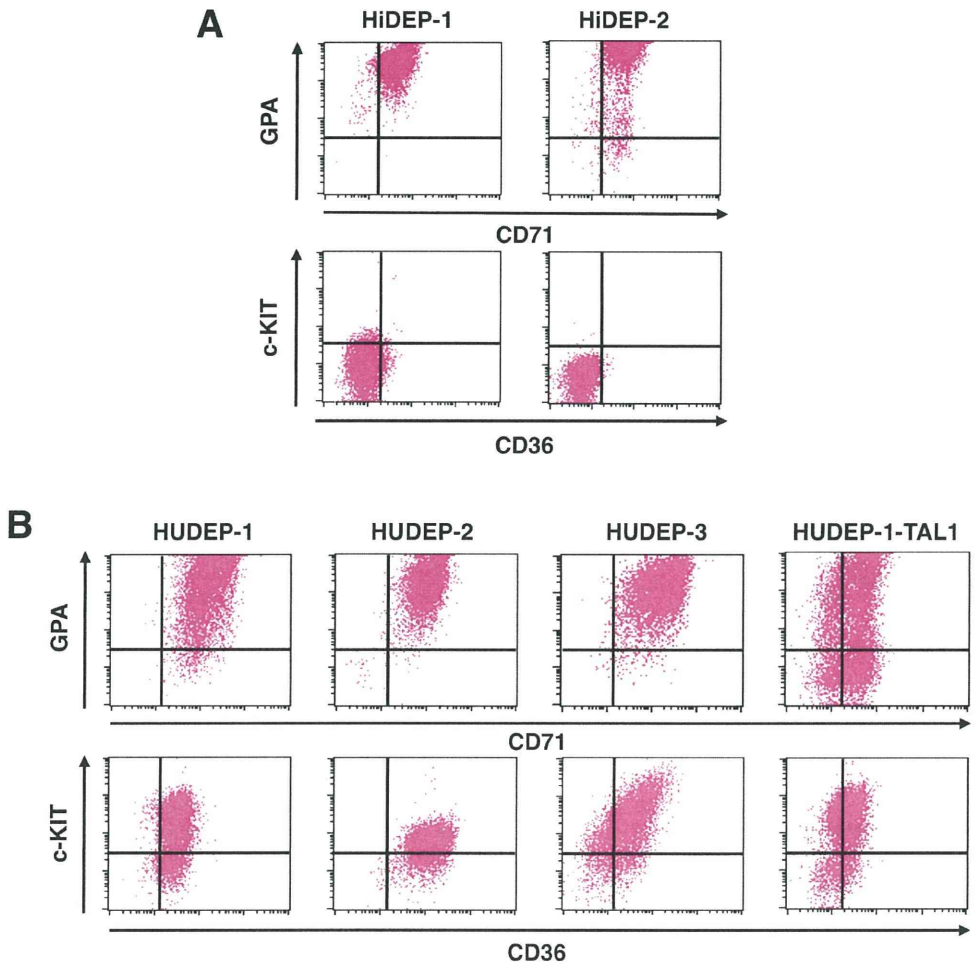

Figure 4. Flow cytometer analyses of the established erythroid progenitor cell lines. (A) Representative results of HiDEP cells. (B) Representative results of HUDEP cells and HUDEP-1 expressing TAL1, HUDEP-1-TAL1. (A, B) GPA, glycophorin A. CD71, transferrin receptor. c-KIT, the receptor of SCF. CD36, a marker of immature erythroid cells.
doi:10.1371/journal.pone.0059890.g004

Morphological Changes during Differentiation

Before the induction of differentiation, both HiDEP-1 and HUDEP-1 cells had round, erythroblast-like morphologies (Figure 9A, B). After the induction of differentiation in HiDEP-1 cells, enucleating cells (arrowheads in Figure 9A) and enucleated cells (black arrows in Figure 9A) were observed from about 7 days after differentiation. Since the cultures contained various types of cell and cell components such as enucleated cells, extruded nuclei after the enucleation process, nucleated cells, dead cells, and cell debris, it was very difficult to accurately calculate the frequency of enucleated cells even by flow cytometrical and morphological analyses. However, at approximately 12 days after the induction of differentiation a considerable number of enucleated cells were present (Figure 9A). The second cell line, HiDEP-2, could also differentiate into more mature cells and produce enucleated RBCs after the induction of differentiation; however, the efficiency of differentiation was lower compared to HiDEP-1 (data not shown).

After the induction of differentiation in HUDEP-1 cells, the morphology of the cells clearly changed and mature and enucleating cells (arrowheads in Figure 9B) and enucleated cells (black arrows in Figure 9B) were observed from about day 6 of differentiation. Although the numbers of enucleated cells were much lower than those induced from HiDEP-1 cells, we did observe reproducible production of enucleated cells. The other HUDEP cell lines, HUDEP-2 and HUDEP-3, could also differentiate into more mature cells and produce enucleated RBCs after the induction of differentiation; the efficiencies of differentiation were almost identical to HUDEP-1 (data not shown).

Confirmation of Enucleated Cells

In general, in vitro produced enucleated RBCs are spherical reticulocytes and not fully mature biconcave cells. To confirm that these enucleated cells were reticulocytes, we performed supravital staining, immunostaining and benzidine staining. The supravital staining demonstrated the presence of reticulocytes (arrows in Figure 10A). In addition, we observed GPA-positive enucleated

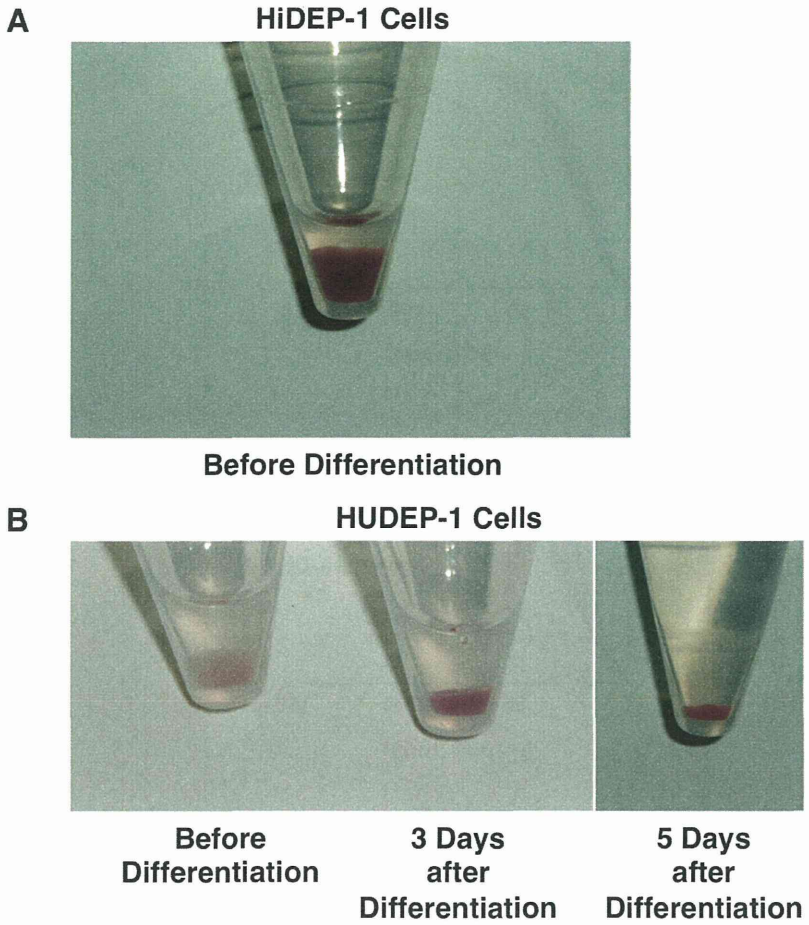

Figure 5. Cell pellets of the established erythroid progenitor cell lines. (A) HiDEP-1 cells before the induction of differentiation. (B) HUDEP-1 cells before and after the induction of differentiation. HUDEP-1 cells were cultured in erythroid differentiation medium on OP9 feeder cells to maintain cell viability during the differentiation process. All other cell lines also showed red cell pellets after the induction of differentiation.
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cells (pink cells in Figure 10B) and benzidine-positive enucleated cells (brown cells in Figure 10C).

Discussion

We previously succeeded in establishing MEDEP cell lines from mouse ES cells and were able to induce enucleated RBC production in these cell lines. However, the protocol developed for mouse cell lines failed to establish immortalized erythroid progenitor cell lines from human ES cells and human iPS cells. This difficulty may be related to the fact that in general it is more difficult to establish immortalized human cell lines than immortalized mouse cell lines. The failure of the original protocol led us to alter our strategy for establishing human erythroid progenitor cell lines.

The principal change to our strategy was to use inducible expression of HPV16-E6/E7 in transfected hematopoietic stem/progenitor cells. Through this approach, we developed a method that enabled establishment of immortalized human erythroid progenitor cell lines that possess the ability to differentiate. In iPS cells, the enforced expression of TAL1 supported the process of obtaining cell lines with hematopoietic potential by increasing the number of induced erythroid cells. To date, we have successfully established immortalized cell lines in all trials with this method. All of the immortalized cell lines we described in this study could differentiate into more mature RBCs including enucleated RBCs. The hemoglobin produced in these cell lines possessed similar oxygen binding properties to the hemoglobin in normal RBCs produced in vivo. To our knowledge, this is the first report describing the establishment of immortalized human erythroid progenitor cell lines able to produce enucleated RBCs ex vivo. Although erythroid progenitor cell lines have previously been established using HPV16-E6/E7, these cell lines did not produce enucleated RBCs [17]. One possible explanation for this failure is the continuous expression of HPV16-E6/E7 in these cells. Since efficient differentiation of cells generally requires cell cycle arrest, the continuous expression of HPV16-E6/E7 might have inhibited terminal differentiation of the cells.

The cell lines obtained in this study displayed different efficiencies for producing enucleated RBCs in vitro as is illustrated by comparison of HiDEP and HUDEP cells. We previously found that the efficiency of enucleated RBC production by MEDEP cells improved in vivo after transplantation [8]. Therefore, it is highly

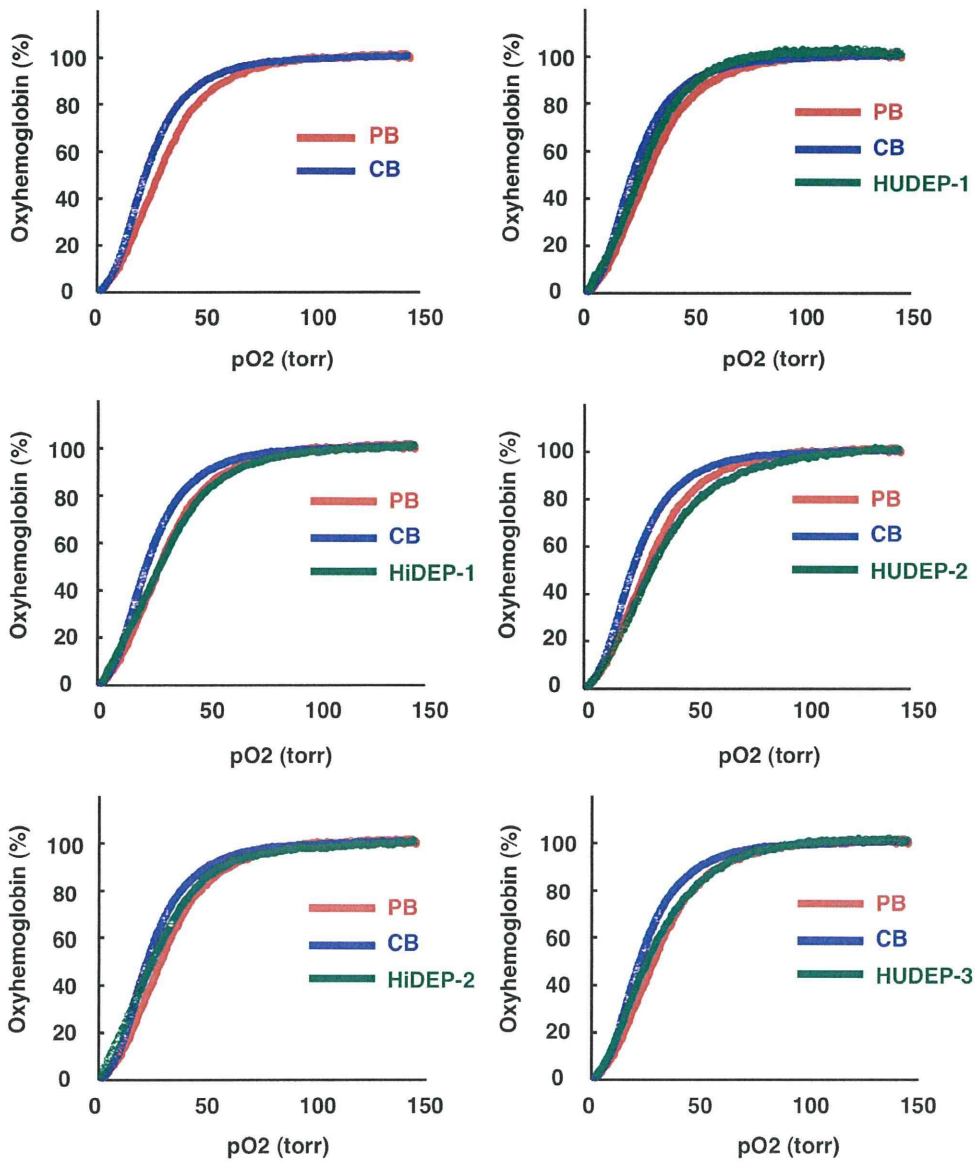

Figure 6. Oxygen-carrying abilities of hemoglobin produced in the established erythroid progenitor cell lines. Oxygen equilibrium curves were determined using an automated apparatus. Following the induction of differentiation, the cells were subjected to the analyses. CB, umbilical cord blood. PB, peripheral blood of adult.
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likely that HiDEP and HUDEP cells would also be able to produce enucleated RBCs more efficiently in vivo than in vitro.

Although we suspect that the cell lines established here might be able to produce enucleated RBCs more abundantly in vivo, the main goal of our work is to develop a means of producing abundant enucleated RBCs ex vivo. There are two reasons for this emphasis. First, enucleated RBCs pose no risk of tumorigenicity and, therefore, can be transfused without hazard into the recipient. This is not the case for transplantation of nucleated cells derived from immortalized cells such as ES cells and iPS cells. Enucleated RBCs can be selected by size, e.g., by filtration, and contaminating nucleated cells can be eliminated by irradiation without affecting the structure and function of RBCs. Currently, such irradiation is routinely used in the clinic before transfusion of RBCs in order to eliminate any lymphocytes. Second, transplantation of progenitor cells requires compatibility for major histocompatibility antigens [8]. This is not the case for enucleated RBCs, which mainly require the compatibility of ABO and RhD blood phenotypes. One of the HUDEP cell lines developed here, HUDEP-3, possesses an O/RhD(+) phenotype (Figure S4, S5 and Table S2); thus, RBCs produced ex vivo from this cell line could potentially be transfused into the vast majority of the Japanese population, since approximately 99% of Japanese possess an RhD(+) blood phenotype. Needless to say, cell lines that produce O/RhD(−) RBCs will have more widespread utility as these RBCs

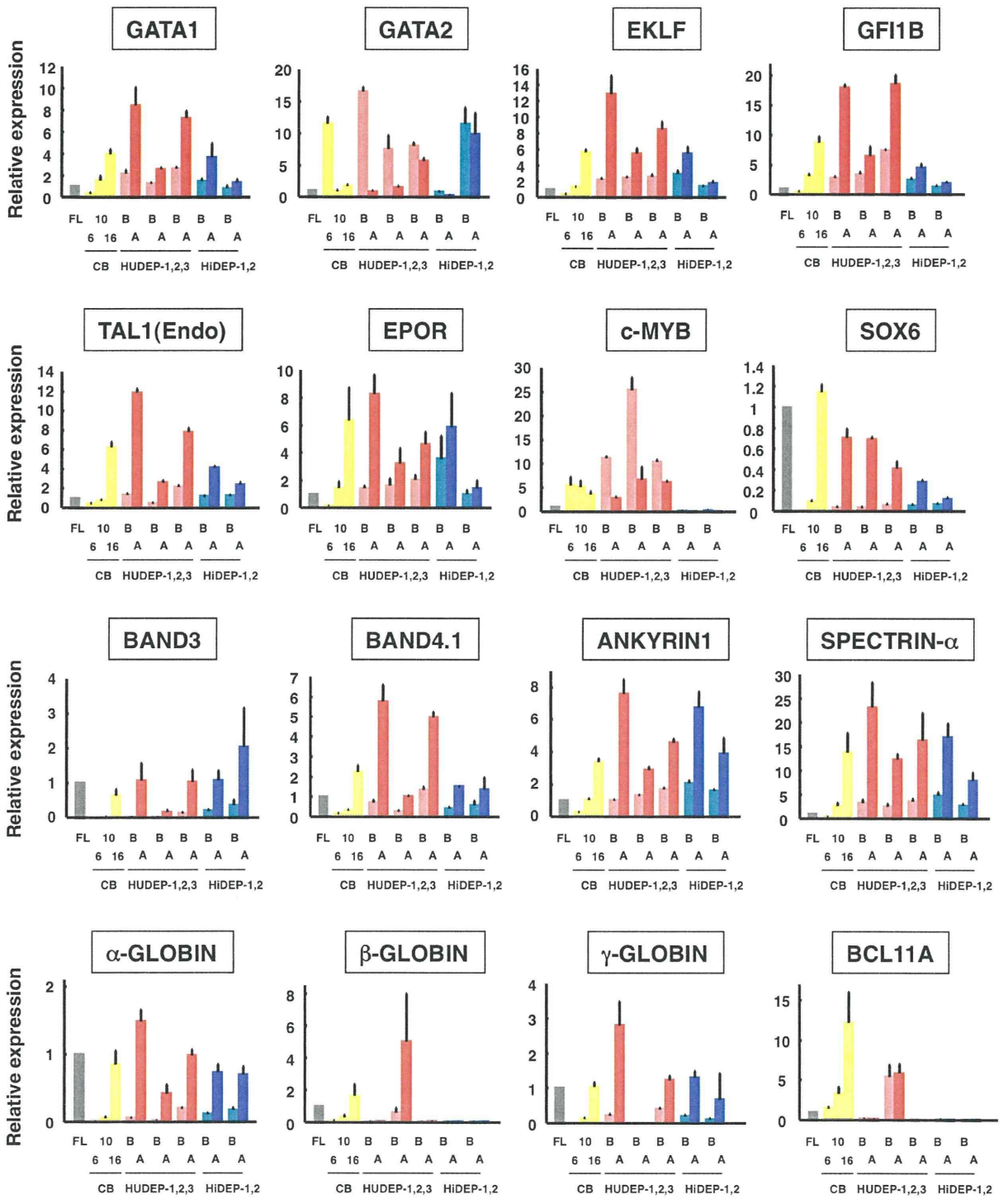

Figure 7. Gene expression profiles of the established erythroid progenitor cell lines estimated by quantitative RT-PCR analysis. Analyzed genes are indicated. FL, results with cDNA derived from human fetal liver. Relative expression was evaluated compared to that of FL. CB, cord blood. CB 6, 10 and 16, results with cDNA derived from cultured erythroid cells, i.e., CD34-positive cells in CB were induced to differentiate into mature erythroid cells for 6, 10 and 16 days, respectively, using the previously reported method [4]. B and A, Before and 2 days after induction of differentiation of HiDEP and HUDEP cells.
doi:10.1371/journal.pone.0059890.g007

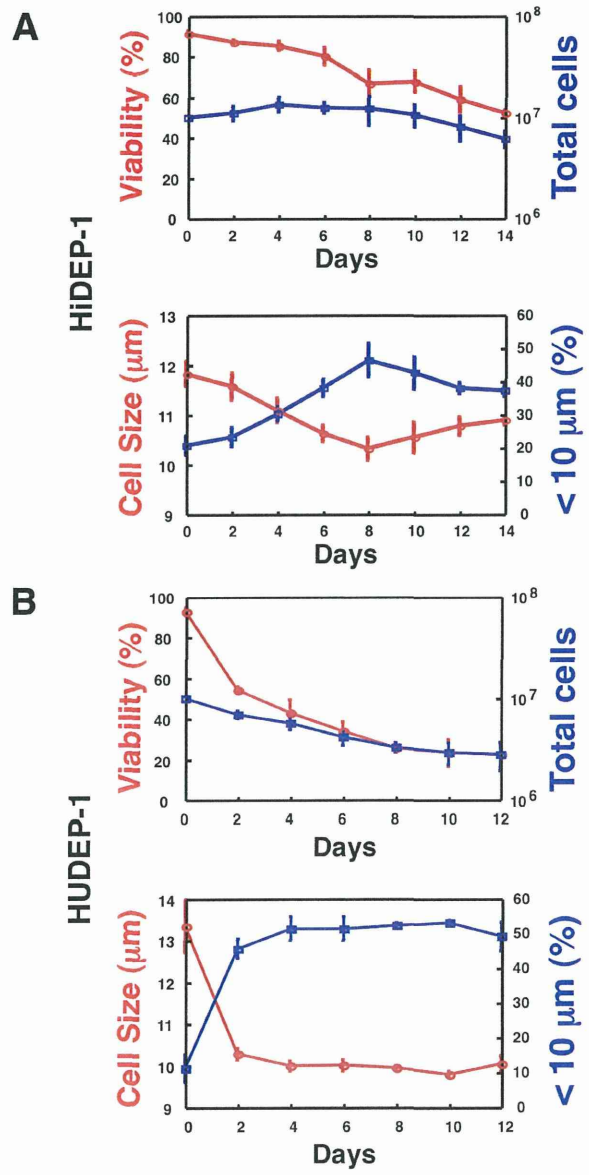

Figure 8. Analyses of cell viability and cell size during the induction of differentiation of the established erythroid progenitor cell lines. Cell viability and cell size were estimated by an automated cell counter. (A) Results of HiDEP-1 cells. (B) Results of HUDEP-1 cells. (A, B) <10 μm (%), the percentages of cells with a diameter less than 10 μm. Among all HiDEP and HUDEP cell lines, HiDEP-1 cells most efficiently differentiated into more mature cells.
doi:10.1371/journal.pone.0059890.g008

can be transfused into the vast majority of people around the world.

Our next aim is to establish cell lines that have a high yield (>90%) of enucleated RBCs after induction of differentiation. The underlying basis for the different characteristics of the various cell lines, such as the efficiency of enucleated RBC production, remains uncertain. Possibly, they may have been immortalized at different stages of erythroid differentiation; the mechanisms of immortalization at different stages of differentiation remain to be studied. Until these mechanisms are elucidated, our experience with the generation of MEDEP, HiDEP and HUDEP cell lines suggests that establishment of useful cell lines will necessitate numerous trials. At present, we do not think that hematopoietic cells derived from iPS cells are a better source than cord blood cells for establishing cell lines. Indeed, HiDEP-1 cells demonstrated a high efficiency of production of enucleated RBCs, while HUDEP-2 cells could produce adult type hemoglobin consisting of α and β globin. We certainly believe it will be feasible to establish HiDEP and/or HUDEP cell lines able to produce adult type hemoglobin and also to produce enucleated RBCs with a high efficiency. With respect to the production of adult type hemoglobin, the results shown in Figure 7 suggested that enforced expression of BCL11A in the source cells may support the establishment of cell lines that produce adult type hemoglobin. In a similar manner to the exogenous expression of TAL1 and HPV16-E6/E7, the introduction of BCL11A will have no consequence for clinical applications if the ultimately established cell lines can produce abundant enucleated RBCs.

Of note, the enucleated cells produced in vitro from HiDEP and HUDEP cells were spherical (Figure 9) and were reticulocytes (Figure 10A). We previously reported that the enucleated RBCs produced from hematopoietic stem/progenitor cells present in cord blood in vitro were also spherical reticulocytes [4]. Generally, the majority of in vitro produced RBCs seem to be reticulocytes; however, it has recently been reported that reticulocytes produced in vitro can complete maturation in vivo, i.e., following transplantation, into biconcave cells [27]. Therefore, it is likely that the reticulocytes produced in vitro from HiDEP and HUDEP cells could also complete maturation in vivo after transfusion.

The method developed to establish the HiDEP cell line involved use of the mouse derived-OP9 cell line as feeder cells during the early stages of establishment and also use of fetal bovine serum (FBS). These xenogeneic factors need to be eliminated before the application of cells in the clinic. There are two reasons for believing this elimination is possible. First, the xenogeneic factors derived from OP9 cells will disappear after long term culture in the absence of OP9 cells. The HiDEP cells ceased to have a requirement for OP9 cells after two or three months in culture and they could be maintained in the presence of humoral factors alone. Notably, HUDEP cell lines could be established in feeder cell-free conditions, i.e., without use of OP9 cells. Second, as we have previously demonstrated, FBS can be replaced with human serum without compromising the production of abundant enucleated RBCs from hematopoietic stem/progenitor cells in cord blood [4]. In addition, all HiDEP and HUDEP cells could be maintained in serum-free conditions using a commercially available medium (see Materials and methods). Thus, with respect to HUDEP cell lines they can be established in a feeder cell-free and serum-free conditions throughout all procedures.

Changes to the characteristics of immortalized cell lines after long term culture can sometimes be an obstacle to their use. However, it is also a fact that many immortalized cell lines, such as human cancer cell lines, stably maintain their characteristic properties even after long term culture. Of note, MEDEP-BRC5, one of the MEDEP cell lines we previously reported [8], has maintained the ability for high rates of production of enucleated RBCs in vitro even after continuous culture for nearly 2 years; in this cell line, more than 50% of the cells are enucleated RBCs after induction of differentiation in vitro [28].

We are now confident that the method described in this study can reproducibly and robustly establish immortalized human erythroid progenitor cell lines, such as the HiDEP and HUDEP

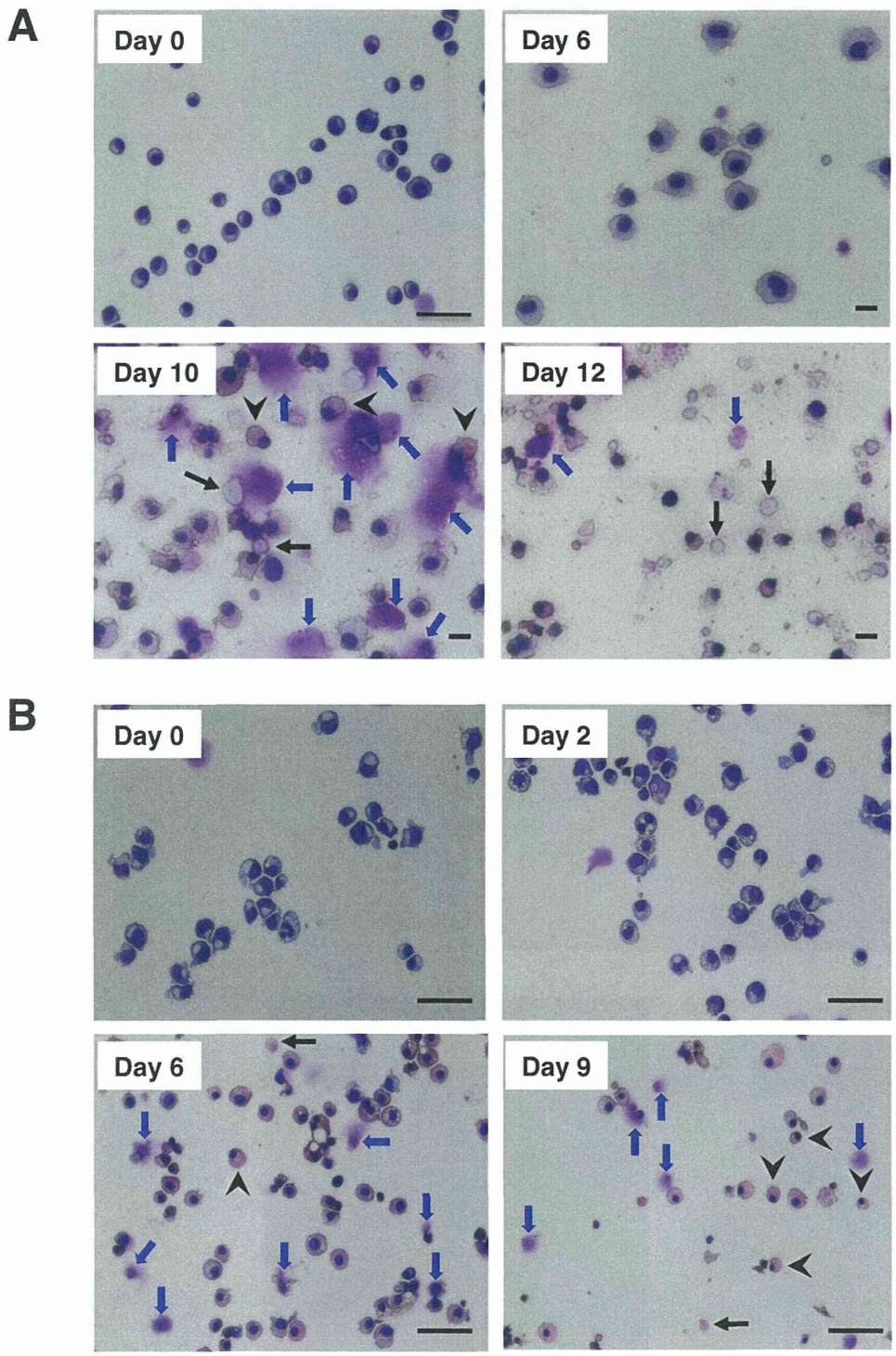

Figure 9. Morphological analyses during the induction of differentiation of the established erythroid progenitor cell lines. (A) HiDEP-1 cells before (Day 0) and 6, 10 and 12 days after the induction of differentiation (Day 6, 10 and 12). Scale bar in Day 0 indicates 50 μm and other scale bars indicate 10 μm. (B) HUDEP-1 cells before (Day 0) and 2, 6 and 9 days after the induction of differentiation (Day 2, 6 and 9). HUDEP-1 cells were cultured in erythroid differentiation medium on OP9 feeder cells to maintain cell viability during the differentiation process. All scale bars indicate 50 μm. (A, B) Black arrows and arrowheads show enucleated and enucleating cells, respectively. Blue arrows indicate cell debris. Among all HiDEP and HUDEP cell lines, HiDEP-1 cells most efficiently produced enucleated cells.
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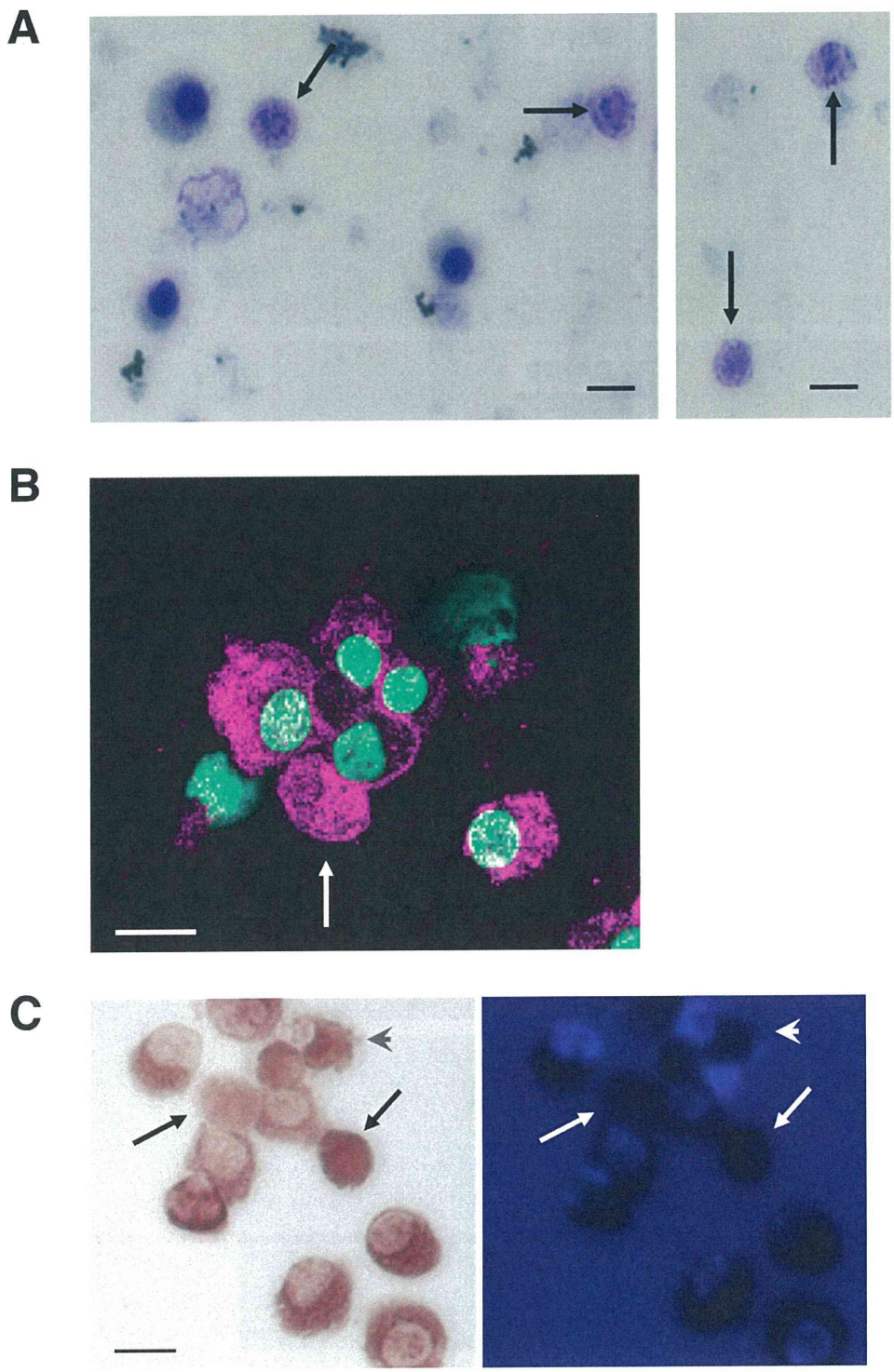

Figure 10. Confirmation of enucleated cells. As a representative, the cells induced from HiDEP-1 cells were subjected to the analyses. (A) Supravital staining. Arrows indicate reticulocytes. (B) Immunohistostaining with glycophorin A antibody (pink) 12 days after the induction of differentiation. Nuclei are stained with SYTO16 (green). An arrow shows an enucleated cell. (C) Benzidine staining 10 days after the induction of differentiation (brown, left panel). After benzidine staining, cell nuclei were labeled with DAPI to distinguish nucleated and enucleated cells (blue stained nuclei in right panel). Arrows or arrowheads show enucleated and enucleating cells, respectively. (A–C) Scale bars indicate 10 μm.
doi:10.1371/journal.pone.0059890.g010

cell lines, able to produce enucleated RBCs ex vivo. Establishment of HiDEP cell lines from iPS cells derived from people possessing very rare blood phenotypes, such as Rh-null, may open a way to produce such rare types of RBCs ex vivo.

Supporting Information

Figure S1 Map of the CSII-EF-RfA lentiviral vector plasmid.
(TIF)

Figure S2 Map of the CSIV-TRE-RfA-UbC-KT lentiviral vector plasmid.
(TIF)

Figure S3 Summary of flow cytometry analyses. Expression of the indicated markers was analyzed. c-KIT, the receptor of SCF. GPA, glycophorin A. (A) Results from HiDEP cells. (B) Results from HUDEP cells.
(TIF)

Figure S4 Analysis of blood types in HUDEP and HiDEP cells. (A) The results with respect to the ABO gene. (B) The results with respect to the RhD gene. (A, B) A detailed description of the typing of the cells can be found in Lu et al., Blood 112; 4475–4484 (2008).
(TIF)

Figure S5 Characterization of phenotype with respect to RhD antigen. Expression of RhD antigen was clearly detected in HiDEP-1-derived cells. In contrast, HUDEP-3-derived cells did not show abundant expression of RhD antigens. These results indicated that RhD antigens might be induced depending on the stage of maturation of the cells since HiDEP-1 produced mature red blood cells at a higher rate (see manuscript).
(TIF)

Table S1 Factor dependency of iPS and cord blood-derived erythroid progenitor cell lines.
(DOC)

Table S2 Blood phenotypes of the established erythroid progenitor cell lines.
(DOC)

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Author Contributions

Conceived and designed the experiments: RK KT YN. Performed the experiments: RK NS. Analyzed the data: RK KS KM TH YN. Contributed reagents/materials/analysis tools: HM. Wrote the paper: RK YN.

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Wnt3a stimulates maturation of impaired neutrophils developed from severe congenital neutropenia patient-derived pluripotent stem cells

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The derivation of induced pluripotent stem (iPS) cells from individuals of genetic disorders offers new opportunities for basic research into these diseases and the development of therapeutic compounds. Severe congenital neutropenia (SCN) is a serious disorder characterized by severe neutropenia at birth. SCN is associated with heterozygous mutations in the neutrophil elastase [elastase, neutrophil-expressed (ELANE)] gene, but the mechanisms that disrupt neutrophil development have not yet been clarified because of the current lack of an appropriate disease model. Here, we generated iPS cells from an individual with SCN (SCN-iPS cells). Granulopoiesis from SCN-iPS cells revealed neutrophil maturation arrest and little sensitivity to granulocyte-colony stimulating factor, reflecting a disease status of SCN. Molecular analysis of the granulopoiesis from the SCN-iPS cells vs. control iPS cells showed reduced expression of genes related to the wingless-type mmtv integration site family, member 3a (Wnt3a)/β-catenin pathway [e.g., lymphoid enhancer-binding factor 1], whereas Wnt3a administration induced elevation lymphoid enhancer-binding factor 1-expression and the maturation of SCN-iPS cell-derived neutrophils. These results indicate that SCN-iPS cells provide a useful disease model for SCN, and the activation of the Wnt3a/β-catenin pathway may offer a novel therapy for SCN with ELANE mutation.

apoptosis | unfolded protein response | SCN disease model

Severe congenital neutropenia (SCN) is a heterogeneous bone marrow (BM) failure syndrome characterized by severe neutropenia at birth, leading to recurrent infections by bacteria or fungi (1). SCN patients reveal an arrest in neutrophil differentiation in the BM at the promyelocyte or myelocyte stage (1), as well as a propensity to develop myelodysplastic syndrome and acute myeloid leukemia (2). Current treatment by high-dose granulocyte-colony stimulating factor (G-CSF) administration induces an increase in the number of mature neutrophils in the peripheral blood of most SCN patients (3). Although this treatment is curative for the severe infections, there is a concern that high-dose G-CSF may increase the risk of hematologic malignancy in these individuals (4).

Several genetic mutations have been identified in SCN patients. Approximately 50% of autosomal-dominant SCN cases were shown to have various heterozygous mutations in the gene encoding neutrophil elastase [elastase, neutrophil-expressed (ELANE)] (5, 6), a monomeric, 218-amino acid (25 kDa) chymotryptic serine protease (7) that is synthesized during the early stages of primary granule production in promyelocytes (8, 9). However, the mechanism(s) causing impaired neutrophil maturation in SCN patients remains unclear due to the current lack of an appropriate disease model.

Results and Discussion

In the present study, we generated induced pluripotent stem (iPS) cells from the BM cells obtained from an SCN patient with a heterologous ELANE gene mutation (exon 5, 707 region, C194X) (SCN-iPS cells) to provide the basis for an SCN disease model. The patient who donated BM cells recurrently suffered from severe infections without exogenous G-CSF administration, but the G-CSF administration once a week prevented his repeated infection. The SCN-iPS cells continued to show embryonic stem cell morphology after >20 passages and also expressed pluripotent markers (Fig. S1*A*). The silencing of exogenous genes and the capability to differentiate into three germ layers by teratoma formation were confirmed for each of the three SCN-iPS cell clones (Fig. S1 *B* and *C*). Furthermore, the same ELANE gene mutation that was present in the patient persisted in the SCN-iPS cells (Fig. S1*D*). The SCN-iPS cells, as well as control iPS cells that were generated from healthy donors, had the normal karyotype (Fig. S1*E*) (10, 11) and no mutations in the mutation-sensitive region of the G-CSF receptor gene (12).

We first compared the hematopoietic differentiation from SCN-iPS cells with that from control iPS cells that were generated from healthy donors. SCN-iPS and control iPS cells were cocultured with a 15-Gy-irradiated murine stromal cell line (the AGM-S3 cell line), as reported (13). After 12 d, the cocultured cells were harvested, and the $CD34^+$ cells separated from these cells (SCN-iPS–$CD34^+$ and control iPS–$CD34^+$ cells, respectively) were cultured in a hematopoietic colony assay by using a cytokine mixture (*Materials and Methods*). The number and size of the erythroid (E) and mixed-lineage (Mix) colonies derived from SCN-iPS–$CD34^+$ cells (1×10^4 cells) were nearly identical to those of the corresponding colonies derived from control iPS–$CD34^+$ cells (E colonies: SCN-iPS cells, 11.0 ± 3.0, and control iPS cells, 11.4 ± 3.9; Mix colonies: SCN-iPS cells, 25.1 ± 7.2, and control iPS cells, 17.4 ± 4.0) (Fig. 1 *B* and *C* and Fig. S2 *A* and *B*). However, the number of myeloid colonies derived from SCN-iPS–$CD34^+$ vs. control iPS–$CD34^+$ cells was significantly lower (SCN-iPS cells, 47.4 ± 19.5; control iPS cells, 127.8 ± 17.9; $P < 0.01$), and the size of the colonies was also smaller (Fig. 1 *A*

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