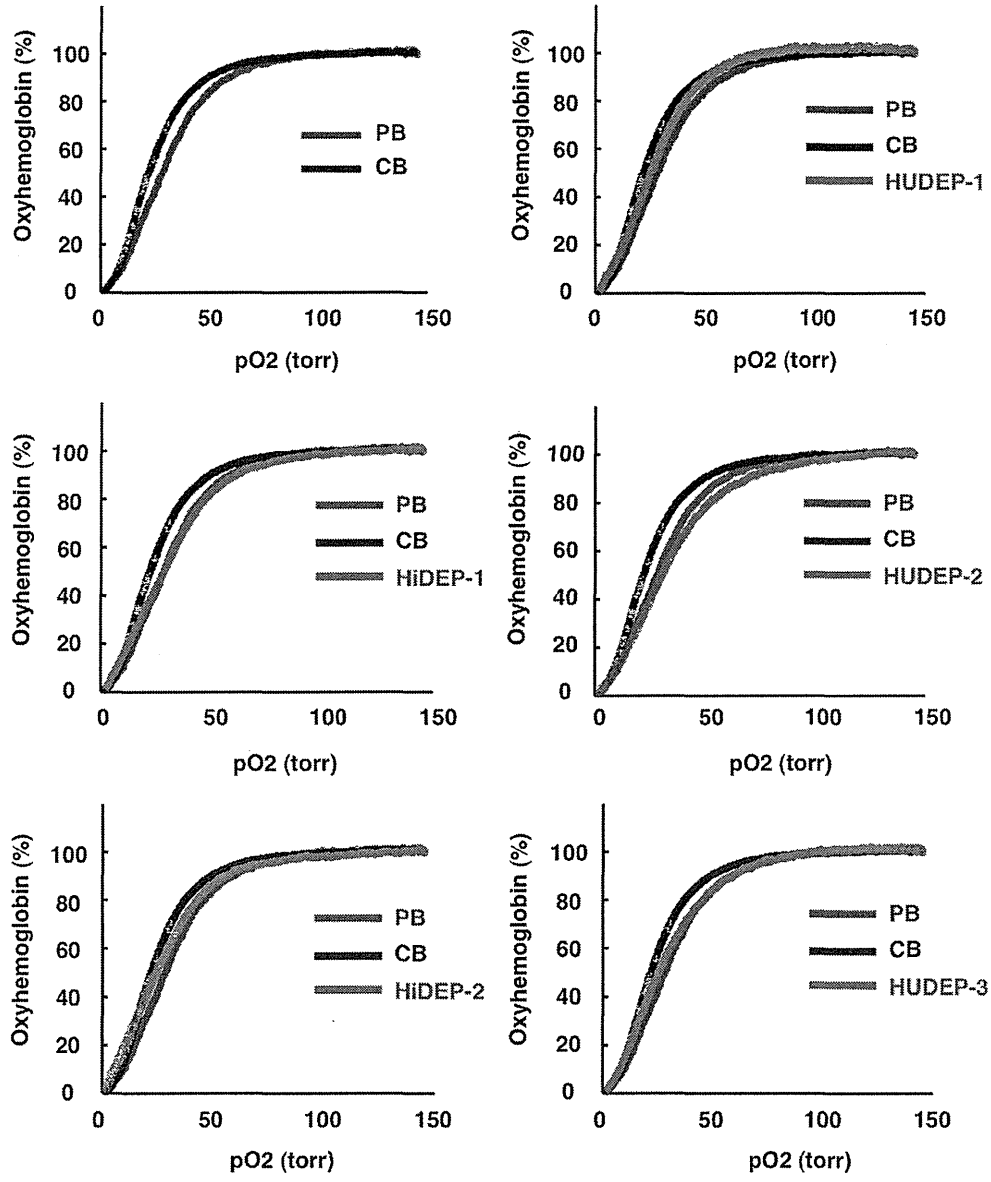

Figure 6. Oxygen-carrying abilities of hemoglobin produced in the established erythroid progenitor cell lines. Oxygen equilibrium curves were determined using an automated apparatus. Following the induction of differentiation, the cells were subjected to the analyses. CB, umbilical cord blood. PB, peripheral 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

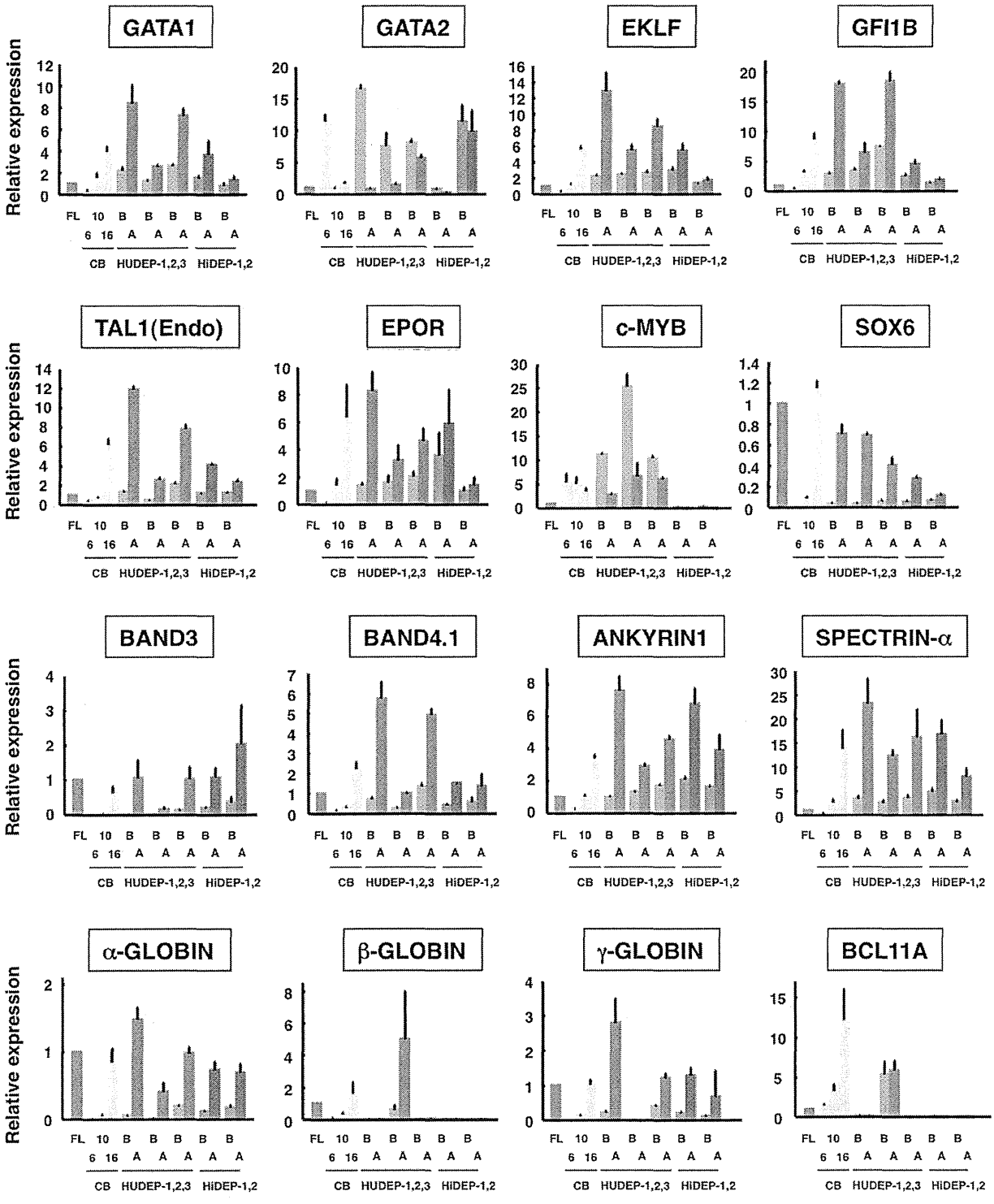

Figure 7. Gene expression profiles of the established erythroid progenitor cell lines estimated by quantitative RT-PCR analysis. Analyzed genes are indicated. FL, results with cDNA derived from human fetal liver. Relative expression was evaluated compared to that of FL. CB, cord blood. CB 6, 10 and 16, 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

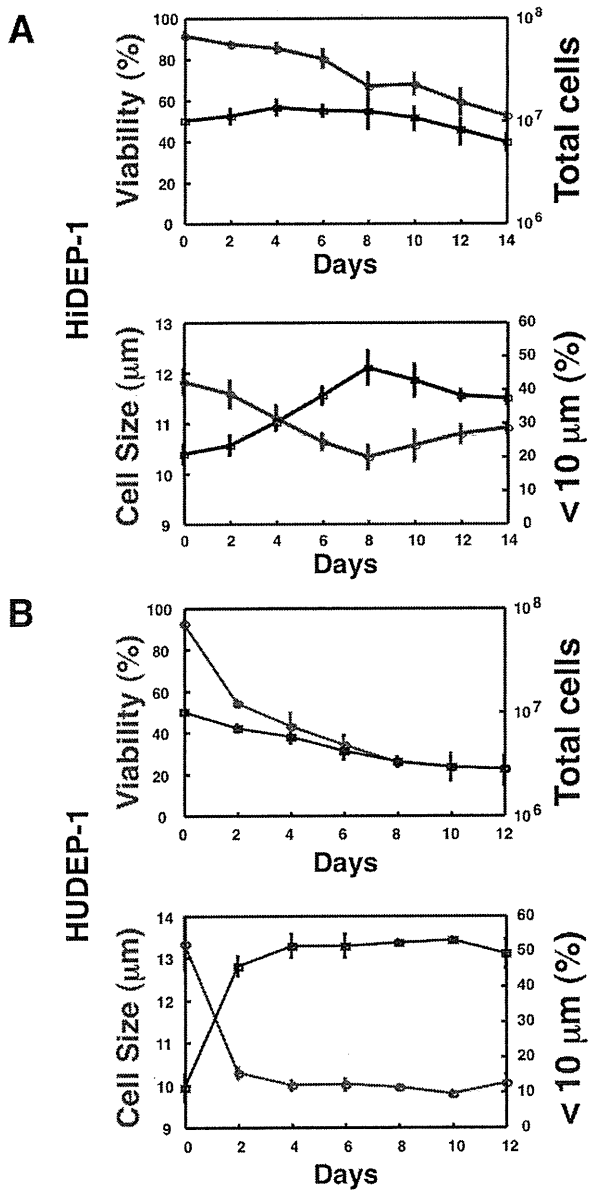

Figure 8. Analyses of cell viability and cell size during the induction of differentiation of the established erythroid progenitor cell lines. Cell viability and cell size were estimated by an automated cell counter. (A) Results of HiDEP-1 cells. (B) Results of HUDEP-1 cells. (A, B) <10 μm (%), the percentages 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

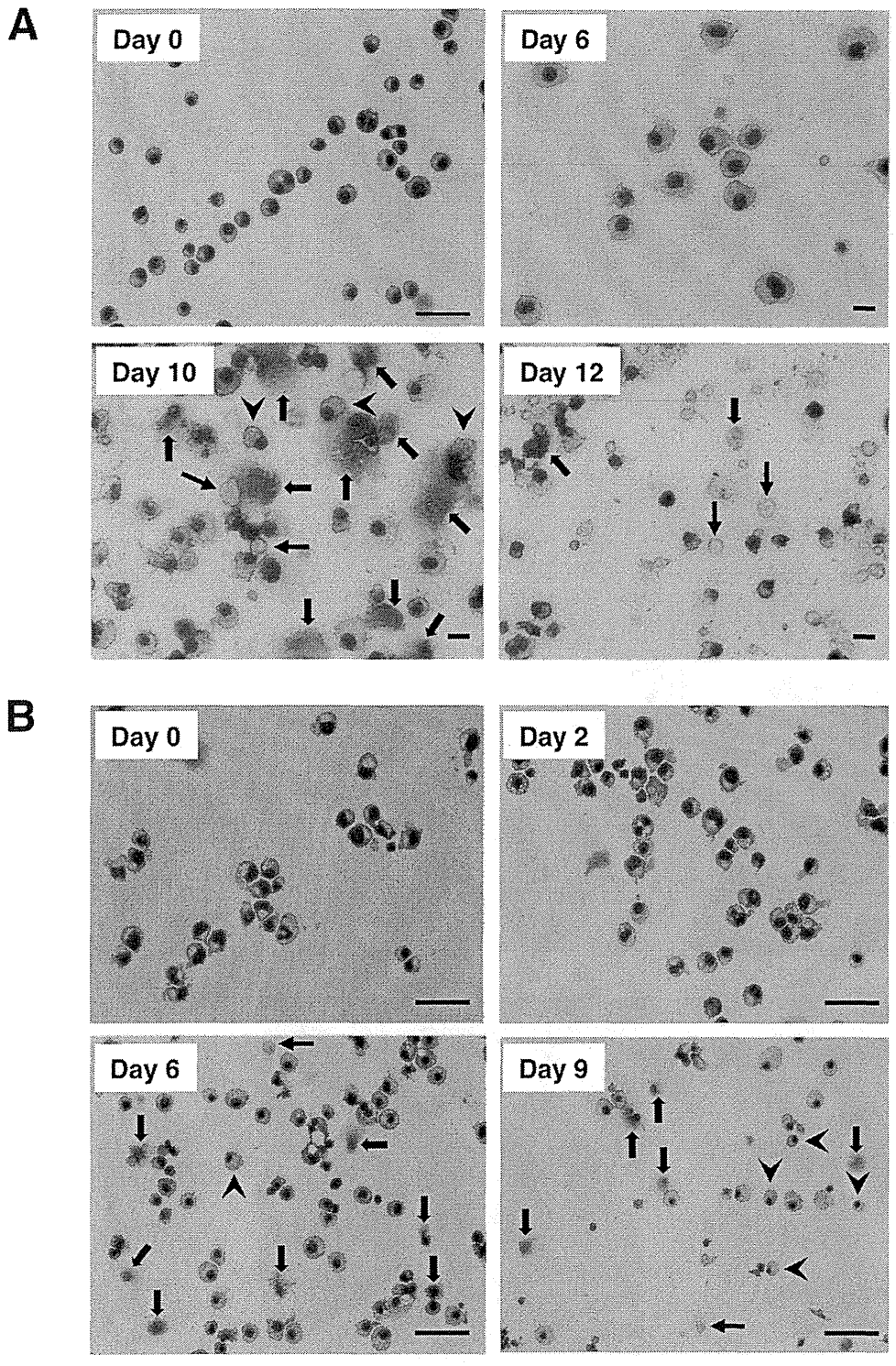

Figure 9. Morphological analyses during the induction of differentiation of the established erythroid progenitor cell lines. (A) HiDEP-1 cells before (Day 0) and 6, 10 and 12 days after the induction of differentiation (Day 6, 10 and 12). Scale bar in Day 0 indicates 50 μm and other scale bars indicate 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.
doi:10.1371/journal.pone.0059890.g009

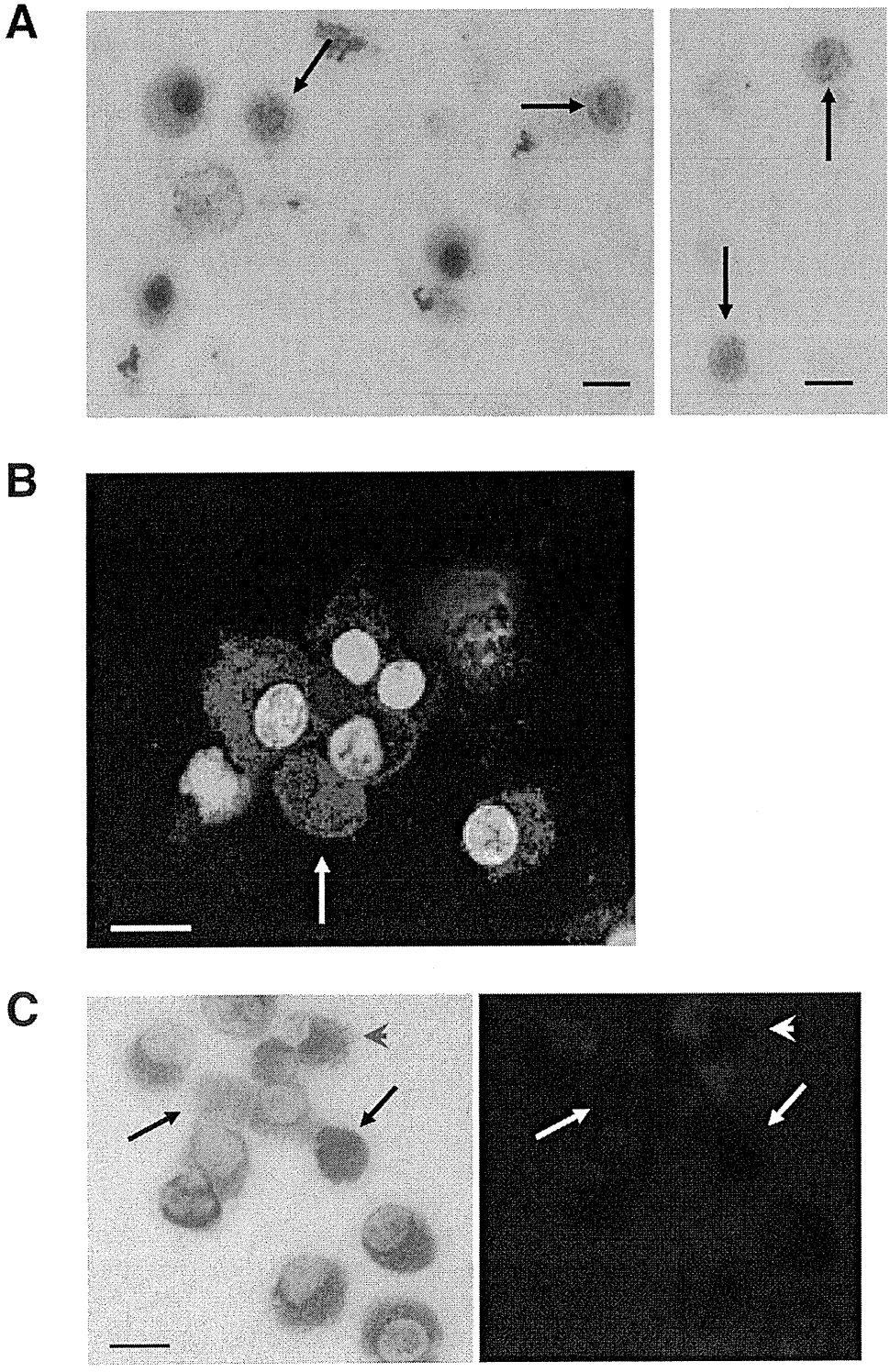

Figure 10. Confirmation of enucleated cells. As a representative, the cells induced from HiDEP-1 cells were subjected to the analyses. (A) Supravital staining. Arrows indicate reticulocytes. (B) Immunohistostaining with glycophorin A antibody (pink) 12 days after the induction of differentiation. Nuclei 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*

Author contributions: T.H., Y.E., K.Y., S.M., S.Y., Y.F., K. Tani, H.N., M.K., and K. Tsuji designed research; T.H., Y.M., K.N., and K.Y. performed research; T.H., Y.E., Y.M., K.N., K.Y., K.U., N.N., S.M., S.Y., M.N., and K. Tsuji analyzed data; and T.H., Y.E., and K. Tsuji wrote the paper.

The authors declare no conflict of interest.

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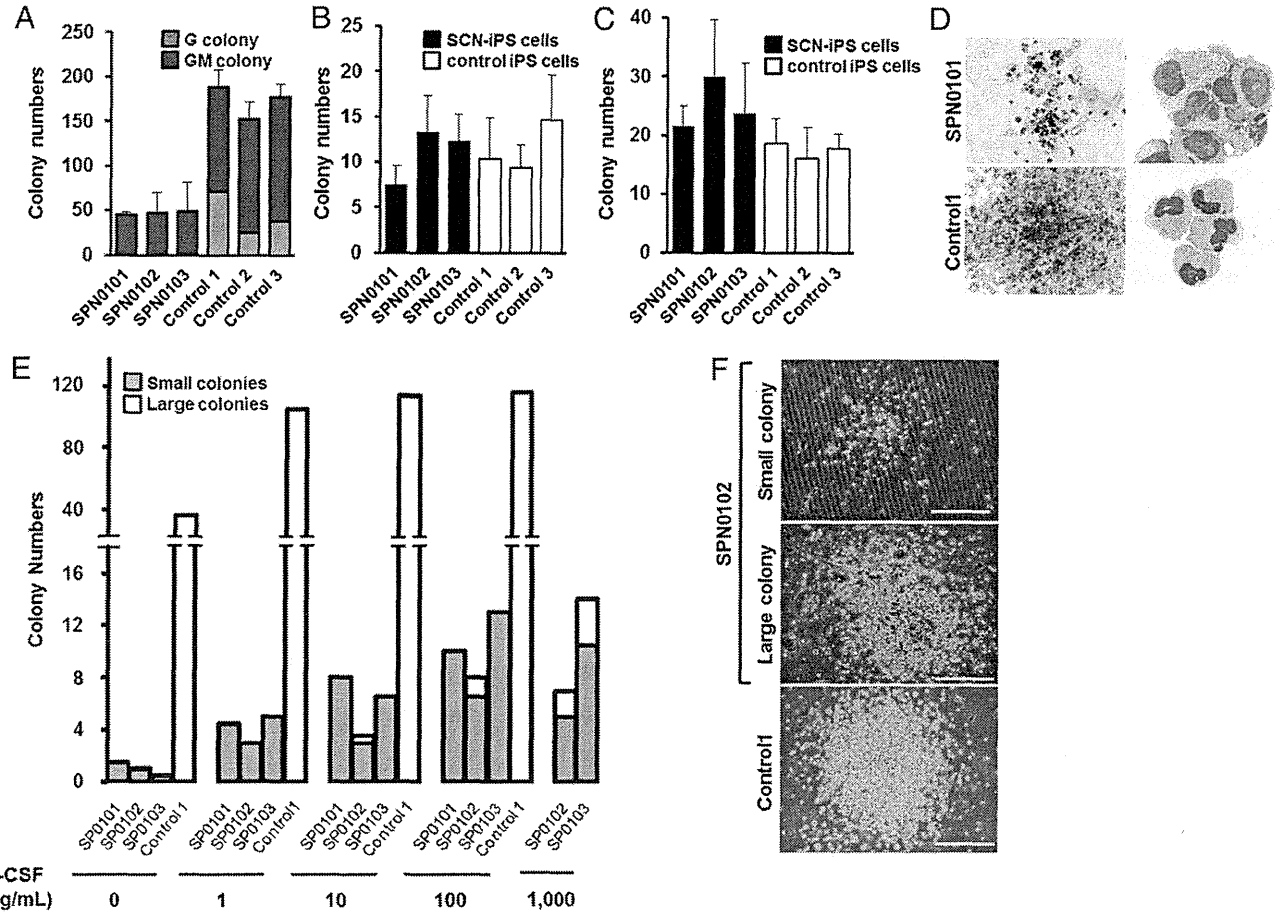

Fig. 1. Impaired neutrophil development from SCN-iPS cells. (*A*–*C*) A hematopoietic colony assay was performed by using 1×10^4 $CD34^+$ cells derived from three SCN-iPS cell clones (SPN0101, SPN0102, and SPN0103) and three control iPS cell clones (controls 1, 2, and 3) in the presence of a cytokine mixture. Colonies were sorted as myeloid (*A*), erythroid (*B*), and mixed-lineage (Mix) (*C*). Data are shown as mean ± SD. (*D*) Photographs of colonies (*Left*; 100×) and cells in a GM colony (*Right*; 400×; May–Grünwald–Giemsa staining). (*E*) A hematopoietic colony assay with dose escalation of G-CSF was performed by using 1×10^5 $CD34^+$ cells derived from SCN-iPS and control iPS cells. Filled and open bars indicate small colonies consisting of <100 cells and large colonies consisting of >100 cells, respectively. Data are shown as the average of three independent experiments. (*F*) Photographs of a small colony derived from SCN-iPS cells (SPN0102) in the presence of 10 ng/mL G-CSF, large colonies derived from SCN-iPS cells in the presence of 1,000 ng/mL G-CSF, and large colonies derived from control iPS cells (control 1) in the presence of 10 ng/mL G-CSF. (Scale bars, 200 μm.)

and *D*). In particular, only a few SCN-iPS cell-derived granulocyte (G) colonies—myeloid colonies consisting of only granulocytes—were detected (Fig. 1*A*). SCN-iPS cell-derived granulocyte–macrophage (GM) colonies—myeloid colonies consisting of macrophages/monocytes with/without granulocytes—contained a few immature myeloid cells in addition to macrophages/monocytes, whereas control iPS cell-derived GM colonies included a substantial number of mature, segmented, and band neutrophils (Fig. 1*D*).

We also found that Mix colonies derived from SCN-iPS cells, but not control iPS cells, contained immature myeloid cells and few mature neutrophils (Fig. S2 *C* and *D*). Next, we conducted a hematopoietic colony assay using various concentrations of G-CSF alone instead of the cytokine mixture to examine the G-CSF dose dependency of neutrophil differentiation from SCN-iPS and control iPS–$CD34^+$ cells. For all concentrations of G-CSF used (1–1,000 ng/mL), the SCN-iPS cell-derived myeloid colonies were significantly lower in number and smaller in size than the control iPS cell-derived myeloid colonies (Fig. 1*E*). Myeloid colony formation from control iPS cells reached a plateau at ~1–10 ng/mL G-CSF, whereas the number and size of those from SCN-iPS cells gradually increased with increasing concentrations of G-CSF. However, the values observed for SCN-iPS cells did not reach those for the control iPS cells, even at the highest dose of G-CSF used (1,000 ng/mL). Furthermore, large colonies consisting of >100 cells derived from SCN-iPS cells were only found with higher concentrations of G-CSF (Fig. 1*F*). Thus, granulopoiesis initiated from SCN-iPS cells was relatively insensitive to G-CSF, reflecting the inadequate in vivo response of neutrophils to G-CSF in SCN patients (14, 15). Therefore, these results support the applicability of the SCN-iPS cells established herein as a disease model for SCN.

To examine neutrophil development from SCN-iPS cells in more detail, SCN-iPS and control iPS–$CD34^+$ cells (1×10^4 cells each) were cocultured in suspension with AGM-S3 cells in the presence of neutrophil differentiation medium (*SI Materials and Methods*). The number of nonadherent cells derived from SCN-iPS–$CD34^+$ cells was lower than that from control iPS–$CD34^+$ cells on day 14 of culture (SCN-iPS cells, $9.77 \times 10^4 \pm 1.65 \times 10^4$ cells; control iPS cells, $52.48 \times 10^4 \pm 23.13 \times 10^4$ cells; $P < 0.05$) (Fig. 2*A*). The proportion of mature neutrophils among the nonadherent cells was also significantly lower for SCN-iPS cells relative to control iPS cells on day 14 (SPN-iPS cells, 15.53% ± 4.33%; control iPS cells, 71.285 ± 3.30%; $P < 0.05$) (Fig. 2 *B* and *C*), indicating that myeloid cells derived from SCN-iPS cells revealed the maturation arrest in the neutrophil development. We then examined a possibility that the maturation arrest in SCN-

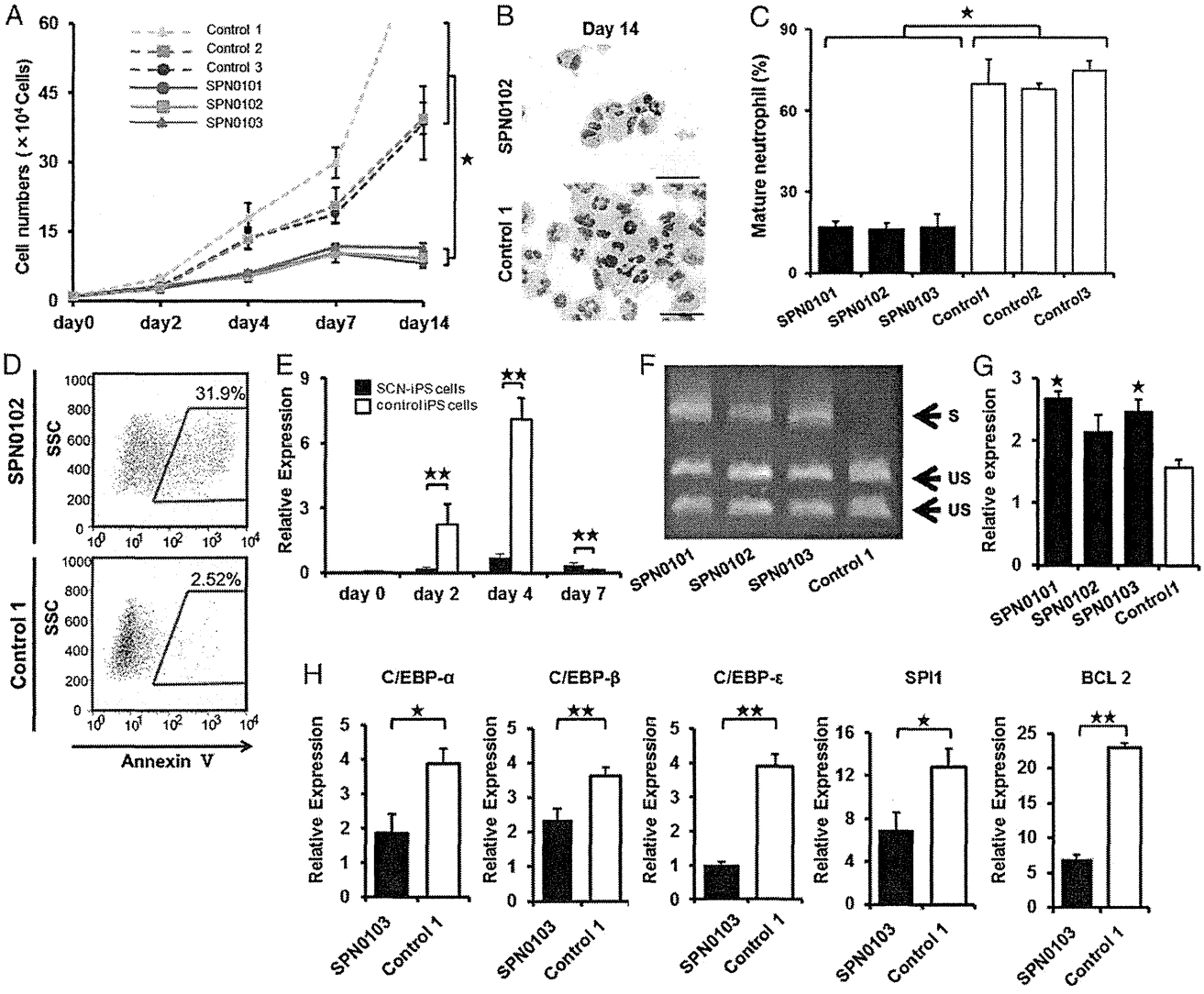

Fig. 2. Analysis of impaired neutrophil development from SCN-iPS cells. (*A*) Total number of nonadherent cells in the suspension culture of 1×10^4 $CD34^+$ cells derived from SCN-iPS and control iPS cells. Data are shown as mean ± SD. $^*P < 0.01$. (*B*) Photographs of nonadherent cells derived from SCN-iPS (SPN0103) and control iPS cells (control 1) on day 14 of culture (400×; May–Grünwald–Giemsa staining; scale bars, 50 μm.) (*C*) Filled and open bars show the proportion of mature neutrophils among the cells derived from SCN-iPS (filled bars) and control iPS (open bars) cells on day 14 of suspension culture. Data are shown as mean ± SD. $^*P < 0.05$. (*D*) Flow cytometric analysis of annexin V expression on cultured cells from SCN-iPS cells (SPN0102) or control iPS cells (control 1) on day 7. (*E*) Sequential qRT-PCR analysis of the relative expression of ELANE mRNA [ELANE/hypoxanthine–guanine phosphoribosyltransferase (HPRT) expression]. Data obtained from independent experiments using three SCN-iPS cell clones (SPN0101, SPN0102, and SPN0103) and three control iPS cell clones are shown as mean ± SD. $^{**}P < 0.01$. (*F* and *G*) $CD34^+$ cells derived from SCN-iPS or control iPS cells were cultured in neutrophil differentiation medium (see text). On day 7, nonadherent cells were collected and analyzed. (*F*) Representative gel showing spliced (S) and unspliced (US) XBP-1 bands on day 7. (*G*) qRT-PCR analysis of the relative mRNA expression (target/HPRT expression) of BiP on day 7. Data are shown as mean ± SD. $^*P < 0.05$; different from control 1). (*H*) qRT-PCR analysis of the relative mRNA expression (target / HPRT expression) of C/EBP-α, C/EBP-β, C/EBP-ε, SPI1, and BCL2 genes in non-adherent cells derived from SCN-iPS cells (filled bars, SPN0103) and control iPS cells (open bars, control 1) on day 2 of suspension culture. Data are shown as the mean ± the s.d. ($^{**}P < 0.01$, $^*P < 0.05$).

iPS cell-derived myeloid cells might be caused by their apoptosis. In flow cytometric analysis, SCN-iPS cell-derived myeloid cells contained a significantly higher proportion of annexin V-positive cells than control iPS-derived myeloid cells on day 7 of culture, suggesting that the maturation arrest in myeloid cells derived from SCN-iPS cells might be caused by their apoptosis (Fig. 2*D*).

We next examined ELANE mRNA expression levels in nonadherent cells derived from SCN-iPS vs. control iPS cells (Fig. 2*E*). ELANE expression was significantly lower in nonadherent cells derived from SCN-iPS vs. control iPS cells on days 2 and 4 of culture ($P < 0.01$), as reported (16, 17). However, the former was a little higher than the latter on day 7 ($P < 0.01$). This result may be explained by the existence of SCN-iPS cell-derived myeloid cells arrested at an early stage along the neutrophil differentiation pathway even on day 7 of culture. We also examined the expression of proteinase 3 and azurocidin, which comprise a family of closely related genes encoding neutrophil granule proteins along with ELANE, and found these genes were more highly expressed on day 4 (Fig. S3).

It has been reported that induction of the endoplasmic reticulum stress (ER) response and the unfolded protein response (UPR) has been advanced as a potential explanation for the molecular pathogenesis of SCN (18, 19). Thus, we examined activation of the UPR by X-box binding protein 1 (XBP-1) mRNA splicing on day 7. As shown in Fig. 2*F*, SPN-iPS cells induced XBP-1 mRNA splicing. We also found the up-regulation of BiP

(also known as GRP78 or HSPA5) (Fig. 2*G*). These results suggested that ER stress response and UPR might be involved in the pathogenesis in SCN.

To examine further the differences in gene expression between the two cell types, a microarray analysis was carried out by using $CD34^+$ cells derived from SCN-iPS and control iPS cells (three clones of each) in suspension culture on day 2. At this early time point, differences in cell number and morphology were not yet readily discernible between SCN-iPS and control iPS cells, as shown in Fig. 2*A*. However, the microarray analysis revealed a differential expression of various genes between the two cell types. Transcription factor genes, which were related to neutrophil development [e.g., CCAAT/enhancer-binding protein (C/EBP)-α (20), C/EBP-β (21), C/EBP-ε (22), and SPI1 (also known as PU.1) (23)], were all down-regulated in SCN-iPS cells. B-cell chronic lymphocytic leukemia/lymphoma 2, which regulates cell death under ER stress through the core mitochondrial apoptosis pathway (24), was also down-regulated (Fig. 3*A*). These findings were confirmed by quantitative reverse-transcriptional PCR (qRT-PCR), as shown in Fig. 2*H*.

Notably, the down-regulation of the genes in SCN-iPS cells related to and regulated by the wingless-type mmtv integration site family, member 3a (Wnt3a)/β-catenin pathway [e.g., Wnt3a, lymphoid enhance-binding factor (LEF)-1, BIRC5 (also known as survivin), and cyclin D1] was also uncovered by microarray analysis and qRT-PCR (Fig. 3 *A–C* and Fig. S4). Therefore, we examined the effect of enhancement of Wnt3a/β-catenin signaling by exogenous Wnt3a addition on the neutrophil development of $CD34^+$ cells derived from SCN-iPS and control iPS cells. Although Wnt3a did not stimulate the survival, proliferation, and differentiation of $CD34^+$ cells derived from both iPS cells in the absence of cytokines stimulating myelopoiesis including G-CSF, the addition of Wnt3a to the neutrophil differentiation medium induced a dose-dependent increase in the percentage of mature neutrophils among the cultured cells, as shown in Fig. 3 *D* and *E*. Furthermore, when Wnt3a was added concurrently with 1,000ng/mL G-CSF, the proportion of mature neutrophils increased more than it did with Wnt3a or 1,000 ng/mL G-CSF alone, reaching a value comparable with that observed for control iPS cells (Fig. 4 *A* and *B*).

The reduced expression of LEF-1 (as regulated by the Wnt3a/β-catenin pathway) reportedly plays a critical role in the defective maturation of neutrophils in SCN patients (25). Therefore, we next examined LEF-1 mRNA expression in SCN-iPS–$CD34^+$ cells cultured in the presence of Wnt3a, G-CSF (1,000 ng/mL), or both. Wnt3a and G-CSF both enhanced LEF-1 mRNA expression, but the most significant increase was observed in the presence of Wnt3a plus G-CSF. LEF-1 expression in SCN-iPS–$CD34^+$ cells in response to Wnt3a plus G-CSF was almost the same as that in control iPS–$CD34^+$ cells (Fig. 4*C*). These results substantiate the importance of LEF-1 in neutrophil development and the pathogenesis of SCN, as shown (25). Moreover the

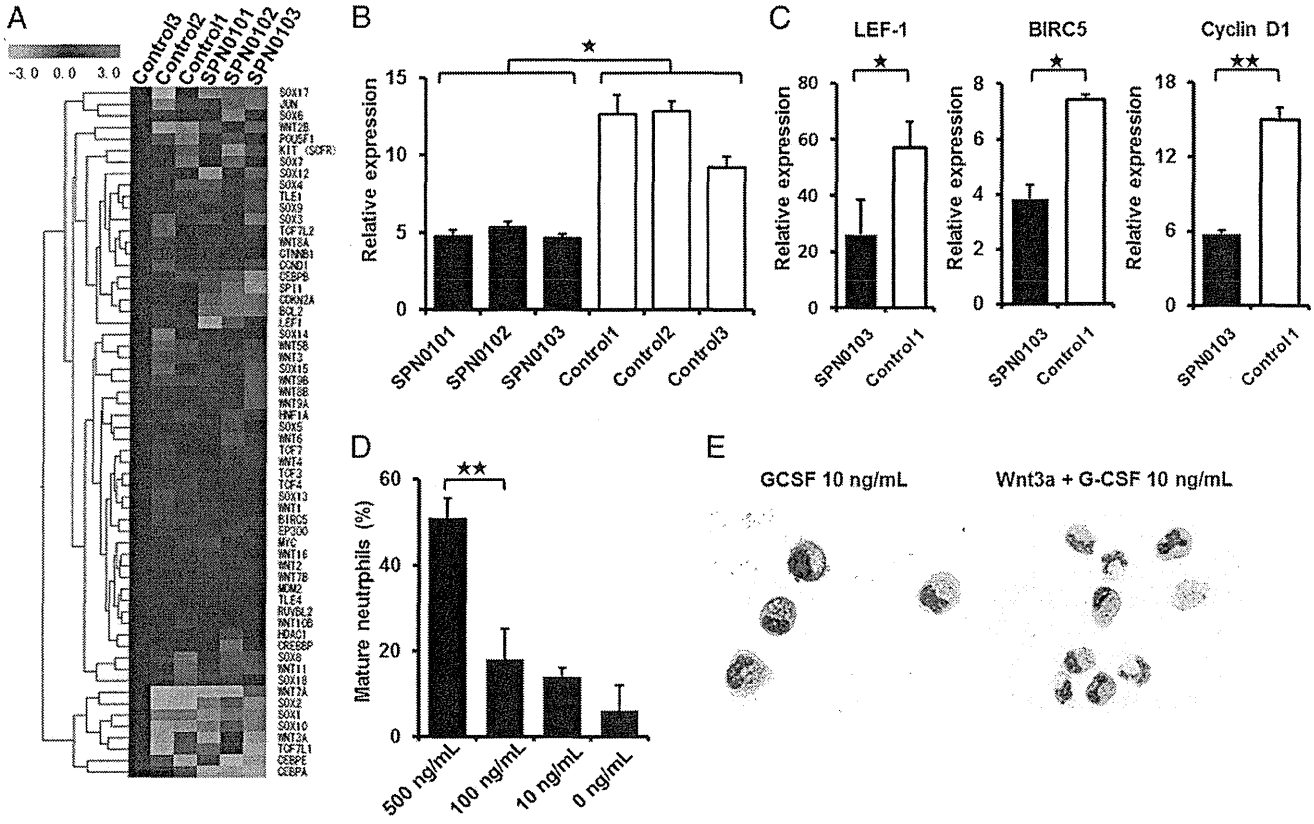

Fig. 3. Effects of Wnt3a on neutrophil development from SCN-iPS cells. (*A*) Heat map showing differential gene expression among SCN-iPS and control iPS cells on day 2. Red, high gene expression; blue, low gene expression compared with gene expression in control 3. (*B*) qRT-PCR analysis of the relative mRNA expression (target/HPRT expression) of Wnt3a on day 2. Filled and open bars indicate experiments using SCN-iPS cells (SPN0101, SPN0102, and SPN0103) and control iPS cells (controls 1, 2, and 3), respectively. Data are shown as mean ± SD. $^*P < 0.05$. (*C*) qRT-PCR analysis of the relative expression (target/HPRT expression) of genes regulated by the Wnt3a/β-catenin pathway (LEF-1, survivin, and cyclin D1) in SCN-iPS cells (filled bars, SPN0103) vs. control iPS cells (open bars, control 1) on day 2 of suspension culture. Data are shown as mean ± SD. $^{**}P < 0.01$; $^*P < 0.05$. (*D*) Proportion of mature neutrophils among the cells derived from SCN-iPS cells (SPN0102) on day 14 of suspension culture with dose escalation of Wnt3a. Data are shown as mean ± SD. $^{**}P < 0.01$. (*E*) Photographs of nonadherent cells on day 7 of suspension culture with or without Wnt3a (500 ng/mL) (400×; May–Grünwald–Giemsa staining).

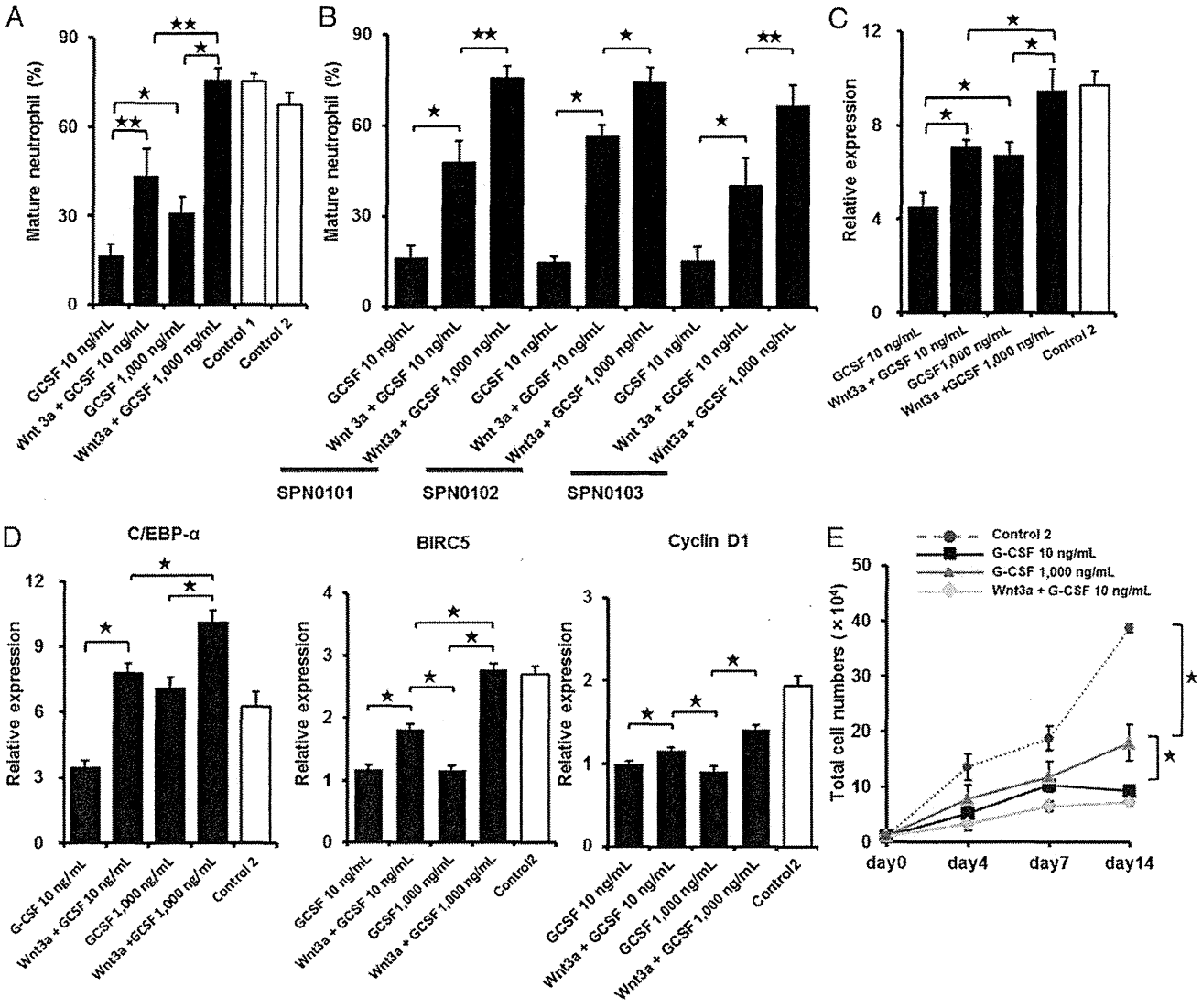

Fig. 4. Effects of Wnt3a in combination with high-dose G-CSF. (*A*) Filled and open bars show the proportion of mature neutrophils among the cells derived from SCN-iPS cells (SPN0101) on day 14 of suspension culture in the presence of neutrophil differentiation medium containing 10 ng/mL G-CSF (GCSF 10 ng/mL); 500 ng/mL Wnt3a and 10 ng/mL G-CSF (Wnt3a+GCSF 10 ng/mL); 1,000 ng/mL G-CSF (GCSF 1,000 ng/mL); or 500 ng/mL Wnt3a and 1,000 ng/mL G-CSF (Wnt3a + GCSF 1,000 ng/mL); and that from control iPS cells (controls 1 and 2) cultured in the neutrophil differentiation medium containing 10 ng/mL G-CSF, respectively. Data are shown as mean ± SD. $^{**}P < 0.01$; $^{*}P < 0.05$. (*B*) The proportion of mature neutrophils among the cells derived from three SCN-iPS cell clones (SPN0101, SPN0102, and SPN0103) on day 14 of suspension culture in the presence of neutrophil differentiation medium containing 10 ng/mL G-CSF (GCSF 10 ng/mL); 500 ng/mL Wnt3a and 10 ng/mL G-CSF (Wnt3a+GCSF 10 ng/mL); or 500 ng/mL Wnt3a and 1,000 ng/mL G-CSF (Wnt3a + GCSF 1,000 ng/mL). Data are shown as mean ± SD. $^{**}P < 0.01$; $^{*}P < 0.05$. (*C*) Filled and open bars show the relative expression (target/HPRT expression) of LEF-1 mRNA in SCN-iPS cells (SPN0101) on day 2 of suspension culture in the presence of differentiation medium containing the same combinations of Wnt3a and G-CSF as shown in *A* and that from control iPS cells (control 2), respectively. Data are shown as mean ± SD. $^{**}P < 0.01$; $^{*}P < 0.05$. (*D*) Filled and open bars show the relative expression (target/HPRT expression) of C/EBP-α, BIRC5, or cyclin D1 mRNA in SCN-iPS cells (SPN0101) on day 2 of suspension culture in the presence of differentiation medium containing the same combinations of Wnt3a and G-CSF as shown in *A* and that from control iPS cells (control 2), respectively. Data are shown as mean ± SD. $^{**}P < 0.01$; $^{*}P < 0.05$. (*E*) Total cell numbers of nonadherent cells in suspension cultures of 1×10^4 $CD34^+$ cells derived from control iPS cells (control 2; red broken line) and SCN-iPS cells (SPN0101) in the presence of neutrophil differentiation medium (black line) and those from SCN-iPS cells in the presence of neutrophil differentiation medium containing 500 ng/mL Wnt3a (yellow line) or 1,000 ng/mL G-CSF (black line). Data are shown as mean ± SD. $^{**}P < 0.05$.

administration of Wnt3a led to up-regulate C/EBP-α, cyclin D1, and BIRC5/survivin in addition to LEF-1 in the presence of G-CSF (Fig. 4*D*). These results suggested that the up-regulation of LEF-1 expression might promote granulopoiesis by increasing the expressions of cyclin D1, BIRC5/survivin, and C/EBP-α and its binding to LEF-1 in accordance with the previous report (25). Interestingly, Wnt3a did not stimulate the proliferation of myeloid cells, whereas 1,000 ng/mL G-CSF did to a certain extent (Fig. 4*E*). Hence, Wnt3a was capable of stimulating the maturation of impaired neutrophils in the presence of G-CSF, but not the proliferation of myeloid cells from SCN-iPS cells.

Importantly, aside from providing new insights into the mechanisms behind impaired neutrophil development in SCN patients, the present study demonstrates that agents activating the Wnt3a/β-catenin pathway are potential candidates for new drugs for SCN with mutations in the ELANE gene. Because endogenous G-CSF is readily increased in SCN patients (26), these activating agents may be viable alternatives to exogenous G-CSF treatment.

Materials and Methods

Additional information is available in SI Materials and Methods.

Generation of Human iPS Cells. BM fibroblasts from a patient with SCN and skin dermal fibroblasts from a healthy donor were acquired after obtaining informed consent after getting the approval by the Ethics Committee of the Institute of Medical Science, University of Tokyo, in accordance with the Declaration of Helsinki. The SCN patient presented with a heterozygous mutation in the ELANE gene in the 707 region of exon 5. SCN-iPS cells were established from the SCN-BM fibroblasts by transfection with the pMX retroviral vector, as described (10). This vector expressed the human transcription factors OCT3/4, SOX2, KLF4, and c-MYC. Control iPS cell clones, control 1 (TkDN4-M) and control 3 (201B7), were gifts from K. Eto and S. Yamanaka (Kyoto University, Kyoto), respectively (10, 11). Control 2 (SPH0101) was newly generated from another healthy donor's skin dermal fibroblasts by using the same methods.

Hematopoietic Colony Assay. A hematopoietic colony assay was performed in an aliquot of culture mixture, which contained 1.2% methylcellulose (Shin-Etsu Chemical), 30% (vol/vol) FBS, 1% (vol/vol) deionized fraction V BSA, 0.1 mM 2-mercaptoethanol (2-ME), α-minimum essential medium, and a cytokine mixture consisting of 100 ng/mL human stem cell factor (hSCF) (Wako), 100 ng/mL fusion protein 6 [FP6; a fusion protein of interleukin (IL)-6 and IL-6 receptor] (a gift from Tosoh), 10 ng/mL human IL-3 (hIL-3) (a gift from Kirin Brewery), 10 ng/mL human thrombopoietin (hTPO) (a gift from Kirin Brewery), 10 ng/mL human G-CSF (a gift from Chugai Pharmaceutical), and 5 U/mL human erythropoietin (a gift from Kirin Brewery). For dose escalation experiments, various concentrations (0, 1, 10, 100, and 1,000 ng/mL) of G-CSF were used instead of the cytokine mixture described above. Colony types were determined according to established criteria on day 14 of culture by in situ observations under an inverted microscope (IX70; Olympus) (27).

Suspension Culture and Neutrophil Differentiation Assay. $CD34^+$ cells (1×10^4 cells) were cocultured with irradiate confluent AGM-S3 cells in neutrophil differentiation medium containing Iscove's modified Dulbecco's medium, 10% FBS, 3 mM L-glutamine, 1×10^{-4} M 2-ME, 1×10^{-4} M nonessential amino acids solution, 100 ng/mL hSCF, 100 ng/mL FP6, 10 ng/mL hIL-3, 10 ng/mL hTPO, and 10 or 1,000 ng/mL human G-CSF. Wnt3a (10, 100, or 500 ng/mL) (R&D) was then added. The medium was replaced with an equivalent volume of fresh medium every 4 d. Living, nonadherent cells were counted following 0.4% trypan blue staining.

PCR primer. All primer sets used in this study are shown in Table S1.

Statistical Analysis. All data are presented as mean ± SD. $P < 0.05$ was considered significant. Statistical analyses were performed by using Prism software (GraphPad).

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